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New Rapid Scheme for Distinguishing the Subspecies of the *Mycobacterium abscessus* Group and Identifying *Mycobacterium massiliense* Isolates with Inducible Clarithromycin Resistance

Shamira J. Shallom,[a] Paul J. Gardina,[b] Timothy G. Myers,[b] Yinong Sebastian,[b] Patricia Conville,[a] Leslie B. Calhoun,[a] Hervé Tettelin,[c] Kenneth N. Olivier,[d] Gulbu Uzel,[d] Elizabeth P. Sampaio,[d,e] Steven M. Holland,[d] Adrian M. Zelazny[a,d]

Microbiology Service, Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, Maryland, USA[a]; Genomic Technologies Section, Research Technologies Branch, Division of Intramural Research, NIAID, National Institutes of Health, Bethesda, Maryland, USA[b]; Institute for Genome Sciences, Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland, USA[c]; Laboratory of Clinical Infectious Diseases, NIAID, National Institutes of Health, Bethesda, Maryland, USA[d]; Leprosy Laboratory, Oswaldo Cruz Institute, Rio de Janeiro, Brazil[e]

***Mycobacterium abscessus* (*M. abscessus* sensu lato, or the *M. abscessus* group) comprises three closely related taxa whose taxonomic statuses are under revision, i.e., *M. abscessus* sensu stricto, *Mycobacterium bolletii*, and *Mycobacterium massiliense*. We describe here a simple, robust, and cost-effective PCR-based method for distinguishing among *M. abscessus*, *M. massiliense*, and *M. bolletii*. Based on the *M. abscessus* ATCC 19977^T genome, regions that discriminated between *M. abscessus* and *M. massiliense* were identified through array-based comparative genomic hybridization. A typing scheme using PCR primers designed for four of these locations was applied to 46 well-characterized clinical isolates comprising 29 *M. abscessus*, 15 *M. massiliense*, and 2 *M. bolletii* isolates previously identified by multitarget sequencing. Interestingly, 2 isolates unequivocally identified as *M. massiliense* were shown to have a full-length *erm*(41) gene instead of the expected gene deletion and showed inducible clarithromycin resistance after 14 days. We propose using this PCR-based typing scheme combined with *erm*(41) PCR for straightforward identification of *M. abscessus*, *M. massiliense*, and *M. bolletii* and the assessment of inducible clarithromycin resistance. This method can be easily integrated into a routine workflow to provide subspecies-level identification within 24 h after isolation of the *M. abscessus* group.**

Rapidly growing mycobacteria (RGM) are ubiquitous environmental microorganisms and a significant cause of human disease (1). The prevalence of lung disease due to RGM is increasing and in many areas of the United States exceeds that of *Mycobacterium tuberculosis* (2). Within the RGM, the *Mycobacterium abscessus* group is a prominent cause of lung infections in patients with cystic fibrosis and chronic pulmonary disease (bronchiectasis, nodules, and cavitations) and of posttraumatic and postsurgical infections (1, 3). Infections with organisms in the *M. abscessus* group are difficult to treat, due to both natural broad-spectrum resistance and acquired resistance, with various antibiotic susceptibility patterns being observed among isolates (4). *M. abscessus* sensu lato, or the *M. abscessus* group, was recently divided into three closely related taxa, i.e., *M. abscessus* sensu stricto (hereinafter referred to as *M. abscessus*), *Mycobacterium massiliense*, and *Mycobacterium bolletii*. *M. massiliense* has been recognized increasingly as an emerging pathogen causing postsurgical wound infection outbreaks (5), and recently it was identified as a cause of respiratory outbreaks in two cystic fibrosis centers, with evidence of transmission between patients (6, 7).

The taxonomic status of the *M. abscessus* group remains controversial. *M. massiliense* and *M. bolletii* were initially proposed as new species mainly on the basis of divergence of their *rpoB* sequences. However, further studies showed that these organisms could not be separated by biochemical tests and mycolic acid pattern analysis (8) and showed less genomic divergence than would be expected for separate species (8, 9). It was recently proposed to unite *M. massiliense* and *M. bolletii* as *Mycobacterium abscessus* subsp. *bolletii* and to designate the new taxon *Mycobacterium abscessus* subsp. *abscessus* for *M. abscessus* isolates (10). However, recent phylogenetic analysis based on whole-genome sequencing data supports the separation of the *M. abscessus* group into three taxa, namely, *Mycobacterium abscessus* subsp *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (7).

Due to their high levels of relatedness, members of the *M. abscessus* group cannot be differentiated on the basis of single-gene sequencing (11, 12). Zelazny et al. combined typing through repetitive sequence-based PCR and pulsed-field gel electrophoresis (PFGE) of macrorestricted DNA with three-locus sequence analysis (*hsp65*, *rpoB*, and *secA1*) to resolve ambiguous identification of *M. abscessus* isolates as *M. abscessus*, *M. massiliense*, or *M. bolletii* (13). Macheras et al. developed a multilocus sequence-based phylogenomic analysis based on eight housekeeping genes (9) and reported the occurrence of horizontal gene transfer among members of the *M. abscessus* group, including the exchange of alleles of genes used for identification and typing. Recently, Choi et al. (14) used two tandem-repeat loci for identification of the *M. abscessus* group, and Wong et al. (15) reported variable-number tandem-repeat (VNTR) analysis for molecular

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Address correspondence to Adrian M. Zelazny, azelazny@mail.nih.gov.
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typing of *M. abscessus*. More recently, Sassi et al. (16) proposed a multispacer typing scheme using 8 intergenic spacers, which are thought to undergo less evolutionary pressure and thus are more variable than housekeeping genes. The recent incorporation of matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) in clinical laboratories has led to increased speed and accuracy in the identification of mycobacteria (17–20). However, closely related strains such as *M. abscessus* and *M. massiliense* could not be distinguished by this methodology (17, 18).

Macrolides, such as clarithromycin and azithromycin, are frequently the only oral antibiotics that are active against *M. abscessus* and *M. massiliense*. However, some *M. abscessus* isolates are resistant to clarithromycin due to point mutations at positions A2058 and A2059 in the region of the *rrl* gene encoding the peptidyltransferase domain of 23S rRNA (21). A second mechanism confers inducible resistance via a functional erythromycin ribosomal methylase gene, *erm*(41). This inducible resistance can be assessed *in vitro* by extended incubation of the organism with clarithromycin (22). The *M. massiliense erm*(41) gene contains mutations, including a large C-terminal deletion that renders it nonfunctional. Based on size differences of the product, *erm*(41) PCR was proposed as a simple method to differentiate *M. massiliense* from *M. abscessus* and *M. bolletii* (23, 24).

Recent publications provide evidence that individual subspecies or even strains of the *M. abscessus* complex are associated with specific patient populations and with differing clinical implications and outcomes (4, 6, 7, 25). Therefore, timely accurate identification of the members of the *M. abscessus* group is important for patient management and epidemiological purposes.

We describe here a simple, robust, and cost-effective PCR-based method for distinguishing among *M. abscessus*, *M. massiliense*, and *M. bolletii* based on discriminatory regions identified using comparative genomic hybridization (CGH) analysis with *M. abscessus* strain ATCC 19977^T. In addition, we report the presence of a full-length *erm*(41) gene in *M. massiliense* clinical isolates that show inducible clarithromycin resistance.

MATERIALS AND METHODS

DNA isolation from reference strains and clinical isolates. Forty-three clinical isolates belonging to the *M. abscessus* group were obtained from sputum, bronchoalveolar lavage fluid, blood culture, skin, or lymph node specimens, as described in a previous publication by Zelazny et al. (13). Three additional clinical isolates were obtained from sputum, bronchoalveolar lavage fluid, and blood samples from patients with bronchiectasis, cystic fibrosis, and interleukin 21 receptor gene-related primary immunodeficiency, respectively. In addition, three reference strains, i.e., *M. abscessus* ATCC 19977^T, *M. massiliense* CCUG 48898^T, and *M. bolletii* CCUG 50184^T, were included. The bacterial strains were stored at −70°C in Tween-albumin broth (Remel, Lenexa, KS). Prior to use, the strains were subcultured on Middlebrook 7H11 agar (Remel, Lenexa, KS). DNA was extracted from a 10-μl loopful of each mycobacterial colony by use of an UltraClean microbial DNA isolation kit (Mo Bio Laboratories, Solana Beach, CA), according to the manufacturer's instructions.

Microarray design and hybridization. The *Mycobacterium abscessus* (ATCC 19977^T) genome sequence was downloaded from the NCBI database (GenBank accession number CU458896.1) and was analyzed with DustMasker to identify low-complexity regions. Microarray probe sequences were selected by "tiling" 60-mers end to end (zero overlap) for 100% coverage. A total of 84,452 probe sequences were uploaded to the Agilent eArray website for fabrication of SurePrint microarrays in the 4x180K format (custom array design number 025760), with each probe spotted in duplicate on each array.

Genomic DNA was labeled using the Agilent genomic DNA enzymatic labeling kit, using Cy3 dye for test samples and Cy5 for the reference strain (*M. abscessus* ATCC 19977^T). Arrays were hybridized using the Agilent Oligo CGH hybridization kit and were scanned using an Agilent microarray scanner at 3-μm resolution, using an extended dynamic range setting (XDR) of 100% and 10% photomultiplier tube voltage (PMT). Agilent Feature Extraction version 10.7.3.1 software, protocol CGH_107_Sep09, was used for image analysis.

CGH microarray data analysis. Normalized $\log_2$ signal ratios were calculated for every microarray probe for each of the test strains, with the value for the reference strain (*M. abscessus* ATCC 19977^T) as the denominator. The log ratios therefore represent the genomic contents of the test strands relative to the reference at each 60-mer tiled across the reference genome. The test strains used included *M. abscessus* clinical isolate CI79, the *M. massiliense* type strain (CCUG 48898), and *M. massiliense* clinical isolates CI76, CI11, and CI154.

Chromosomal segments with altered copy numbers (copy number variations [CNVs]), relative to the reference strain, were identified by applying the circular binary segmentation (CBS) algorithm (26) to normalized log ratios ordered by chromosome position. Array data normalization, detection of CNVs, and genomic visualization were performed using JMP/Genomics software (SAS version 4.1). Genomic segments that differed between the *M. massiliense* and *M. abscessus* genomes were selected based on the expected product sizes, aiming for 1,000 to 2,000 bp for the full-length PCR product and a difference of at least 300 bp for the strain with deletions (to aid in unambiguous detection).

Generation of primer pairs for genotyping and sequencing. PCR primers for genotyping were designed using the *Mycobacterium abscessus* ATCC 19977^T sequence (GenBank accession number CU458896.1) upstream and downstream of the differential CGH segments (27, 28). PCR was performed using 4 different primer pairs with 46 clinical isolates and three reference strains, using Qiagen *Taq* polymerase (Qiagen, Carlsbad, CA) in a 25-μl total reaction mixture volume with 12.5 μl of master mix composed of buffer, dNTPs, *Taq* polymerase, 2 to 3 μl (~50 ng) of extracted DNA, and water to achieve a final volume of 25 μl. Additionally, the region of the *erm*(41) gene was amplified with the primers *erm*F (5′-GAC CGG GGC CTT CTT CGT GAT-3′) and *erm*R1 (5′-GAC TTC CCC GCA CCG ATT CC-3′) (22, 24). The PCR cycling conditions were initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 62°C or 66°C (specified below) for 1 min, and extension at 72°C for 1.5 min, with final extension at 72°C for 10 min. An annealing temperature of 62°C was used for PCRs with the *erm*(41) gene and MAB_4751 primers, while 66°C was used for PCRs with MAB_2396, MAB_2697c, and MAB_4792 primers. PCR products were visualized by 1% agarose gel electrophoresis. PCR products from the *erm*(41) gene were purified for sequencing using 0.5-ml Amicon Ultra 100K centrifugal filters (Millipore Ltd., Ireland). *In silico* PCR was done using NCBI BLASTn (http://blast.ncbi.nlm.nih.gov/) with this set of primer pairs and the publicly available *M. abscessus* ATCC 19977^T and *M. massiliense* CCUG 48898^T genome sequences to obtain the expected PCR product size for each taxon. The *M. bolletii* genome was not used since the available sequences were located in more than 20 contigs. Sequencing of PCR amplicons was performed with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Carlsbad, CA), and data were assembled using Lasergene SeqMan Pro technology (DNASTAR Inc., Madison, WI). Sequences were compared against the GenBank database using NCBI BLASTn (27) (http://blast.ncbi.nlm.nih.gov/).

Clarithromycin susceptibility testing. Clarithromycin MIC values were determined in Mueller-Hinton medium by the broth microdilution method, using Sensititre RGMYCO plates (Trek Diagnosis, Cleveland, OH). Plates were evaluated after 3 to 5 days and were further incubated for 14 days at 30°C for a final reading to ensure detection of inducible resis-

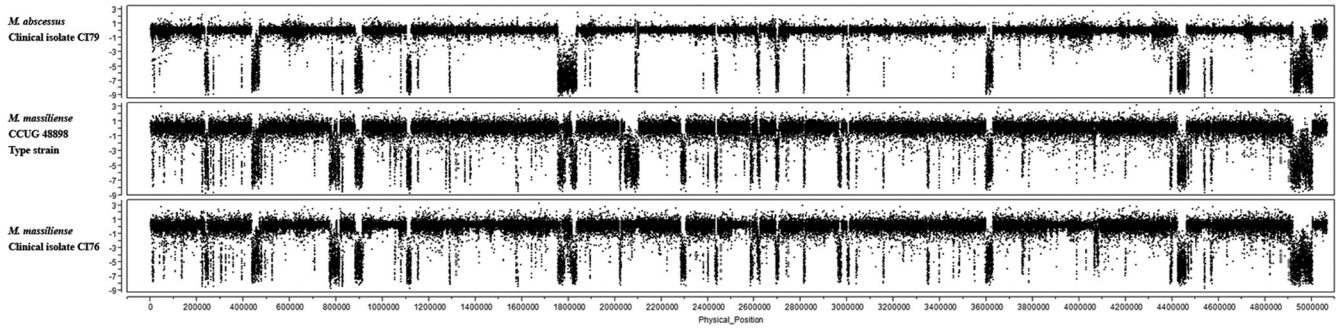

FIG 1 Whole-genome comparative genome hybridization (CGH) patterns of mycobacterial isolates relative to the *M. abscessus* type strain ATCC 19977. The normalized $\log_2$ signal ratios (*y* axis) for individual probes tiled across the genome (*x* axis) are shown for *M. abscessus* clinical isolate CI79 (top panel), the *M. massiliense* type strain CCUG 48898 (middle panel), and *M. massiliense* clinical isolate CI76 (bottom panel). Log ratios near 0 indicate genomic signal intensity similar to that of the *M. abscessus* reference genome, whereas contiguous regions of highly negative ratios indicate putative deletion segments (i.e., signal was absent due to lack of homologous DNA in the test strain).

tance, and the interpretative breakpoints used were those recommended by the CLSI (29).

Accession numbers. Microarray data files were deposited in the NCBI GEO archive (accession number GSE43629). The *erm*(41), *hsp65*, *rpoB*, and *secA1* partial gene sequences from strains CI2040 and CI8182 were deposited in the GenBank database under accession numbers KF360854 to KF360861.

RESULTS

CNV detection and selection for use in genotyping. We performed CGH to identify chromosomal aberrations by copy number variation (CNV) to distinguish *M. abscessus* from *M. massiliense*, using the type strain ATCC 19977 and clinical isolate CI79 as representatives of *M. abscessus* and the type strain CCUG 48898 and clinical isolates CI11, CI76, and CI154 as representatives of *M. massiliense*. Figure 1 shows CGH results against the *M. abscessus* reference genome for several representative test strains. The $\log_2$ ratios (*y* axis) are plotted against the genomic position of each tiling probe (*x* axis) for *M. abscessus* clinical isolate CI79 (Fig. 1, top panel), the *M. massiliense* type strain CCUG 48898 (Fig. 1, middle panel), and *M. massiliense* clinical isolate CI76 (Fig. 1, bottom panel). Log ratios close to 0 indicate genomic signal intensity similar to that of the *M. abscessus* ATCC 19977^T reference genome (assumed to have a copy number of 1 for all regions targeted by the tiling probes), while contiguous regions with highly negative ratios indicate putative deletion segments (i.e., signal was absent due to lack of homologous DNA in the test strain). The *M. abscessus* isolate CI79 generally had the tightest signal ratio (least deviation from 0), consistent with greater sequence homology to probes designed from the *M. abscessus* ATCC 19977 type strain; however, several apparent deletions relative to the type strain were observed (Fig. 1, top panel). *M. massiliense* strain CCUG 48898^T, and clinical isolate CI76 showed even more numerous deviations relative to the *M. abscessus* ATCC 19977^T reference genome (Fig. 1, middle and bottom panels, respectively). The largest putative deletion segments approached 80 kb and thus were too large for straightforward analysis by PCR; CNVs that can be exploited in PCR assays were drawn from the population of smaller deletions.

A detailed view of the CGH patterns of four putative CNVs is shown in Fig. 2 for MAB_2396, MAB_2697c, MAB_4751, and MAB_4792 genes, which were among the candidate loci to distinguish *M. abscessus* from *M. massiliense*. The borders (as determined by the CBS algorithm) of putative CNVs occurring in the *M. massiliense* strains relative to the *M. abscessus* type strain are indicated by dashed vertical lines. These regions reflect genomic losses in *M. massiliense* strains CI11, CI154, CCUG 48898^T, and CI76 but are present in *M. abscessus* CI79 and ATCC 19977^T. Table 1 describes the predicted discriminatory segments that were selected for PCR primer design after filtering for CNVs that were consistent, unambiguous, and in the desired size range. The CGH segments were labeled according to the locus tag of the 5′ most affected gene in that segment region.

Design and testing of a PCR-based genotyping scheme for the *M. abscessus* group. Primers were designed from flanking regions upstream and downstream of four candidate differential regions (Table 2) by using the *Mycobacterium abscessus* ATCC 19977^T sequence (GenBank accession number CU458896.1) as a template. Primers at four locations, i.e., MAB_2396, MAB_2697c, MAB_4751, and MAB_4792, and the *erm*(41) locus (22, 24) were selected for a large-scale survey of clinical isolates. *In silico* PCR was performed with the *M. abscessus* ATCC 19977^T and *M. massiliense* CCUG 48898^T genomes using BLAST (27), to confirm the expected sizes of PCR products from the respective genomes (Table 3).

PCR genotyping patterns of 46 clinical isolates were compared to those of *M. abscessus* ATCC 19977^T, *M. massiliense* CCUG 48898^T, and *M. bolletii* CCUG 50184^T. A detailed description of the PCR products obtained for each of the clinical isolates, including 29 *M. abscessus*, 15 *M. massiliense*, and 2 *M. bolletii* isolates, is shown in Table S1 in the supplemental material. PCR products from almost all of the clinical isolates obtained with primers for MAB_2396 and MAB_2697c matched the patterns observed for the reference isolates. Interestingly, PCR primers targeting MAB_4751 produced either a 450-bp or 375-bp amplicon for the *M. massiliense* clinical isolates. The MAB_4751 region was found to be useful not only for the identification of *M. massiliense* but also for separation of this taxon into two distinct subgroups, subgroup I including CI510, CI71, CI72, CI73, CI76, CI1210, CI2040, and CI8182 and subgroup II including CI614, CI615, CI74, CI75, and CI122 (see Table S1 in the supplemental material). Primers targeting MAB_4792 also differentiated between the *M. abscessus* and *M. massiliense* isolates. In addition, *M. abscessus* clinical isolates were separated into two subgroups; subgroup I (CI63, CI65, CI82, CI128, CI1216, CI1219, and CI11220) showed a band at 650

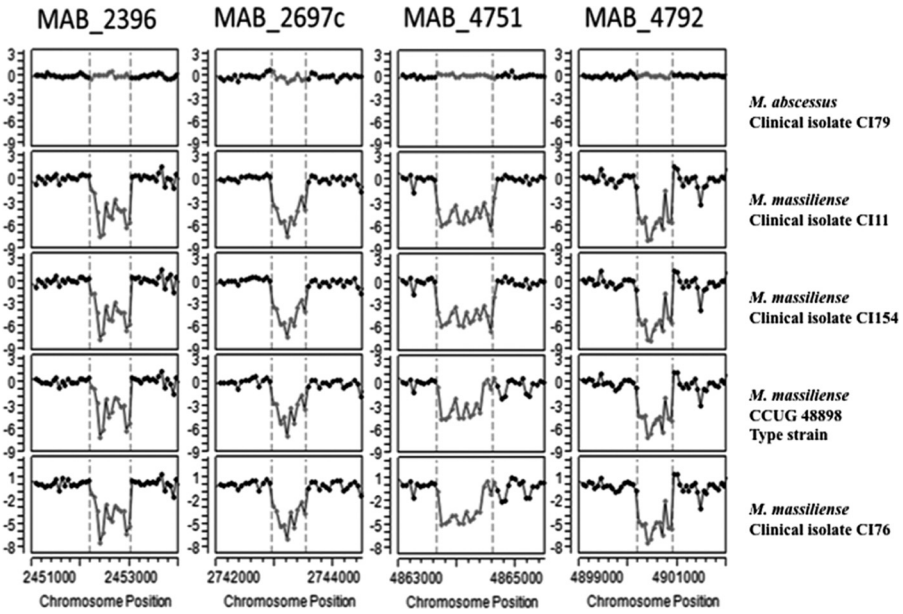

FIG 2 CGH patterns for four putative deletions that were selected as candidates to distinguish *M. massiliense* from *M. abscessus*. The normalized $\log_2$ signal ratios for individual probes in selected genomic regions are shown for *M. abscessus* and *M. massiliense* strains. Consistent log ratios (*y* axis) near 0 indicate that genomic DNA is present throughout these regions in *M. abscessus* clinical isolate CI79 and in the reference genome (*M. abscessus* type strain ATCC 19977). In contrast, the *M. massiliense* type strain CCUG 48898 and *M. massiliense* clinical isolates CI11, CI154, and CI76 show regions with negative ratios, suggesting the absence of genomic DNA at those locations.

bp, instead of the 1,200-bp product observed for the type strain and the remaining (subgroup II) clinical isolates, using primers targeting MAB_4792 (see Table S1 in the supplemental material). For *M. bolletii*, the type strain and clinical isolates CI59 and CI78 yielded PCR fragment sizes identical to those for *M. abscessus* with primer sets for MAB_2697c and MAB_4792 and fragment sizes identical to those for *M. massiliense* with the primers for MAB_2396 (see Table S1 in the supplemental material). The primers for MAB_4751 showed 500-bp and 400-bp bands for *M. bolletii* type strain and isolate CI59 and a 500-bp band for isolate CI78.

Identification of the tested clinical samples (see Table S1 in the supplemental material) was considered unambiguous when the products obtained with all four primer pairs matched the patterns expected for the taxon. Our rapid PCR scheme was unambiguous in identification of the majority of the clinical isolates tested; however, two isolates showed product sizes not expected for the taxon in one of the PCRs. *M. abscessus* isolate CI1214 showed a 750-bp band for locus MAB_2396 (similar to *M. massiliense* or *M. bolletii*), and *M. abscessus* isolate CI121 showed 500- and 400-bp products (similar to *M. bolletii*) for locus MAB_4751.

Full-length *erm*(41) gene and inducible clarithromycin resistance among *M. massiliense* isolates. A region of the *erm*(41) gene was amplified with primers that yielded a full-size product (~700 bp) for the *M. abscessus* and *M. bolletii* type strains and a shorter product (~350 bp) for the *M. massiliense* type strain, consistent with a previously described gene truncation (22, 24). Thirteen of 15 clinical isolates of *M. massiliense* showed the expected truncated *erm*(41) gene product of ~350 bp. Surprisingly, two isolates (CI2040 and CI8182) showed products with sizes similar to the *M. abscessus* full-length *erm*(41) gene product size of ~700 bp (see Table S1 in the supplemental material).

TABLE 1 Distinguishing gene segments in *M. massiliense* discovered by array CGH against the *M. abscessus* genome

M. abscessus ATCC 19977^T locus	Gene annotation	CNV in: *M. abscessus* isolate CI79	CNV in: *M. massiliense* CCUG 48898^T and isolates CI11, CI154, and CI76	Position of segment at: Start	Position of segment at: End	CGH segment length (estimated loss) (bp)
MAB_357	Conserved hypothetical protein	None	Deletion	354,601	355,621	1,020
MAB_0705	Conserved hypothetical protein	None	Deletion	706,861	707,881	1,020
MAB_1313	Probable transcriptional regulator, *AraC* family	None	Deletion	1,313,941	1,314,721	780
MAB_2396	Probable acetyltransferase	None	Deletion	2,452,201	2,452,981	780
MAB_2697c	Hypothetical protein	None	Deletion	2,742,961	2,743,501	540
MAB_4751	Conserved hypothetical protein (patatin-like)	None	Deletion	4,863,661	4,864,561	900
MAB_4792	Putative transcriptional regulator	None	Deletion	4,900,201	4,900,861	660

TABLE 2 PCR primers targeting regions that differ between *M. abscessus* and *M. massiliense* isolates

M. abscessus ATCC 19977^T locus	Gene annotation	Primers	
		Forward	Reverse
MAB_2396	Probable acetyltransferase	5′-AGGCGGCCACCGACGTCGCGATGGA-3′	5′-TGCGCCCGCCCAGCGCGTATCCG-3′
MAB_2697c	Hypothetical protein	5′-GACTCCGGTGGCCGCGGCGA-3′	5′-GCCGGAGCGCTGGGTGGGCT-3′
MAB_4751	Conserved hypothetical protein (patatin-like)	5′-CCCGCATGCAGCTGGCCGCGCA-3′	5′-GCGCCAGTGGTGGGGCCACCCGT-3′
MAB_4792	Putative transcriptional regulator	5′-GCGGTGACGACCGCGGGGGCGAT-3′	5′-TCGGGGCAGGCCAGGGCGCCTA-3′
erm(41)	Erythromycin ribosomal methylase	5′-GACCGGGGCCTTCTTCGTGAT-3′	5′-GACTTCCCCGCACCGATTCC-3′

Sequencing of the *erm*(41) PCR product from *M. massiliense* strains CI2040 and CI8182 revealed 99.8% and 98.5% similarity, respectively, to the *erm*(41) gene of *M. abscessus* M93, 98.9% and 98.1% similarity, respectively, to the *erm*(41) gene of *M. abscessus* ATCC 19977^T, and 98.7% similarity (for both strains) to the *erm*(41) gene of *M. bolletii* CCUG 50184^T. In addition, these two novel *erm*(41) genes were 98.7% identical to each other. Sequence analysis showed the presence of the nucleotide T at position 28 in the *erm*(41) gene, which has been described for *M. abscessus* strains showing inducible clarithromycin resistance (30).

Inducible resistance was assessed *in vitro* by extended incubation of the organisms with clarithromycin (22). Clarithromycin susceptibility testing for these strains (CI2040 and CI8182) showed MIC values of 2 μg/ml (susceptible) at 5 days and >16 μg/ml (resistant) after 14 days, consistent with the presence of inducible resistance seen in the *M. abscessus* ATCC 19977 type strain. Partial amplification and sequencing of the *rrl* 23S rRNA gene from these isolates showed no mutations associated with clarithromycin resistance (22).

DISCUSSION

Differentiation among *M. abscessus*, *M. massiliense*, and *M. bolletii* is challenging for clinical microbiology laboratories. Recent work proved the inaccuracy of single-target sequencing for separating these three taxa within the *M. abscessus* group (11, 12). Sequencing of several targets (such as *hsp65*, *rpoB*, and *secA1*), combined with phylogenomic analysis, has been shown to increase identification accuracy among these different taxa (13). More recently, a multilocus sequence analysis targeting 8 housekeeping genes and a multispacer sequence analysis were reported (9, 16). However, all of these methods require genomic sequencing, which is relatively costly and time-consuming and may not be available in all clinical microbiology laboratories. We describe here a simple, accurate, robust, and cost-effective PCR-based method for distinguishing *M. abscessus* from *M. massiliense* and *M. bolletii* and subtyping *M. abscessus* and *M. massiliense* isolates. Although an array-based genomic approach was initially used to determine the features differentiating *M. abscessus* from *M. massiliense* and to design the assay, the actual genotyping method is PCR-based, requiring low-cost laboratory equipment, and was designed specifically for use by clinical laboratories.

Our genotyping method was able to rapidly and accurately identify 46 clinical isolates as *M. abscessus*, *M. massiliense*, or *M. bolletii* in agreement with sequence-based identification results (13). The robustness of the method is demonstrated by the combination of product sizes observed for each taxon. For example, primers for MAB_2396 and MAB_2697c were found to be useful in differentiating *M. massiliense* (750-bp and 450-bp products, respectively) from *M. abscessus*. The presence of a 375-bp or 450-bp product with primers for MAB_4751 not only identifies *M. massiliense* but also subtypes this taxon into two groups. A combination of differentiating primers for MAB_2396, MAB_2697, and MAB_4751, yielding product sizes of 750 bp, 1,000 bp, and 500 bp or 500/400 bp, respectively, separated *M. bolletii* from the other taxa within the *M. abscessus* group. One limitation of this study is that we did not have a large number of *M. bolletii* isolates to further validate the findings for this group.

Our previous work on these isolates (13) demonstrated at least two groups within *M. massiliense*, as determined by targeted sequencing and repetitive sequence-based PCR typing. We and other groups have shown that *M. massiliense* seems to be a genomically heterogeneous entity. Our genotyping scheme separated *M. massiliense* into two subgroups according to the product size of 450 bp or 375 bp obtained with the primer set for

TABLE 3 PCR products from differential regions for *M. abscessus*, *M. massiliense*, and *M. bolletii* clinical isolates and type strains

M. abscessus locus	PCR product size (bp)							
	Predicted		Observed					
	M. abscessus ATCC 19977^T	*M. massiliense* CCUG 48898^T	*M. abscessus*		*M. massiliense*		*M. bolletii*	
			ATCC 19977^T	Clinical isolates	CCUG 48898^T	Clinical isolates	CCUG 50184^T	Clinical isolates
MAB_2396	1,486	740	~1,500 and 500	~1,500 and 500	~750	~750	~750	~750
MAB_2697c	1,049	449	~1,000	~1,000 and/or 500 faint	~450	~450	~1,000	~1,000
MAB_4751	1,338	444	~1,400 faint and 500	~1,400 faint and/or 500	~450	~450 or 375	~500 and 400	~500 and 400 or ~500
MAB_4792	1,124	605	~1,200	~1,200 or 650	~600	~600	~1,200	~1,200
erm(41)	700	350	~700	~700	~350	~350 or 700	~700	~700

MAB_4751. This size difference is visually represented in Fig. 2 as a narrower dip segment (smaller loss of genome) in *M. massiliense* type strain CCUG 48898 and isolate CI76, yielding a PCR product of 450 bp, than in *M. massiliense* CI11 and CI154, which have a wider dip segment (larger loss of genome), yielding a PCR product of 375 bp. Interestingly, these two subgroups matched the two *M. massiliense* clusters in the phylogenomic analysis derived from concatenated *hsp65*, *rpoB*, and *secA1* sequences in our previous study (13). Similarly, *M. abscessus* clinical isolates were separated into two subgroups based on the PCR product size for locus MAB_4792, which in general followed the clustering of the phylogenomic tree (13). Our rapid PCR scheme was unambiguous in the identification of the majority of the clinical isolates tested; however, two isolates showed product sizes not expected for the taxon in one of the PCRs. The *M. abscessus* isolate CI1214 showed a 750-bp band for locus MAB_2396 (similar to *M. massiliense* or *M. bolletii*). This strain was previously shown to be more divergent than most the *M. abscessus* isolates on the phylogenomic tree (13). *M. abscessus* isolate CI121 showed the 500- and 400-bp doublet products (similar to *M. bolletii*) for locus MAB_4751. These results should not be surprising, considering the requirement to sequence multiple targets for a consensus identification result using multilocus sequence analysis. They are also consistent with the observed phylogenetic trees derived from concatenated multilocus and whole-genome sequences of the *M. abscessus* group (7, 9), with some strains appearing "between" defined taxa, possibly due to horizontal gene transfer among members in the group.

Distinguishing *M. massiliense* from *M. abscessus* is clinically important because of their unique susceptibility patterns and treatment outcomes. The rates of clinical responses to therapy, including clarithromycin, are higher for patients with *M. massiliense* lung disease than for patients with *M. abscessus* lung disease (4). Clinically acquired resistance to clarithromycin in *M. abscessus* isolates occurs via point mutations to G at positions A2058 and A2059 of the *rrl* gene encoding the peptidyltransferase domain of the 23S rRNA (21) or by a drug-inducible mechanism via a functional *erm*(41) gene product (22). Partial sequencing of the *rrl* gene of the *M. massiliense* clinical isolates CI2040 and CI8182 revealed no mutations at these positions. On the other hand, sequence analysis of the *erm*(41) gene revealed a full-length gene product, which has not been previously reported for *M. massiliense*. In addition, it showed the characteristic nucleotide T (instead of C) at nucleotide position 28 previously described for *M. abscessus* with inducible clarithromycin resistance (30). Most importantly, susceptibility testing confirmed the presence of inducible clarithromycin resistance in both *M. massiliense* strains.

The *M. massiliense erm*(41) gene includes several mutations, including a large C-terminal deletion that renders it nonfunctional. Analysis of the size differences of the products obtained with *erm*(41) PCR was proposed as a simple method to differentiate *M. massiliense* from *M. abscessus* and *M. bolletii* species (23, 24). Our genotyping scheme combined with *erm*(41) PCR revealed two *M. massiliense* isolates, CI2040 and CI8182, harboring full-length *erm*(41) PCR products. We conclude that *erm*(41) PCR alone is insufficient to differentiate *M. massiliense* from *M. abscessus*. It is tempting to speculate regarding possible explanations for a full-length *erm* gene in clinical isolates of *M. massiliense*. Horizontal gene transfer of *rpoB* sequences has been shown to occur within members of the *M. abscessus* group (9), so one intriguing possibility is that the *erm*(41) gene was transferred from *M. abscessus* or *M. bolletii* into *M. massiliense*, resulting in a similar phenotype. Consistent with horizontal gene transfer is the presence of a full-length *erm*(41) gene in CI2040 and CI8182 clinical isolates which are most similar to the *erm*(41) genes of *M. abscessus* M93 and ATCC 19977^T, while *hsp65*, *rpoB*, and *secA1* sequences are most similar to those of *M. massiliense* type strain CCUG 48898. Also consistent with horizontal gene transfer is the presence of the 750-bp band for locus MAB_2396 (similar to *M. massiliense* or *M. bolletii*) for *M. abscessus* isolate CI1214 and the 500- and 400-bp products (similar to *M. bolletii*) for locus MAB_4751 in *M. abscessus* isolate CI121. While the molecular mechanisms used by these organisms to recombine foreign DNA into their genomes are not completely understood, the occurrence of different members of the *M. abscessus* group, as well as other species, coinfecting the same patient provides opportunities for direct exchange of genetic material. The use of a small set of core genes for typing cannot reveal all horizontal gene transfer events that occur, but including key determinants such as antibiotic resistance determinants within the typing scheme can help track transfer events that directly affect therapy.

Our PCR-based genotyping method using four primer pairs for the identification of *M. abscessus*, *M. massiliense*, and *M. bolletii*, combined with detection of full-length or truncated *erm*(41) for inducible clarithromycin resistance, can provide useful information for physicians. Moreover, the genotyping protocol provides initial subtyping information for *M. massiliense* and *M. abscessus* and highlights possible events of horizontal gene transfer, both of which can be further explored by genomic sequencing. The incorporation of this assay into the workflow of the mycobacteriology laboratory has led to a decrease in the need for multitarget genome sequencing for identification and typing of the *M. abscessus* group. Our protocol does not require an expensive platform such as a sequencing system or MALDI-TOF MS instrument. It is a relatively simple technique with direct visual results. The turnaround time is about 3 to 4 h from isolation of the organism. The estimated cost of PCR and running the PCR product on an agarose gel is less than $2 per isolate.

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