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Molecular Characterization of *Mycobacterium avium* Complex Isolates Giving Discordant Results in AccuProbe Tests by PCR-Restriction Enzyme Analysis, 16S rRNA Gene Sequencing, and DT1-DT6 PCR

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Based on cultural and biochemical tests, a total of 84 strains (72 clinical and 12 environmental isolates from the Caribbean Isles, Europe, and the Indian subcontinent) were identified as members of the *Mycobacterium avium* complex (MAC). They were further characterized with MAC, *M. avium,* and *M. intracellulare* probes of the AccuProbe system, and this was followed by selective amplification of DT6 and DT1 sequences. Seventy isolates gave concordant results; 63 were identified as *M. avium,* 5 were identified as *M. intracellulare,* and 24 remained untypeable by both methods. Fourteen isolates gave discrepant results, as they were DTl positive but gave negative results by the *M. intracellulare* AccuProbe test. Consequently, a detailed molecular analysis of all DT1-positive isolates (14 discrepant strains plus 5 ***M. intracellulare*** **strains) was performed by PCR-restriction analysis (PRA) of the *hsp65* gene and 16S rRNA gene sequencing. The results confirmed the reported heterogeneity of *M. intracellulare,* as only 6 of 19 isolates (32%) gave PRA results compatible with published *M. intracellulare* profiles while the rest of the isolates were grouped in four previously unpublished profiles. 16S rRNA gene sequencing showed that only 8 of 19 isolates (4270) were related to *M. intracellulare* IWGMT 90247 (EMBL accession no. X88917), the rest being related to MCRO19 (EMBL accession no. X93030) and MIWGTMR10 (EMBL accession no. X88915). In conclusion, we have characterized a significant number of MAC isolates which were not identified by the AccuProbe test, PRA, or 16S rRNA sequencing. However, all of them were identifiable by DTl-DT6 PCR (they were DT6 negative and DTl positive) and could be tentatively identified as *M. intracellufure* based on preliously published observations. It is noteworthy that the majority of such isolates (14 of 19) were from the Indian subcontinent, with 12 of 14 being environmental isolates. Our study confirms the marked heterogeneity of-V. *intrucellulare* isolates and shows the utility of in-house DTl PCR to detect this group of isolates, which would otherwise have been missed by the AccuProbe system in a routine clinical microbiology laboratory.**

The *Mycobactetium avium* complex (MAC), which includes II. Nviunz and M. intracellulare. is a major opportunistic infection in AIDS patients (22, 23). The diagnosis of MAC organisms remains lengthy due to their slow growth, and results for identification by cultural and biochemical tests may take as long as 1 month. Furthermore, additional tests are needed to further discriminate between the two MAC species and include hybridization with DNA probes (5, 13, 24, 31), PCR with spec~fic primers (4, 17, 21), amplification of conserved mycobacterial sequences followed by either hybridization with species-specific probes to variable regions within the amplified target (2, 14) or restriction enzyme analysis (33), and 16s rRNA gene sequencing (11, 16).

The use of the commercialized AccuProbe Culture Identification Test (Gen-Probe Inc., San Diego, Calif.) is particularly suitable for the clinical mycobacteriology laboratory because of its case and rapidity (12, 38). Although the sensitivity and specificity of the *M. avium* probe are reportedly high, many isolates do not react with the MAC and/or *M. intracellulare* probes (18,40,41). Therefore, there is a need for a simple and rapid method that is applicable in the routine clinical microbiology laboratory and able to identify a majority of MAC isolates. In this context, both PCR-restriction analysis (PRA) of the *hsp65* gene (33) and DT1-DT6 PCR (8, 34-36) seem particularly useful.

We recently compared the DT1-DT6 PCR method with the AccuProbe system and reported the usefulness of DT6 PCR for easily identiping *M. avium* isolates (8). In addition, we also described MAC isolates not reacting with the *M. avium* and *M. intracellulare* probes of the AccuProbe system, which could be tentatively identified as *M. intracellulare* by DT1 PCR (8). In the present study. we extend this information to various other isolates from the Caribbean Isles and the Indian subcontinent and report on their molecular characterization with various probes of the AccuProbe system and by DT1-DT6 PCR, PRA of a 439-bp region of the *hsp65* gene, and 16S rRNA gene sequencing.

MATERIALS AND METHODS

Origin of isolates. A total of 84 strains (72 clinical and 12 environmental isolates from the Caribbean Isles, Europe, and the Indian suhcontinent), which were identified as members of the MAC on the basis of cultural and biochemical tests (6), were used in this investigation. The Caribbean strains were isolated from clinical specimens at the Institut Pasteur of Guadeloupe, the Indian strains were isolated in an *M. bovis* BCG trial area in South India (15), and the European isolates were from the Institut Pasteur of Paris. All the isolates were cultured on Löwenstein-Jensen medium.

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TABLE 1. Results of molecular identification tests on Indian and Caribbean isolates[a]

Strain	Biochemical test result	AccuProhe results[b,c]			PCR result[c]		PRA result (profile)	16S rRNA result most closely related to (EMBL accession no.)[d]:
		MAC	MAV	MIN	DT6	DT1		
Gp 94025	MAC	+	-	+		+	*M. intracellulare* (D)	*M. intracellulare* (X88917)
Gp 94052	MAC	+	-	-		+	New (C)	*M. intracellulare* (X88917)
Gp 94064	MAC	+	-	-		+	New (C)	*M. intracellulare* (X88917)
Gp 94070	MAC	+	-	+		+	*M. intracellulare* (D)	*M. intracellulare* (X88917)
Gp 94145	MAC	+	-	+	-	+	*M. intracellulare* (D)	*M. intracellulare* (X88917)
In 1	MAC	+	-	-		+	New (E)	MCR019 (X93030)
In 3	MAC	+	-	-		+	New (E)	MCRO19 (X93030)
In 4	MAC					+	New (A)	MIWGTMR10 (X88915)
In 5	MAC	-	-			+	New (B)	MCRO19 (X93030)
In 6	MAC	+	-	+		+	*M. intracellulare* (D)	*M. intracellulare* (X88917)
In 7	MAC	-	-	-		+	New (B)	MCR019 (X93030)
In 8	MAC					+	New (B)	MCRO19 (X93030)
In 9	MAC					+	New (A)	MCR019 (X93030)
In 10	MAC	-	-	-		+	New (C)	MCRO19 (X93030)
In 11	MAC	-	-	-		+	New (C)	MCR019 (X93030)
In 13	MAC	+	-	-		+	*M. intracellulare* (D)	*M. intracellulare* (X88917)
In 14	MAC					+	New (C)	MCR019 (X93030)
In 15	MAC	-	-	-		+	New (B)	MCR019 (X93030)
In 18	MAC	+	-	+		+	*M. intracellulare* (D)	*M. intracellulare* (X88917)

[a] Results obtained for Caribbean clinical isolates (designations with Gp) and Indian isolates (In 6 and 18, from sputum: In1, 7, 8, 9, 10, 13, and 14, from dust: In3, 5, and 15, from water: and In4 and 11, from soil) are shown.
[b] AccuProbe tests for MAC, *M. avium* (MAV), and *M. intracellulare* (MIN) were performed.
[c] +, positive; -, negative.
[d] For further details, please refer to Fig. 4.

Preparation of genomic DNA. The bacterial DNA for DTI-DT6 PCR was prepared as recently reported (8). Bacterial DNA for PRA was prepared by a glass bead method: briefly. one loopful of bacteria was suspended in 300 µ1 of TE (10 mM Tris, 1 mM EDTA) and 100 µl of acid-washed glass beads (diameter. <106 µm: Sigma, St. Louis, Mo.), heated at 94°C for 15 min, and sonicated at 35 kHz for 15 min in a water bath sonicator (Gen-Probe Inc.). An aliquot (5 µl) of the supernatant containing the crude DNA extract was used for PCR.

PRA. Amplification was performed according to the procedure described by Telenti et al. (33) using primers Tb11 (5'-ACCAACGATGGTGTGTCCAT-3') and Tb12 (5'-CITGTCGAACCGCATACCCT-3'). which amplified a 439-bp fragment of the *hsp65* gene. followed by *Bst* EII (Promega, Madison, Wis.) and *Hae* 111 (BioLabs, Inc., Beverly, Mass.) enzyme digestions of the amplification product. After digestion, 12 µl of the restriction digest was loaded on a 4% (wt/vol) NuSieve, 3:1 agarose gel (FMC Bioproducts, Rockland, Maine) and the gel was electrophoresed in 1 x Tris-acetate-EDTA buffer until the digested fragments were well separated. PhiX-174-RF DNA *Hae* III digest (Pharmacia Biotech, Uppsala, Sweden) served as an external molecular size marker and was added to every sixth lane of migration to reduce migration-related errors. Fragments were visualized by ethidium bromide fluorescence. and the lengths were calculated by computer-assisted analysis (28).

The AccuProbe test. MAC, *M. avium,* and *M. intracellulare* probes of the AccuProbe system (Gen-Probe Inc.) were run as reported previously (8, 12, 40). The results were expressed in relative light units (RLUs) on a Leader-50 luminometer. A positive reaction was a result greater than the cut-off value of 30,000 RLUs, with a repeat range of 20.000 to 29.999 RLUs. Parallel positive controls included *M. avium* ATCC 25291 and *M. intracellulare* ATCC 13950. *M. tuberculosis* ATCC 25177 served as a negative control for all three probes used.

DTI-DT6 PCR assays. The method used for DT1-DT6 PCR assays was essentially similar to that described previously, with primers AV6 and AV7 (5'-ATGGCCGGGAGACGATCTATGCCGGCGTAC-3' and 5'-CGTTCGAT CGCAGTITGTGCAGCGCGTACA-3'. respectively) directing the amplification of a 187-bp fragment within the DT6 sequence and primers IN38 and IN41 (5'-GAACGCCCG'ITGGCTGGCCATTCACGAAGGAG-3' and 5'-GCGCA ACACGGTCGGACAGGCCTTCCTCGA-3'. respectively) directing the amplification of a 666-bp fragment within the DTI sequence (8,. 34-36). Each amplification run included a negative control sample without DNA and a positive control sample with 2 ng of *M. avium* ATCC 25291 (serotype 2) for DT6 primers and 3 ng of *M. intracellulare* ATCC 13950 (serotype 16) for the DT1 primers (8, 34-36). Half of the amplification reaction mixture was analyzed by electrophoresis on a 3% NuSieve, 3:1 agarose gel (FMC BioProducts) with the 100-bp ladder (Pharmacia Biotech) as a marker. Gels were stained with ethidium bromide and photographed on a UV transilluminator. Despite the perfect agreement shown between the results of DT1-DT6 PCR and Southern hybridization analysis with the entire DT1 and DT6 probes (8, 28, 36), we further reconfirmed all PCR-negative results by Southern hybridization (8, 28).

16S rRNA gene sequencing. The sequence of the hypervariable fragment A was determined as reported recently (16.29.42) by the dideoxynucleotide chain termination method using the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems Division, Perkin-Elmer Corp., Foster City, Calif.) using a GeneAmp PCR System 9600 (Perkin-Elmer) and a DNA Analysis System 373 Stretch (Applied Biosystems Division, Perkin-Elmer). The results obtained were entered into a computer, compared to known sequences in the GenBank database, and interpreted by using the BlastN algorithm.

RESULTS

Identification by biochemical tests, the AccuProbe system, DT1-DT6 PCR, and Southern hybridization. The results of this investigation are summarized in Table 1 and Fig. 1 to 4. All 54 isolates studied were initially identified as MAC based on their biochemical and cultural characteristics (6). Although concordant data were obtained for 70 isolates (63 *M. avium,. 5 M. intracellulare,* and 2 untypeable isolates [results not shown]), 14 isolates did not give concordant data when typed in parallel by DT1-DT6 PCR and the AccuProbe system. Surprisingly, all 14 of these isolates were DT1 positive (Fig.1) but did not react with the *M. intracellulare* AccuProbe (Table 1). For this reason, we decided to investigate in more detail all the DT1-positive

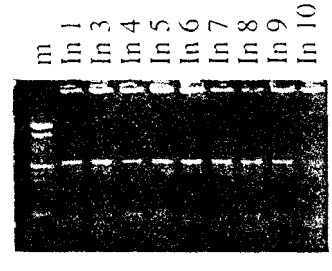

FIG. 1. Representative DTI PCR results of some of the MAC isolates from the Indian subcontinent. Bacterial DNAs were amplified with primers IN38 and IN41. Lane m, molecular weight marker.

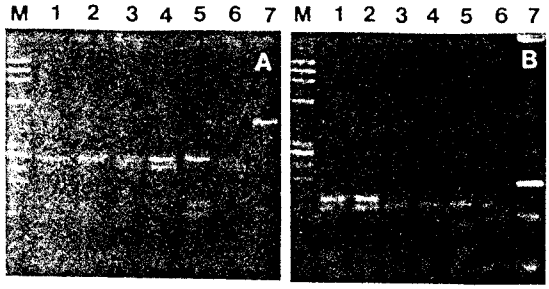

FIG 2. Representative PRA patterns of DT1 PCR-positive MAC isolates obtained upon *Bst* EII (A) and *Hae* III (B) digestion. Lane M, molecular weight markers: lane 1, *M. intracellulare* type strain ATCC 13950, representing PRA pattern D; lane 2, PRA pattern D; lane 3, PRA pattern B; lanes 4 and 6, PRA pattern C: lane 5, PRA pattern E: lane 7, PRA pattern A. Please refer to Table 1 and Fig. 3 for further details concerning the representative PRA patterns.

isolates, which included the 14 latter isolates as well as the 5 isolates identified as *M. intracellulare* by the two initial methods. As shown in Table 1, all 19 of these isolates gave negative results by both the *M. avium* AccuProbe system and DT6 PCR and were not considered identical to *M. avium* (8). Furthermore, only 10 of 19 isolates (53%) were MAC AccuProbe positive.

PRA. PRA, a recently described methodology (33), was validated on type strains of various mycobacterial species. As results similar to those reported by Telenti et al. (33) were obtained under our experimental conditions (data not shown), this methodology was applied to the 19 DT1-positive isolates shown in Table 1. Only 6 of 19 isolates (32%; 3 of 5 Caribbean strains and 3 of 14 Indian strains) presented the restriction profile described by Telenti et al. (33) for *M. intracellulare.* Profiles obtained for other isolates did not correspond to those previously described for any of the mycobacterial species studied and could be grouped into three distinct PRA profiles by *Bst* EII digestion: (i) no digestion. (ii) 245- and 220-bp fragments. and (iii) 245-, 125-, and 100-bp fragments. These groups were further subdivided into four distinct profiles by *Hae* III digestion (patterns A, B, C, and E [Fig. 2 and 3]), in addition to the one typical profile previously reported for *M. intracellulare* (pattern D [Fig. 2 and 3]).

16S rRNA gene sequencing. 16S rRNA gene sequencing of the hypervariable fragment A was performed essentially as reported earlier for mycobacteria (16, 29, 42), and the results obtained are summarized in Fig. 4. Overall, eight isolates (5 Caribbean and 3 Indian) (Table 1) were most closely related to *M. intracellulare* (EMBL accession no. X88917), a strain recently included in a cooperative study by Wayne et al. (42); 10 isolates were most closely related to MCRO19 (EMBL accession no. X93030), a strain identified as MAC by biochemical tests (29); and only a single isolate (strain In 4) was close to MIWGTMR10 (EMBL accession no. X88915), an isolate found to be closely related to *M. interjectum* (42). Therefore, it can be concluded that all the DT1-positive isolates in the present study except one showed a certain degree of genetic homogeneity upon 16S rRNA gene sequencing, constituting two major groups.

interestingly one of these groups *(M. intracellulare* X88917) was constituted of clinical isolates (except a single environmental isolate, In13) and comprised all five isolates from Guadeloupe and three of the Indian isolates (Table 1; Fig. 4). Another group was composed uniquely of Indian environmental isolates related to MCRO19 (29). When the results obtained were entered into a computer and compared to known sequences in the GenBank database, the latter group was also found to be related to isolates as diverse as the unspecified strain IWGMT 90236 (42). *M. scrofulaceum, M. simiae,* and *M. intracellulare* (results not shown). It should be noted that isolates in this group are clearly distinct from two recently described species, *M. lentiflavum* and *M. triplex,* that may resemble MAC or *M. simiae* by biochemical tests (10, 30). particularly as our isolates were nitrate and urease negative and harbored 16S rRNA gene sequences distinct from those reported previously (10. 30).

As the MCRO19 isolate was reportedly closely related to *M. scrofulaceum* and *M. simiae,* we further verified the reported absence of DT1 fragments in these two species (36) by performing DT1 PCR on various type strains and clinical isolates of *M. simiae* and *M. scrofulaceum.* Since all the isolates were devoid of the DT1 fragment (results not shown), it was taken as conclusive evidence to exclude the possibility that our DT1-positive isolates were variants of *M. scrofulaceum* and/or *M. simiae.*

DISCUSSION

Because of the scarceness of biochemical differences between *M. avium* and *M. intracellulare* (9, 19, 25, 37). additional techniques, such as high-performance liquid chromatography (3) and serotyping based on the detection of glycopeptidolipid antigens (7, 39, 40, 43), have been attempted by reference laboratories, with the aim of discriminating *M. avium* from *M. intracellulare;* however, such techniques remain cumbersome and are not easily applicable in most clinical laboratories. Furthermore, serotyping has several drawbacks such as producing inconsistent data among laboratories and inability to type all isolates, and is not an optimal method for MAC identification (11, 24, 32).

Consequently, the aim of the present investigation was to further characterize isolates initially identified as MAC on the basis of cultural and biochemical criteria by the AccuProbe tests. selective amplification of DT6 and DT1 sequences, PRA, and 16S rRNA gene sequencing. The present investigation is, therefore, a logical extension of our previous study. showing a relatively good correlation between DT6 PCR and the *M. avium* AccuProbe test (5). As a straightforward correlation between DT1 PCR and *M. intracellulare* AccuProbe could not

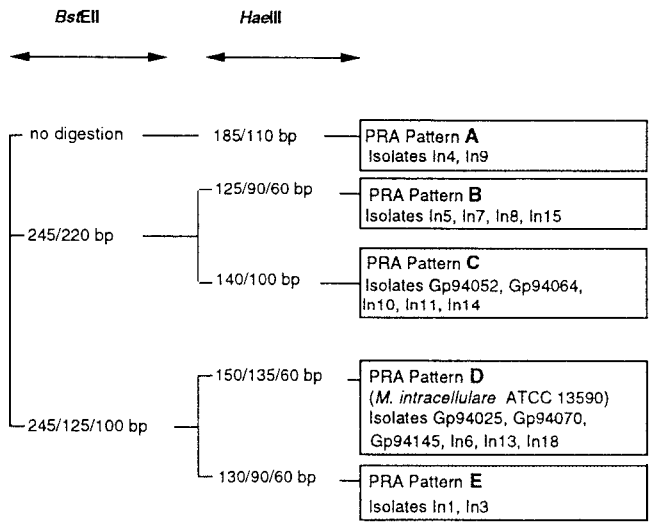

FIG. 3. Algorithm for the differentiation of DT1 PCR-positive MAC isolates from *M. intracellulare* type strain ATCC 13950 is characterized by PRA pattern D. Molecular sizes for each individual band are separated by a slash mark.

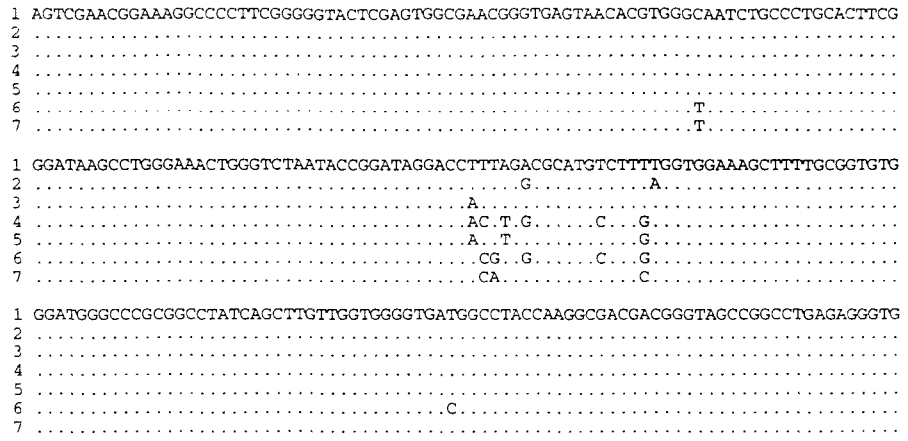

FIG. 4. Alignment of 16S rRNA gene sequences of MAC isolates within the hypervariable region A. Nucleotides different from those of *M. intracellulare* (X88917) are indicated. The first nucleotide corresponds to *Escherichia coli* 16S rRNA position 69. Sequence 1. *M. intacellulare* (X88917); sequence 2, Gp 94025, Gp 94052, Gp 94064, Gp 94070, Gp 94145, and In 6: sequence 3. In 13 and In 18: sequcncc 4, strain MCRO19 (X93030), in 1 In 3, In 5, In 7, In 8, In 10, In 11, In 14, and In 15; sequence 5, In 9, sequence 6, strain MIWGTMR10 (X88915): sequence 7, In 4.

be well established in the latter study because of the paucity of DT1- and/or *M. intracellulare* AccuProbe-positive isolates (only 4 of 69 isolates studied [8]), we further extended this study to include more DT1 PCR- and/or *M. intracellulare* AccuProbe-positive isolates by adding techniques such as PRA and 16S rRNA gene sequencing.

Since the genetic heterogeneity of MAC organisms other than the species *M. avium* is well established (1, 20, 23, 27), it was logical that our aim was not to study the heterogeneity of MAC isolates. Instead. we were interested in investigating whether the DT1 PCR-positive organisms could be included within MAC (and eventually as *M. intracellulare),* as most of them did not react with either the MAC or *M. intracellulare* AccuProbes. Such a reaction would have incited a routine clinical microbiology laboratory performing only the AccuProbe test (as is the case for the majority of labs within the United States and Western Europe) not to classify these isolates as MAC. a possibility which has both clinical and epidemiological implications.

A detailed analysis of all DT1-positive isolates (14 discrepant strains plus 5 *M. intracellulare* strains) was performed by PRA, a method which was recently reported to be useful in identifying several mycobacterial species. including *M. intracellulare* (33). However, the authors used only 12 clinical isolates of *M. intracellulare,* all of which were from Western Europe and had identical PRA profiles (33). In our study, only 6 of 19 isolates (32%) gave PRA results compatible with the previously published *M. intracellulare* profile (33), whereas the remaining isolates were grouped into four previously unpublished profiles (Table 1: Fig. 2 and 3). It is interesting that all the isolates with the published *M. intracellulare* PRA profile (33) were also simultaneously MAC and *M. intracellulare* AccuProbe positive in our study (except a single *M. intracellulare* probe-negative isolate [Table 1]). This observation may simply reflect the fact that Telenti et al. (33) selected uniquely *M. intracellulare* isolates reacting positively with the *M. intracellulare* probe of the AccuProbe system. Facts that may further contribute to the reported heterogeneity of *M. intracellulare* isolates include their geographic origin and the recently reported heterogeneity of the PRA target. Indeed, the sequencing of the 439-bp portion of *hsp65* which is the target of PRA (32) showed the highest number of alleles for *M. intracellulare,* compared to *M. scrofulaceum* and *M. avium* (13 sequences instead of 8 and 7, respectively).

Many strains cannot be included precisely in a taxonomic group by 16S rRNA gene sequencing alone. Wayne et al. stated, "there are no universally applicable criteria for deciding how many base deletions and/or substitutions in 16S rRNA sequences are sufficient to justify establishment of a new species" (42). However, based on criteria defined by Wayne et al. (42), all the isolates closely related to isolate IWGMT 90247 (EMBL accession no. X8S917) (Table 1 and Fig. 4) were MAC organisms that could be considered close to *M. intracellulare.* Indeed, this group included some isolates that reacted with the X probe of Syngene but not with the *M. intracellulare* probe of the AccuProbe system (42). Thus. when analyzed by 16S rRNA gene sequencing, only 8 of 19 isolates (425) were related to *M. intracellulare* IWGMT 90247 (EMBL accession no. X88917) in our study. The remaining 10 of 19 isolates were related to MCRO19 (a strain identified as MAC by biochemical tests [29]). and only a single isolate was close to MIWGTMR10 (an isolate that was difficult to classify and was hypothesized to be a ribovar and/or subspecies of *M. interjectum* [42]).

Thus. if a clinical microbiology laboratory aims to detect all the MAC isolates in a clinical setting. it is clear that contrary to DT1 PCR, which did not fail to detect any of the 19 isolates studied here (considered 100% detection). *M. intracellulare* AccuProbe, PRA, 16S rRNA gene sequencing. and the MAC AccuProbe would detect only 26, 32, 42, and 53% of the isolates, respectively. In conclusion, we have characterized a significant number of MAC isolates which were identified by neither the AccuProbe test, PRA, nor 16S rRNA gene sequencing but were grouped together by DT1-DT6 PCR (they were DT6 negative and DT1 positive) and tentatively identified as *M. intracellulare* based on previously published observations (8, 28, 34-36). It is noteworthy that the majority of such isolates (14 of 19) were from the Indian subcontinent, with 12 of 14 being environmental isolates. Our results therefore confirm the marked heterogeneity of MAC isolates related to *M. intracellulare* (36) and show the utility of in-house DT1 PCR to detect this group of isolates, which would otherwise have been missed by the AccuProbe system in a routine clinical microbiology laboratory.

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