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 DT1 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compatible with published *M. intracellulare* profiles while the rest of the isolates were grouped in four previously unpublished profiles. 16S rRNA gene sequencing showed that only 8 of 19 isolates (42%) were related to *M. intracellulare* (8). In addition, we also described MAC isolates not reacting with the AccuProbe test, PRA, or 16S rRNA sequencing. However, all of them were identifiable by DT1-DT6 PCR (they were DT6 negative and DT1 positive) and could be tentatively identified as *M. intracellular* based on previously 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. intracellulare* isolates and shows the utility of in-house DTI PCR to detect this group of isolates, which would otherwise have been missed by the AccuProbe system in a routine clinical microbiology laboratory.

The *Mycobacterium avium* complex (MAC), which includes II. Nviuzn 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 specific-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 identifying *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 subcontinent), 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 de Paris. All the isolates were cultured on Lowenstein-Jensen medium.
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 µl of TE (10 mM Tris, 1 mM EDTA) and 100 µl of acid-washed glass beads (diameter, <106 µm: Sigma, St. Louis, Mo.) was added to every sixth lane of migration to reduce migration-related errors. Fragments were well separated. PhiX-174-RF DNA (Promega, Madison, Wis.) and 100 µl of acid-washed glass beads (diameter, <106 µm: Sigma, St. Louis, Mo.) were run as reported previously (8, 12, 40). The Agarose gel was electrophoresed in 1 x Tris-acetate-EDTA buffer until the digested fragments were well separated. The 16S rRNA gene. followed by Bst EII (Promega, Madison, Wis.) and Hae III (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. The 16S rRNA gene sequence 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 8900 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, DTI-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 DTI-DT6 PCR and the AccuProbe system. Surprisingly, all 14 of these isolates were DTI 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 DTI-positive isolates.

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.
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* EI digestion: (i) no digestion. (ii) 245- and 220-bp fragments, and (iii) 225-, 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 MIWGTM910 (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 WGMT 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
be well established in the latter study because of the paucity of
MAC isolates. Instead, we were interested in investigating
how many base deletions and/or substitutions in 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 Accu-
Probe test (as is the case for the majority of labs within the
United States and Western Europe) not to classify these iso-
lates as MAC. A possibility which has both clinical and epide-
miological implications.

A detailed analysis of all DT1-positive isolates (14 discrep-
ant 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. intracel-
 lulare (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 previ-
uously published M. intracellulare profile (33), whereas the re-
mainng isolates were grouped into four previously unpub-
lished 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 Accu-
Probe 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. in-
tracellulare isolates reacting positively with the M. intracel-
 lulare 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 re-
ported heterogeneity of the PRA target. Indeed, the sequenc-
ing 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 spe-
cies” (42). However, based on criteria defined by Wayne et al.
(42), all the isolates closely related to isolate IWGMT 90247
(EMBL accession no. X88917) 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 (42%) were related to M.
intracellulare IWGMT 90247 (EMBL accession no. X88917)
in our study. The remaining 10 of 19 isolates were related to
MCR019 (a strain identified as MAC by biochemical tests
[29]), and only a single isolate was close to MIWGMTMR10 (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 iso-
lates, respectively. In conclusion, we have characterized a sig-
nificant 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 iso-
lates (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. intra-
cellulare (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 microbi-
ology laboratory.
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