Mycobacterium colombiense sp. nov., a novel member of the Mycobacterium avium complex and description of MAC-X as a new ITS genetic variant

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Forty-five mycobacterial strains isolated from 23 Colombian HIV-positive patients were identified as members of the Mycobacterium avium complex (MAC) and were characterized using different molecular approaches. Seven of the isolates showed characteristic features that allowed them to be differentiated from other members of the complex. The isolates had a novel 16S–23S rRNA internal transcribed spacer (ITS 1) gene sequence which is described as a new sequevar, MAC-X. All of the seven novel isolates gave a positive result with the MAC-specific AccuProbe (Gen-Probe), but tested negative for Mycobacterium avium and Mycobacterium intracellulare species-specific probes (64 and 100 % of the isolates, respectively). The novel isolates could be differentiated phenotypically from other members of the MAC on the basis of the production of urease and by a consistent mycolic acid pattern. The novel isolates shared some characteristics with M. avium, such as the avium variant I (av-I) pattern of the \textit{hsp}65 gene as determined by PCR restriction analysis and a positive PCR result for the \textit{mig} (macrophage-induced) gene. However, the novel isolates showed a unique 16S rRNA gene sequence. DNA–DNA relatedness values, from 24 to 44 %, confirmed the distinction of the novel isolates from other members of the MAC at the genetic level and their status as members of a separate species. The novel isolates are proposed as representatives of a novel species, \textit{Mycobacterium colombiense} sp. nov., that is closely related to \textit{M. avium} within the MAC. The type strain is 10B\textsuperscript{T} (= CIP 108962\textsuperscript{T} = CECT 3035\textsuperscript{T}).

Members of the \textit{Mycobacterium avium} complex (MAC) are currently identified on the basis of positive results with the commercial MAC-specific probe, AccuProbe (Gen-Probe). Originally, two species were identified within the MAC, namely \textit{Mycobacterium avium} and \textit{Mycobacterium intracellulare}. However, recent studies have drawn attention to the wide diversity of isolates that can be detected in the complex (Mijs et al., 2002; Smole et al., 2002; Lebrun et al., 2005). Analysis of the 16S–23S rRNA internal transcribed spacer (ITS 1) sequence, a molecular approach frequently used for the identification of these bacteria, has revealed the existence of more than 30 different sequevars (Tortoli et al., 2004). Only a few of these sequevars belong to members of \textit{M. avium} (seven sequevars) or \textit{M. intracellulare} (four sequevars). Recently, members of the MAC represented by sequevar MAC-A were described as the novel species \textit{Mycobacterium chimaera}. This novel species has similar characteristics to \textit{M. intracellulare} (Tortoli et al., 2004).

During a study to characterize MAC isolates from HIV-positive Colombian patients, a group of distinct strains was identified. These isolates showed several features that allowed them to be differentiated from other members of the MAC and recognized as a separate novel species.
Seven bacterial strains were isolated from four HIV-positive patients in 1995 (four strains) and 2000 (three strains). One strain was isolated from sputum and the remaining six were isolated from blood samples. Patients were hospitalized in three hospitals in Bogota, Colombia (Table 1). All patients died as a consequence of their underlying immunocompromised clinical status.

The seven bacterial isolates tested positive with the commercial MAC-specific probe and tested negative with the \textit{M. intracellulare} species-specific probe. Four of the isolates also gave a negative result when tested with the \textit{M. avium} species-specific probe (AccuProbe; Gen-Probe) (Table 1). The isolates were also investigated by using the INNO LiPA probe assay (Innogenetics) and were identified as belonging to the \textit{Mycobacterium avium–intracellulare–scrofulaceum} (MAIS) group, but different from \textit{M. avium}, \textit{M. intracellulare} and \textit{Mycobacterium scrofulaceum}.

**Phenotypic identification**

Samples were isolated using a biphasic medium. Ogawa Kudoh agar supplemented with iron citrate was used as the solid phase and Sauton Tween-albumin modified medium was used as the liquid phase. The novel mycobacterial isolates were initially considered to be non-pigmented and slow-growing, but some colonies developed some pigmentation with age. The isolates were unable to grow on MacConkey agar, Sauton agar supplemented with picric acid or Löwenstein–Jensen agar supplemented with NaCl (5%). A set of biochemical tests was performed (Kent & Kubica, 1985) and some variability in results was found when the seven isolates were compared. All isolates were positive for urease activity which was the main biochemical characteristic that enabled the novel strains to be distinguished from other members of the complex (Table 1).

HPLC analysis was performed by employing a standard method (CDC, 1996) on a System Gold instrument (Beckman) equipped with an XL Ultrasphere column (Beckman). The chromatographic patterns were visually compared with the HPLC library (available at http://www.mycobactoscana.it/page4.htm) and, in particular, with the profiles of species belonging to the MAC. Analysis of mycolic acids revealed the three-clustered pattern typical of strains belonging to the species \textit{M. avium}, \textit{M. intracellulare} and \textit{M. chimaera}. In the novel isolates, however, the peaks of the first and second clusters consistently appeared in increasing peak height, which differed from the bell-shaped appearance that is characteristic of other members of the MAC (Fig. 1).

These results, together with the results of the commercial probe tests, did not enable the novel isolates to be identified any further than their recognition as members of the MAC.

### Table 1. Comparison of characteristics of \textit{Mycobacterium colombiense} sp. nov. and other members of the MAC

All isolates were obtained from HIV-positive patients. Patient A came from Hospital Simon Bolivar, patients B and C were from Hospital San Juan de Dios and patient 57B was from the Instituto de Seguro Social. All of these institutions are located in Bogota, Colombia. All strains were able to grow at 37°C and gave a positive result in the MAC AccuProbe test. All strains were negative in tests for niacin production, acid phosphatase activity and Tween hydrolysis. INNO LiPA, Line probe assay; MAIS, \textit{Mycobacterium avium–intracellulare–scrofulaceum}; +, positive; −, negative; +/−, intermediate; UN, unknown.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>\textit{Mycobacterium colombiense} sp. nov. strains</th>
<th>\textit{M. avium}</th>
<th>\textit{M. intracellulare}</th>
<th>\textit{M. chimaera}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient Source</td>
<td>6B 7B 9B 10B T 16B 19B 57B</td>
<td>A A B B C B D</td>
<td>M. avium</td>
<td>M. intracellulare</td>
</tr>
<tr>
<td>INNO LiPA</td>
<td>MAIS MAIS Blood Blood Blood Blood Blood Sputum</td>
<td>M. avium</td>
<td>M. intracellulare i</td>
<td>M. intracellulare ii</td>
</tr>
<tr>
<td>AccuProbe test:</td>
<td>\textit{M. avium}</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>\textit{M. intracellulare}</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Catalase activity:</td>
<td>Room temperature</td>
<td>+ + + + + + + + +</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>68 °C</td>
<td>+ + + + + − + − +</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Urease</td>
<td>+ + + + + + + +</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Aryl sulphatase activity:</td>
<td>Room temperature</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>3 days</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+/−</td>
</tr>
<tr>
<td>14 days</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Growth temperature:</td>
<td>Room temperature</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>45 °C</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>
Genotypic characterization

Several molecular methods were used to characterize the novel strains. Bacterial DNA was purified from liquid cultures as described previously (van Soolingen et al., 1993).

PCR restriction analysis (PRA) and sequencing of the 65 kDa heat-shock protein gene. PRA of the hsp65 gene (Telenti et al., 1993) is a frequently used method for the identification of mycobacteria because it is easy and rapid. Many PRA patterns are already available for recognized members of the genus (Wong et al., 2003; Leao et al., 2005). All of the novel isolates displayed the M. avium variant I (av-I) hsp65 gene restriction pattern (Smole et al., 2002) after digestion with HaeIII and BstEII restriction enzymes. The amplified products were sequenced for a more complete characterization (model 3730 automated sequencer; Applied Biosystems). A BLAST comparison (at the NCBI database) of the sequence showed up to five single mutations when compared with the av-I hsp65 gene reference sequence (GenBank accession number AF126031). In contrast, the sequence displayed 100% similarity with that of the strain recently deposited as MAIS-28 by Lebrun et al. (2005). An alignment of the hsp65 sequences examined is available as Supplementary Fig. 1 in IJSEM Online.

PCR detection of selected targets. Several PCR amplifications were performed to detect other molecular targets previously described as being characteristic of M. avium such as insertion sequences IS1245 (Guerrero et al., 1995) and IS1311 (Roiz et al., 1995) and the macrophage-induced gene (mig Beggs et al., 2000). Two isolates, 9B and 10B1, tested negative for both insertion sequences while the other novel strains were weakly positive for one or both IS elements (data not shown). Possible cross-hybridization with an IS1245/1311-related insertion sequence in these isolates could not be discounted (Keller et al., 2002).

The mig gene has been identified as encoding an acyl-CoA synthetase, a central enzyme in the metabolic pathway of bacteria (Morsczeck et al., 2001). All of the novel isolates showed positive results for this target by PCR (data not shown) and confirmation was obtained by sequencing of the amplified products. The resulting sequences revealed the amplification of a gene encoding an acyl-CoA synthetase that was most closely related to that of M. avium (Beggs et al., 2000; GenBank accession number U43598) with 85.5% identity at the amino acid level (data not shown).

Gene sequencing. The 16S rRNA gene (Kirschner et al., 1993) and the 16S–23S ITS 1 spacer (Novi et al., 2000) were amplified and sequenced by using an automated sequencing system (model 3730; Applied Biosystems). The nucleotide sequences were compared with those in GenBank. The complete ITS 1 and 16S rRNA gene sequences were aligned with other known related mycobacterial sequences using CLUSTAL X version 1.81 software. Two separate phylogenetic trees were constructed for the ITS 1 and 16S rRNA gene sequences, using Mycobacterium tuberculosis and Mycobacterium fortuitum, respectively, as outgroups. Tree topologies were generated by using the neighbour-joining method with the Kimura two-parameter model and bootstrap analysis with 1000 replications (Figs 2 and 3).

The novel isolates displayed a new ITS 1 sequence, named MAC-X, that differed from all other previously described sequences within the complex and that was more closely related to MAC-R (Fig. 2).

A BLAST analysis of the ITS 1 sequence showed 100% nucleotide similarity with the MAC strain MAIS-28 (Lebrun et al., 2005). Strain MAIS-28 also shared some other genomic characteristics with the novel isolates such as the M. avium PRA pattern and the MAIS result by the INNO LiPA probe assay (Lebrun et al., 2005).

The 16S rRNA gene sequence is considered the gold standard molecular methodology for the identification of mycobacteria (Tortoli, 2003; Clarridge, 2004; Devulder et al., 2005). The sequence of the entire 16S rRNA gene was determined for all of the novel isolates. All of the isolates possessed identical 16S rRNA gene sequences. Comparisons of this sequence with other 16S rRNA gene sequences in GenBank indicated that it was more closely related to the sequence of M. avium (Fig. 3). The 16S rRNA gene sequence of the novel isolates was found to be identical to another previously submitted sequence (Mycobacterium sp. HSC 1852; GenBank accession number AY184225). This mycobacterial strain, isolated from a human lymphadenitis, differs from the novel isolates as it gives a negative result with the MAC AccuProbe (Pauls et al., 2003) (Table 1).

Strains with similar ITS 1 and 16S rRNA gene sequences have been isolated from blood samples taken from patients...
in the USA and Thailand (Turenne et al., 2006). This could indicate a putative wider distribution of the novel isolates. Further studies are required to ascertain whether strains MAIS-28 (Lebrun et al., 2005), HSC 1852 (Pauls et al., 2003) and other isolates (Turenne et al., 2006) are also members of our proposed novel species.

**DNA–DNA relatedness**

Global genomic relatedness, as determined by DNA–DNA hybridization, still remains the reference methodology to confirm bacterial distinction from other closely related species (Stackebrandt et al., 2002; Zeigler, 2003). Experiments were performed to analyse the total genomic relationships of the novel isolates with other members of the MAC. DNA–DNA hybridization experiments were performed on membrane filters by using a dot-blot-based procedure as described previously (Jimenez et al., 2004). Genomic DNA from strain 10BT (considered as the type-strain of the proposed novel species) was used as the reference DNA. The results are summarized in Table 2. Levels of DNA–DNA hybridization between the novel isolates and strain 10B$^\text{T}$ were greater than 75%, indicating that they are all members of the same single species. The level of DNA–DNA hybridization between strain 10B$^\text{T}$ and other members of the MAC varied from around 40% with *M. avium* strains to lower values with *M. intracellulare* (24%) and *M. chimaera* (28%). DNA–DNA hybridization with *M. fortuitum* ATCC 6841$^\text{T}$, a rapidly growing mycobacterium, was as low as 12%. These data indicate that our isolates can be distinguished as a separate novel species within the *M. avium* complex, being most closely related to *M. avium*.

Finally, the DNA–DNA relatedness value between strain 10B$^\text{T}$ and strain 57B was at the limit for species differentiation (74%, Table 2). This result could indicate some internal variability within this novel species.

**Genetic typing**

The internal variability of the isolates under study was investigated by molecular typing methods. Some of the isolates showed weakly positive bands by PCR for the two insertion sequences IS1245 and IS1311. None of the isolates showed RFLP patterns under standard conditions and with both IS1245 (van Soolingen et al., 1998) and IS1311 (Roiz et al., 1995) as probes. We were therefore unable to type the isolates by this procedure. To differentiate the strains, randomly amplified polymorphic DNA (RAPD) analysis was performed according to a previously described protocol.
and oligonucleotides INS2 and M13 (Welsh & McClelland, 1990; Linton et al., 1994) were used as primers. Both oligonucleotides revealed similar patterns’ distribution. Up to four different patterns were identified using INS2 as a primer (see Supplementary Fig. S2 in IJSEM Online). As expected, some correlation emerged between the patterns obtained and patient/hospital origin of the strains.

Description of Mycobacterium colombiense sp. nov.

Mycobacterium colombiense (co.lom.bi’en.se. N.L. neut. adj. colombiense pertaining to Colombia, the South American country where the strains were first isolated).

Cells are acid-fast non-motile rods. Visible growth with non-pigmented rough colonies appears in 3 weeks. Growth occurs at 20 and 37 °C. Grows on Löwenstein-Jensen, Ogawa Kudoh and Sauton agars, but does not grow on MacConkey agar. Negative in tests for niacin production, acid phosphatase activity and Tween hydrolysis. Positive for catalase activity at room temperature (approx. 20 °C). Can be differentiated from the other three species within the MAC by the ability to produce urease. The HPLC pattern, although similar to that of M. avium, M. intracellulare and M. chimaera, allows the strains to be identified on the basis of the relative height of the peaks. Results of DNA analysis, such as the unique ITS 1 sequence (MAC-X) and the unique 16S rRNA gene sequence, allow the strains to be differentiated from other members of the MAC. The hsp65 gene PRA pattern is identical to that of M. avium variant I (av-I).

DNA–DNA relatedness clearly differentiates M. colombiense as a separate species within the MAC.

(Zhang et al., 1997) and oligonucleotides INS2 and M13 (Welsh & McClelland, 1990; Linton et al., 1994) were used as primers. Both oligonucleotides revealed similar patterns’ distribution. Up to four different patterns were identified using INS2 as a primer (see Supplementary Fig. S2 in IJSEM Online). As expected, some correlation emerged between the patterns obtained and patient/hospital origin of the strains.

**Table 2. DNA–DNA hybridization between M. colombiense sp. nov. strain 10B and other members of the MAC**

Mean values ± standard deviation from two separate experiments.

<table>
<thead>
<tr>
<th>Strains</th>
<th>DNA–DNA relatedness (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. colombiense 10B T</td>
<td>100</td>
</tr>
<tr>
<td>M. colombiense 6B</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>M. colombiense 7B</td>
<td>118 ± 12</td>
</tr>
<tr>
<td>M. colombiense 9B</td>
<td>81 ± 12</td>
</tr>
<tr>
<td>M. colombiense 16B</td>
<td>82 ± 13</td>
</tr>
<tr>
<td>M. colombiense 19B</td>
<td>107 ± 25</td>
</tr>
<tr>
<td>M. colombiense 57B</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>M. avium ATCC 25291 T</td>
<td>44 ± 6</td>
</tr>
<tr>
<td>M. intracellulare (clinical isolate)</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>M. chimaera FI 01069 T</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>M. fortuitum ATCC 6841 T</td>
<td>12 ± 2 ± 5</td>
</tr>
<tr>
<td>Calf thymus DNA</td>
<td>4 ± 2 ± 0 ± 5</td>
</tr>
</tbody>
</table>
The type-strain, isolate 10B (\(=\) CIP 108962\(^T\) = CECT 3035\(^T\)), was isolated from the blood of an HIV-positive patient in Colombia, South America.

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**References**


