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## Short Communication

# Molecular characterization of nontuberculous mycobacteria isolated from human cases of disseminated disease in the USA, Thailand, Malawi, and Tanzania

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The genus *Mycobacterium* includes more than 130 validated species and subspecies. For accurate identification of NTMs, sequence-based methods are replacing traditional techniques. In this study, 122 clinically significant isolates previously characterized as NTMs by a combination of AccuProbe (GenProbe, Inc.) HPLC, and conventional methods were subjected to 16S rRNA gene and ITS1 sequence analyses. Originally identified as *Mycobacterium avium* complex (MAC) ($n$=107), *M. kansasii* ($n$=2) or another NTM species ($n$=13), this collection is revealed by sequence-based analyses to contain rare and novel species that were previously unrecognized.

All strains were isolated from the blood of patients with disseminated disease in the USA (Crump et al., 2003), Thailand (Archibald et al., 1999), Malawi (Bell et al., 2001) or Tanzania (Archibald et al., 1998) (Table 1). Sample preparation, PCR, DNA sequencing, as well as editing of the 16S rRNA gene and ITS1 sequences have been described previously (Turenne et al., 2001, 2002). Sequence comparisons of both targets were performed using the RIDOM database (Harmsen et al., 2002), in-house data (Turenne et al., 2001) and GenBank sequences at NCBI. Phylogenetic analyses were performed in MEGA 3.1 using the Neighbor-Joining method (Kumar et al., 2004). New ITS1 sequences were deposited in GenBank under accession No. AY701784, AY701785 and AY701786.

16S rRNA gene sequence analysis of the 15 MAC Accuprobe-negative isolates confirmed the original findings that 2 were *M. kansasii* strains. Further testing indicated that they were ITS1 type I, the predominant sequevar among clinical isolates (Alcaide et al., 1997). The remaining isolates included 8 with 100% 16S rRNA gene sequence identity to established type strains including *M. simiae* ($n$=5), *M. abscessus* ($n$=1), and *M. parascrofulaceum* ($n$=2). The five remaining strains did not exhibit sequence identity to established type strains. Blood stream infections of *M. simiae* and *M. abscessus* have been documented previously. *M. parascrofulaceum* is a recently validated species related to *M. simiae* (Turenne et al., 2004).

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Abbreviations: ATCC, American Type Culture Collection; ITS1, 16S-23S internal transcribed spacer 1; NCBI, National Center for Biotechnology Information; NTM, nontuberculous mycobacteria.

Table 1. Sequence-based results and geographical representation of all NTM isolates.

| Original identification | 16S rRNA gene (A) or ITS1 (B) sequence | USA | Thailand | Malawi | Tanzania |
|---|---|---|---|---|---|
| A) MAC Accuprobe negative isolates: | | | | | |
| *M. simiae* (*n*=2); *M. simiae-avium* (*n*=3) | *M. simiae* (T) | — | 3 | 2 | — |
| *M. kansasii* | *M. kansasii*[a] (T) | 2 | — | — | — |
| *M. scrofulaceum* | *M. parascrofulaceum* (T) | — | 2 | — | — |
| *M. abscessus* | *M. abscessus* (T) | 1 | — | — | — |
| *M. simiae* | "*M. sherrisii*" (T) | — | 1 | — | — |
| *M. mucogenicum* | *M. mucogenicum* N248 (AY215289)[b] | 2 | — | — | — |
| *M. chelonae* | *M. chelonae* (var. *niacinogenes*) ATCC 19237[b] | 1 | — | — | — |
| *M. fortuitum* | *M. fortuitum* S358[b] | 1 | — | — | — |
| Subtotal: | | 7 | 6 | 2 | — |
| B) MAC Accuprobe positive isolates: | | | | | |
| Mav-A | | 18 (22%)[c] | 9 (39%) | — | — |
| Mav-B | | 56 (68%) | 10 (43%) | 1 | 1 |
| Mav-F | | 1 | 3 | — | — |
| Mav-H[d] | | 1 | — | — | — |
| Min-A | | 4 | — | — | — |
| MAC-E[e] | | 1 | — | — | — |
| MAC-V[f] | | — | 1 | — | — |
| MAC-W[f] | | 1 | — | — | — |
| Subtotal: | | 82 | 23 | 1 | 1 |
| Total: | | 89 | 29 | 3 | 1 |

A) Results of 16S rRNA gene sequencing for 15 MAC Accuprobe negative isolates, which show 100% sequence identity with the type strain of an established species (T) or with a strain not representative of a type strain (strain is indicated, refer to text for explanation). B) ITS1 sequevars of the MAC isolates.

[a] Final identification as *M. kansasii* was based on photochromogenicity and an ITS1 analysis.

[b] Does not correspond to the type strain of the species indicated; candidate for new species or subspecies. Refer to text.

[c] Percent shown is for the country only.

[d] Previously unpublished sequevar, 16S rRNA gene sequence corresponds to *M. avium*.

[e] 16S rRNA gene sequence reveals a unique sequence. Refer to text.

[f] Previously unpublished sequevar, 16S rRNA gene sequence corresponds to *M. colombiense*.

Most commonly documented as respiratory tract isolates, these isolates are identified as the clinical isolates from blood for only the second time in this report (Tortoli et al., 2005). Although readily identified by our sequencing-based methods, the *M. parascrofulaceum* isolates were misclassified as *M. scrofulaceum* by conventional techniques. Similarly, another isolate originally designated as *M. simiae* was correctly identified by sequencing as "*M. sherrisii*" (Selvarangan et al., 2004), a species not previously associated with disseminated disease. Sequences of two other isolates were identical to strain *M. mucogenicum* N248 from GenBank (AY215289). Although the same species designation was indicated by conventional methods,

the 16S rRNA gene sequences differ from the *M. mucogenicum* ATCC 49650$^T$ type strain by 5 bp and the biochemically atypical strain *M. mucogenicum* ATCC 49649 (Springer et al., 1995) by 1 bp. Closer examination may reveal that these isolates merit distinct subspecies or species designation. The two remaining isolates may also represent novel species or subspecies. One was designated "*M. chelonae* chemovar *niacinogenes*" based on its 100% sequence identity with strain ATCC 19237. The ATCC strain was deposited as *Mycobacterium borstelense* subsp. *niacinogenes* but is also known as *Mycobacterium chelonae* Bergey et al. chemovar *niacinogenes*. The 16S rRNA gene sequence varies from the type strain of *M. chelonae* by 3 bases and from *M. abscessus* by 1 base. The 16S rRNA gene sequence of our final NTM isolate corresponds to that of *M. fortuitum* S358 in RIDOM, but differs from the type strain of *M. fortuitum* by 9 bases. It is more similar to *M. neworleansense* ATCC 49404$^T$, exhibiting a single bp difference.

The isolates identified as MAC by AccuProbe were further characterized by ITS1 sequencing. To date, 31 MAC ITS1 sequevars have been documented: MavA–G for *M. avium*, MinA–D for *M. intracellulare*, and MAC A–U for isolates not assigned to either species and therefore candidates for new species (De Smet et al.,

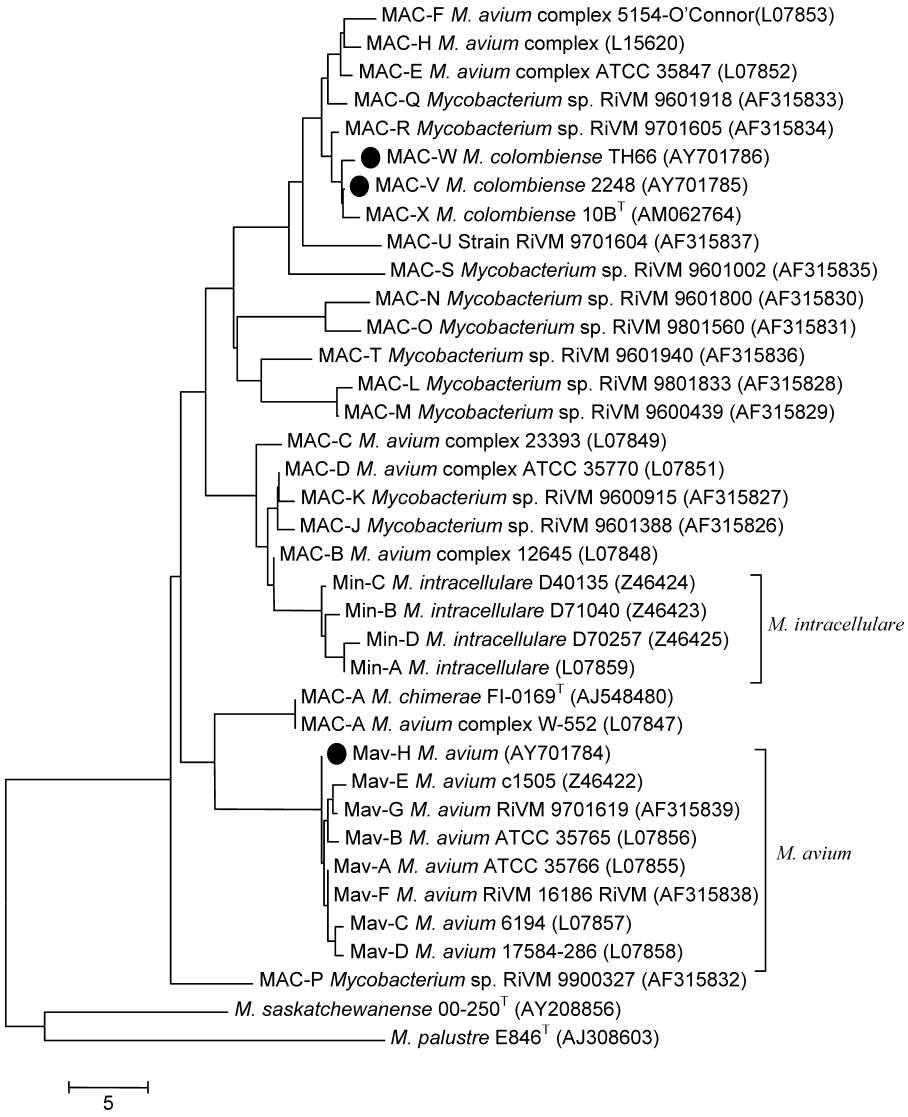


Fig. 1. Phylogenetic representation of MAC ITS1 sequevars to date, including the 3 new sequevars identified in this study (indicated by a black circle), as well as the 4 established MAC Accuprobe positive species: *M. chimaera* and *M. colombiense* (belonging to the MAC); *M. palustre* and *M. saskatchewanense* (not belonging to the MAC).

GenBank accession numbers are indicated in parentheses. The bar represents the number of nucleotide variation.

1995; Frothingham and Wilson, 1993; Mijs et al., 2002). Three MAC sequevars have since been assigned to independent species: MAC-A in *M. chimaera* (Tortoli et al., 2004), MAC-G in *M. parascrofulaceum* (Turenne et al., 2004), and MAC-X in the newly described *M. colombiense* (Murcia et al., 2006). Of our 107 isolates, the majority corresponded to the established *M. avium* sequevars: Mav-B ($n=68$; 64%), Mav–A ($n=27$; 25%); and Mav-F ($n=4$; 3%). Four isolates were Min-A, identical to the type strain of *M. intracellulare.* The remaining 4 isolates included one identified as MAC-E, and three novel sequevars. By 16S rRNA, the MAC-E isolate revealed an identical match with a strain in RIDOM identified as *M. intracellulare* ATCC 35847, which does not represent the type strain. In fact, this strain reveals 5 base variations from the type strain of *M. intracellulare* and likely represents a new species within the MAC. Of the novel ITS1 sequevars, one isolates had a 16S rRNA gene sequence 100% identical to *M. avium* ATCC 25291$^T$. Its ITS1 sequence exhibited a single base deletion relative to Mav-A, was designated Mav-H, and is considered a new sequevar within the *M. avium* species. The remaining pair of isolates have novel ITS1 sequences that differ by only 1 bp and have been designated MAC-V and MAC-W. As depicted in Fig. 1, they cluster with sequevars MAC-R and MAC-X. By 16S rRNA gene sequencing, these isolates were identical to each other as well as *M. colombiense* (i.e. MAC-X). This data indicates that MAC-V and MAC-W are sequevars of *M. colombiense*. The ITS1 differences may reflect the geographic diversity of these isolates. Although all are blood isolates, the MAC-V strain is from Thailand, MAC-W from the US, and MAC-X from South America. The RIDOM database contains an additional member of this group: although deposited as *M. intracellulare* S350, the 16S rRNA gene is identical to *M. colombiense* while the ITS1 clusters with MAC-V and MAC-X.

This study reaffirms the fact that, within the genus *Mycobacterium*, both the number of species and intraspecies diversity, have been highly underestimated by traditional identification methods. By applying sequence-based methods, we corrected the species designation of seven isolates, and identified three novel MAC ITS1 sequevars. Our analyses suggest that several strains are good candidates for new species. We also report a first case of blood stream infection caused by "*M. sherrisii*." All were deemed clinically significant on the basis that they were isolated from blood. This study emphasizes the advantage of using sequence-based identification to recognize new or rare species of mycobacteria that can be involved in pathogenesis which may otherwise go unnoticed using conventional methods.

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