Mycobacterium arupense, Mycobacterium heraklionense, and a newly proposed species, “Mycobacterium virginiense” sp. nov., but not Mycobacterium nonchromogenicum, as species of the Mycobacterium terrae complex causing tenosynovitis and osteomyelitis

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**Recommended Citation**  
Vasireddy, Ravikiran; Vasireddy, Sruthi; Brown-Elliott, Barbara A.; Wengenack, Nancy L.; Eke, Uzoamaka A.; Benwill, Jeana L.; Turenne, Christine; and Wallace, Richard J. Jr., “Mycobacterium arupense, Mycobacterium heraklionense, and a newly proposed species, “Mycobacterium virginiense” sp. nov., but not Mycobacterium nonchromogenicum, as species of the Mycobacterium terrae complex causing tenosynovitis and osteomyelitis.” Journal of Clinical Microbiology.54,5. 1340-1351. (2016).  
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Mycobacterium arupense, Mycobacterium heraklionense, and a Newly Proposed Species, “Mycobacterium virginiense” sp. nov., but Not Mycobacterium nonchromogenicum, as Species of the Mycobacterium terrae Complex Causing Tenosynovitis and Osteomyelitis


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Mycobacterium terrae complex has been recognized as a cause of tenosynovitis, with M. terrae and Mycobacterium nonchromogenicum reported as the primary etiologic pathogens. The molecular taxonomy of the M. terrae complex causing tenosynovitis has not been established despite approximately 50 previously reported cases. We evaluated 26 isolates of the M. terrae complex associated with tenosynovitis or osteomyelitis recovered between 1984 and 2014 from 13 states, including 5 isolates reported in 1991 as M. nonchromogenicum by nonmolecular methods. The isolates belonged to three validated species, one new proposed species, and two novel related strains. The majority of isolates (20/26, or 77%) belonged to M. terrae, making establishment of species pathogenicity uncertain (3).

The complex is recognized as an environmental contaminant of sputum and a cause of tenosynovitis and osteomyelitis primarily of the fingers and wrist (3–35). Whether one or more members of the complex are true respiratory pathogens has not been established (1, 22).

The first published case report of tenosynovitis caused by the MTC was by Hirata and Tomiyama in 1976 (4). There have been approximately 34 additional case reports published since then, identified using nonmolecular methods (3–25), with 14 cases identified using molecular methods (26–28, 30–36) (Tables 1 and 2). With the exception of four isolates of M. arupense, including the original description of M. arupense (28), details of the methods and/or explicitly stating a 100% 16S rRNA gene sequence identity to recognized species for the remaining cases with molecular identifications have been absent (Table 2).

An excellent history and species update of the M. terrae complex based on multigene sequencing targets was published by Tortoli et al. (1). He noted that the presence of a two nucleotide insertion in helix 18 of the 16S rRNA gene (bp ~430 to 500; hypervariable region B or region V3) provided a consistent signature sequence for members of the MTC compared to other slowly growing mycobacteria (37, 38). He also characterized several new species in the complex, including Mycobacterium heraklionense and Mycobacterium engbaekii (1).

The greater availability of DNA sequencing (59) has resulted in a “boom” of new species of MTC, beginning with Mycobacterium hiberniae (39). An additional eight new species have been validly published since 2006: Mycobacterium arupense (28), Mycobacterium kumamotonense (36), Mycobacterium heraklionense (1), Mycobacterium senueense (40), Mycobacterium minnesotense (41), Mycobacterium virginiense “sp. nov., but not Mycobacterium nonchromogenicum, as species of the Mycobacterium terrae complex causing tenosynovitis and osteomyelitis. J Clin Microbiol 54:1340–1351. doi:10.1128/JCM.00198-16.

Editor: G. A. Land
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M. arupense isolates of have been respiratory except for the two previously mentioned (43), which were associated with tenosynovitis or hand infections (28). The distribution of these 11 newer species of the complex as well as the two established members (M. terrae and M. nonchromogenicum) among clinical isolates from synovial fluid, tissue, or bone based on DNA sequencing is not known. Thus, we collected tendon, synovial, and bone isolates of the M. terrae complex recovered over 30 years, including isolates previously identified as M. nonchromogenicum using nonmolecular methods, and subjected them to molecular identification (3). We also present a

cobacterium longobardum (1), Mycobacterium algericum (42), and Mycobacterium enghaenic (1). Two other MTC species have been described that currently have no standing in nomenclature: “Mycobacterium paraterrae” (43) and “Mycobacterium sinense” (44).

Almost all published cases of these 11 new species of the MTC have been respiratory except for the two previously mentioned isolates of M. arupense (including the type strain, ATCC BAA-1242), which were associated with tenosynovitis or hand infections (28). The distribution of these 11 newer species of the complex as well as the two established members (M. terrae and M. nonchromogenicum) among clinical isolates from synovial fluid, tissue, or bone based on DNA sequencing is not known. Thus, we collected tendon, synovial, and bone isolates of the M. terrae complex recovered over 30 years, including isolates previously identified as M. nonchromogenicum using nonmolecular methods, and subjected them to molecular identification (3). We also present a
TABLE 2. Characteristics of 14 previously reported cases of tenosynovitis due to the complex based on DNA sequencing

<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Site of tenosynovitis</th>
<th>Underlying disease or source of trauma</th>
<th>Treatment</th>
<th>DNA Sequence</th>
<th>Species</th>
<th>Granulomatous inflammation</th>
<th>Surgery</th>
<th>Gram-positive bacilli</th>
<th>DNA-DNA homology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67/F</td>
<td>Right 2nd finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Norway/2006</td>
</tr>
<tr>
<td>2</td>
<td>19/M</td>
<td>Right index finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>3</td>
<td>60/F</td>
<td>Right 2nd finger</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>4</td>
<td>60/F</td>
<td>Right 3rd finger</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>5</td>
<td>54/M</td>
<td>Left 3rd finger, palm</td>
<td>Diabetes, motorcycle</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>6</td>
<td>58/M</td>
<td>Right index finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>7</td>
<td>54/F</td>
<td>Right 3rd finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>8</td>
<td>35/M</td>
<td>Right 2nd finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>9</td>
<td>71/M</td>
<td>Left forearm</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>10</td>
<td>37/M</td>
<td>Right 2nd finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>11</td>
<td>56/F</td>
<td>Right 2nd finger</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Texas/2006</td>
</tr>
<tr>
<td>12</td>
<td>68/M</td>
<td>Right 3rd finger</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>M. arupense</td>
<td>Granulomatous</td>
<td>NA</td>
<td>+</td>
<td>M. arupense</td>
<td>Norway/2011</td>
</tr>
</tbody>
</table>

M, male; F, female; com 16S, complete 16S sequence; "16S," base pair not specified; "hsp65," base pair not specified.

**CASE REPORT**

The patient is a 75-year-old previously healthy male who sustained a chainsaw injury to his leg. He developed a secondary wound infection and suspected osteomyelitis of the underlying tibia. He underwent an incision and debridement of the leg. Gram stain and routine cultures were negative. Special stains, including acid-fast bacillus (AFB) stains, were negative, but AFB cultures were positive in broth for a nonpigmented slowly growing organism initially identified by high-performance liquid chromatography (HPLC) patterns and phenotypic characteristics as being in the *M. terrae* complex. Histopathology on the tissue was not performed. The patient was treated initially with clarithromycin and doxycycline because of a history of a rash with sulfonamides. Subsequent susceptibility tests showed the isolate to be susceptible to clarithromycin, ethambutol, rifabutin, linezolid, and trimethoprim-sulfamethoxazole (TMP-SMX) but resistant to doxycycline. The patient was treated with clarithromycin and ethambutol, and his wound healed without incident. The isolate (MO-4693) was subsequently shown by complete 16S rRNA gene and partial *rpoB* gene sequencing to be a previously unrecognized member of the *M. terrae* complex.

**MATERIALS AND METHODS**

*Previous cases.* All previously published cases of the association of *M. terrae* complex with tenosynovitis or osteomyelitis were sought in the medical literature. Identification obtained by nonsequence-based versus sequence-based methods was highlighted.

*Current isolates.* All synovial tissue, joint fluid, or bone biopsy isolates of the *M. terrae* group submitted to the Mycobacteria/Nocardia Research Laboratory at The University of Texas Health Science Center at Tyler (UTHSCT) between 1984 and 2014 for identification and/or susceptibility testing were sought. This number included five isolates identified as *M. nonchromogenicum* based on phenotypic features and HPLC patterns from a 1991 publication (3), including strain MO-233. Clinical information was reviewed at the time of presentation. Isolates had been stored at −70°C in tryptic soy broth with 15% glycerol and were subcultured to Middlebrook 7H10 agar for molecular testing.

Reference strains included in the study were *M. terrae* ATCC 15755T, *M. nonchromogenicum* ATCC 19350T, *M. kumamotoense* DSM 45093T, *M. arupense* ATCC BAA-1242T, and "*M. paraterrae*" DSM 45127T. This study was approved by the Institutional Review Board of UTHSCT.

**DNA extraction.** A small loopful of bacteria from isolated colonies was suspended in 100 μL of preparation reagent (PrepMan Ultra, Life Technologies, Carlsbad, CA). Samples were held for 30 s and then heat killed for 10 min at 100°C, and then the samples were cooled down to room temperature for 2 min and centrifuged at maximum speed in a microcentrifuge for 2 min. The DNA was extracted by transferring 50 μL of the supernatant.

**16S rRNA complete gene PCR and sequencing.** 16S rRNA complete gene sequencing was performed as previously described by Edwards et al. (45).

PCR was performed in a 20-μL reaction mixture using a 10 μM concentration of each of the primers (pA and pH) (45), 1 × FailSafe Premix I, and 1.25 U of FailSafe enzyme mix (Epitect, Madison, WI). The PCR product (5 μL) was run on a 2% agarose gel (Promega, Madison, WI) with EZ safe stain (1 μL) (eEnzyme, Gaithersburg, MD) and visualized under UV light using a SYBR gold emission filter.

After purifying the amplicon using USB ExoSap-IT reagent (Affymetrix, Santa Clara, CA), sequencing was performed using primers pC,
pD, and pE (45) and a BigDye Terminator v3.1 cycle sequencing kit on an ABI 3500 genetic analyzer according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA).

Gene sequence analysis was performed using RipSeq software (Isentio AS, Bergen, Norway). Primer regions were excluded, yielding a final sequence of 1,489 bp for all strains (as is the case for members of the *M. terrae* complex). Sequences were compared to those for validated type strains and all available sequences using RipSeq and NCBI BLAST version 2.3.0+. Three separate NCBI databases were used for BLAST analyses: (i) 16S rRNA sequences (*Bacteria* and *Archaea*), (ii) the nucleotide collection (nr/nt), and (iii) whole-genome shotgun contigs (wgs). A separate BLAST analysis using only the first 500 bp from the 5 new species was performed to better assess the presence of similar sequences in the public domain. Interpretation was in accordance with the Clinical and Laboratory Standards Institute (CLSI) interpretive criteria for DNA target sequencing (46) (Table 3).

### TABLE 3 Twenty-six clinical cases of tenosynovitis or osteomyelitis due to *M. terrae* complex and their causative species based on complete 16S rRNA gene sequence

<table>
<thead>
<tr>
<th>Organism and isolate no.</th>
<th>Strain name</th>
<th>Source</th>
<th>Complete 16S rRNA gene sequence base pair match</th>
<th>% identity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Location/yr</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M.</em> arupense (<em>n = 10</em>)</td>
<td>MO-3556</td>
<td>Right elbow synovial fluid</td>
<td>1,475/1,475</td>
<td>100</td>
<td>Missouri/2010</td>
</tr>
<tr>
<td>2</td>
<td>MO-2220</td>
<td>Fourth finger fluid</td>
<td>1,475/1,475</td>
<td>100</td>
<td>Iowa/2006</td>
</tr>
<tr>
<td>3</td>
<td>MO-1791</td>
<td>Right wrist</td>
<td>1,475/1,475</td>
<td>100</td>
<td>Texas/2004</td>
</tr>
<tr>
<td>4</td>
<td>MO-4448</td>
<td>Finger</td>
<td>1,482/1,482</td>
<td>100</td>
<td>Arkansas/1999</td>
</tr>
<tr>
<td>5</td>
<td>MO-1082, pigmented</td>
<td>Right elbow synovial fluid</td>
<td>1,475/1,475</td>
<td>100</td>
<td>Florida/1999</td>
</tr>
<tr>
<td>6</td>
<td>MO-1089</td>
<td>Synovial fluid</td>
<td>1,482/1,482</td>
<td>100</td>
<td>Kansas/1998</td>
</tr>
<tr>
<td>7</td>
<td>MO-49</td>
<td>Hand</td>
<td>1,482/1,482</td>
<td>100</td>
<td>Florida/1985</td>
</tr>
<tr>
<td>8</td>
<td>MO-86, pigmented</td>
<td>Left wrist, synovium</td>
<td>1,474/1,474</td>
<td>100</td>
<td>Maine/1986</td>
</tr>
<tr>
<td>9</td>
<td>MO-3744</td>
<td>Left index finger</td>
<td>1,474/1,474</td>
<td>100</td>
<td>Missouri/2011</td>
</tr>
<tr>
<td>10</td>
<td>MO-4781</td>
<td>Hand</td>
<td>1,465/1,465</td>
<td>100</td>
<td>Texas/2013</td>
</tr>
<tr>
<td><em>M.</em> heraklionense (<em>n = 10</em>)</td>
<td>MO-3474</td>
<td>Right index finger</td>
<td>1,427/1,427</td>
<td>100</td>
<td>Texas/2010</td>
</tr>
<tr>
<td>2</td>
<td>MO-4449</td>
<td>Tissue, left hand</td>
<td>1,427/1,427</td>
<td>100</td>
<td>Massachusetts/1996</td>
</tr>
<tr>
<td>3</td>
<td>MO-786</td>
<td>Finger, tissue</td>
<td>1,427/1,427</td>
<td>100</td>
<td>North Carolina/1996</td>
</tr>
<tr>
<td>4</td>
<td>MO-778</td>
<td>Finger</td>
<td>1,427/1,427</td>
<td>100</td>
<td>California/1996</td>
</tr>
<tr>
<td>5</td>
<td>MO-7</td>
<td>Right hand, synovium</td>
<td>1,427/1,427</td>
<td>100</td>
<td>Texas/1984</td>
</tr>
<tr>
<td>6</td>
<td>MO-51</td>
<td>Hand</td>
<td>1,427/1,427</td>
<td>100</td>
<td>California/1985</td>
</tr>
<tr>
<td>7</td>
<td>MO-4967</td>
<td>Right index finger</td>
<td>1,422/1,422</td>
<td>100</td>
<td>Washington/2014</td>
</tr>
<tr>
<td>8</td>
<td>MO-5013</td>
<td>Right index finger</td>
<td>1,422/1,422</td>
<td>100</td>
<td>Texas/2013</td>
</tr>
<tr>
<td>9</td>
<td>MO-5024</td>
<td>Right index finger</td>
<td>1,422/1,422</td>
<td>100</td>
<td>Illinois/2014</td>
</tr>
<tr>
<td>10</td>
<td>MO-5209</td>
<td>Index finger</td>
<td>1,422/1,422</td>
<td>100</td>
<td>Washington/2015</td>
</tr>
<tr>
<td><em>M.</em> kunamotoense (<em>n = 1</em>)</td>
<td>MO 2762</td>
<td>Right hand tendon</td>
<td>1,423/1,424</td>
<td>99.93</td>
<td>Massachusetts/2008</td>
</tr>
<tr>
<td>Proposed new species (<em>n = 5</em>)</td>
<td>MO 1300</td>
<td>Knee</td>
<td>1,474/1,474</td>
<td>100</td>
<td>Florida/2001</td>
</tr>
<tr>
<td>2</td>
<td>MO-233&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Flexor tendon</td>
<td>1,474/1,474</td>
<td>100</td>
<td>Virginia/1991</td>
</tr>
<tr>
<td>3</td>
<td>MO-5116</td>
<td>Elbow</td>
<td>1,474/1,474</td>
<td>100</td>
<td>North Carolina/2014</td>
</tr>
<tr>
<td>4</td>
<td>MO-3559</td>
<td>Flexor tendon</td>
<td>1,359/1,359</td>
<td>100</td>
<td>Unknown</td>
</tr>
<tr>
<td>5</td>
<td>MO-4693</td>
<td>Tibia</td>
<td>1,359/1,359</td>
<td>100</td>
<td>Missouri/2013</td>
</tr>
</tbody>
</table>

<sup>a</sup> % identity is the identity of organism against the corresponding type strain. In the case of the final two values, the strains both have same single base pair mismatch.

<sup>b</sup> Proposed type strain.

### hsp65 partial gene sequencing
A 441-bp region of the *hsp65* gene was amplified (49) and used for molecular analysis of a 401-bp sequence (excluding the primer regions). Sequencing was done on some of the isolates that are not 100% to a validated type strain by 16S rRNA gene sequencing using the BigDye Terminator v3.1 cycle sequencing kit on an ABI 3500 genetic analyzer. Both the nucleotide collection (nr/nt) and whole-genome shotgun contig (wgs) NCBI databases were used for BLAST analyses.

### Phylogenetic analyses
For all gene targets, sequencing alignments of strains from this study and chosen sequences from GenBank were created and phylogenetic analyses were conducted using MEGA version 6 (50). A web-accessible database of *hsp65* sequences from *Mycobacterium* reference strains was also used to populate the *hsp65* alignment (51).

Members of the *M. terrae* complex are known to harbor two copies of the ribosomal operon that may contain differences in the 16S rRNA gene (52, 53), seen as ambiguous bases in sequence electropherograms. Variations in positions of ambiguity were not considered in 16S phylogenetic analyses in this study.

### Susceptibility testing
Susceptibility testing of the *M. terrae* complex isolates was performed using broth microdilution according to CLSI guidelines (54).

RESULTS

Previous reported cases. A total of 35 previously published cases of tenosynovitis and osteomyelitis due to members of the M. terrae complex and identified using nonmolecular methods since 1976 were reviewed. Biochemical testing and HPLC were primarily used in the species identification. All cases were previously reported to be due to M. terrae or M. nonchromogenicum. Most (80%) cases involved the hand, fingers, or wrists, with histopathologic findings of granuloma and growth of the organism from operative materials (Table 1).

A total of 14 cases of tenosynovitis and osteomyelitis due to the M. terrae complex identified using molecular methods were investigated (Table 2). In three cases (cases 1, 9, and 12) 16S rRNA gene sequencing was used but did not provide a percent match with a type strain, in one case (case 7) DNA-DNA hybridization was used, and in three cases (cases 2, 3, and 11) no molecular details were given. The first three cases (cases 1 to 3) (Table 2) were all reported as M. nonchromogenicum. Two isolates (cases 4 and 5, Table 2) underwent complete 16S rRNA gene sequencing as well as secondary gene target sequencing as part of the original study describing M. arupense by Cloud et al. in 2006 (28). One of these (AR 30097) is the recognized type strain of M. arupense, and both isolates were a 100% match of the complete 16S rRNA gene to each other and other identified sputum isolates (28). In two other cases partial 16S rRNA gene sequencing was used, with 100% match to the type strain of M. arupense (cases 8 and 13) (31, 35). Two additional case isolates underwent 16S rRNA partial gene sequencing (cases 6 and 10); one reported as M. arupense and the other as M. longobardum, although both were only a 99.2% match to the type strain. The isolate in the last case (no. 14) underwent complete 16S sequencing and was a 99.8% (1,429/1,432 bp) match to M. arupense (36). By current CLSI standards these last three isolates are grouped as “most closely related”) (46, 55). Overall, based on the provided information, only four isolates met current CLSI sequencing criteria for a specific species identification (cases 4, 5, 8, and 13), and all were identified as M. arupense (46).

Current isolates. A total of 26 patients with available isolates for study were identified (Table 3). Five of these isolates were identified in the presequencing era (all as M. nonchromogenicum) (3), and 21 were new isolates. The 25 patients from known locations were from 13 states: Texas (5), Missouri (3), Florida (3), Massachusetts (2), California (2), Washington (2), Iowa (1), Arkansas (1), Kansas (1), Maine (1), North Carolina (2), Illinois (1), and Virginia (1). Isolates were from the finger, hand, or wrist (18) (69%), elbow (3) (11.5%), knee (1) (4%), flexor tendon (2) (8%), tibia (1) (4%), and synovial fluid (1) (4%).

16S rRNA complete gene sequencing. By complete 16S rRNA gene sequencing, a 100% identity to a validated type strain sequence (Table 3) was obtained for three species: M. arupense (10 isolates or 38%), M. heraklionense (10 isolates or 38%), and M. kumamotonense (one isolate or 4%). There were no matches to M. terrae or M. nonchromogenicum. Of note, strain MO-2762, identified as M. kumamotonense, presented with a gap in the 3′ end compared to the type strain sequence. For a clinical respiratory isolate of M. kumamotonense from Canada (unpublished data; strain B0621B018392 [Fig. 1]), a sequence electropherogram of this region revealed the presence of two 16S copies where one sequence contained the gap and the other did not. This resulted in a single base pair shift at that position, making all subsequent sequence data uninterpretable. We considered, then, that this gap could be a feature of some strains of M. kumamotonense and therefore was not considered a true base pair difference.

There were three isolates (MO-233, MO-1300, and MO-5116) with 100% identity to each other but no match to any validated species or other GenBank sequence (“new species”) using the top 250 matches from nucleotide collection database. The closest established species were M. arupense, with a mismatch of 5 bp (99.7%), followed by M. nonchromogenicum and M. heraklionense, with mismatches of 9 bp (99.4%) and 10 bp (99.3%), respectively. These isolates and their relationship to other isolates of the M. terrae complex using the complete 16S rRNA gene are shown in Fig. 1.

The two other isolates, MO-3559 and MO-4693, did not match any known species or the proposed new species and (after excluding positions of ambiguity) have 100% identity with each other. The closest validated species for MO-3559 is M. arupense (7-bp mismatch [99.5%]) followed by M. nonchromogenicum and M. heraklionense, with mismatches of 9 bp (99.4%) and 10 bp (99.3%), respectively. For MO-4693, the closest validated species is M. arupense (5-bp mismatch [99.7%]), followed by M. nonchromogenicum and M. heraklionense, with mismatches of 6 bp (99.6%) and 8 bp (99.4%), respectively. MO-3559 has two base pair differences compared to the proposed new species MO-233, and MO-4693 presents with only one base pair change from MO-233 after excluding ambiguous bases (the second base pair change in 16S rRNA gene between MO-3559 and MO-233 is an ambiguous base in the 16S rRNA gene sequence of MO-4693). This indicates that some of the 5 ambiguous bases in the 16S rRNA gene of MO-4693 might be true base pair mismatches with MO-3559, which is also supported by significant sequence variations observed between the two isolates by other genes (rpoB and hsp65).

The isolate MO-4693 presented with 5 ambiguous bases that could not be resolved upon repeat sequencing from a single colony and are presumed to be due to 2 differing copies of the 16S rRNA gene.

Comparing against nonvalidated species, the closest match for the 5 new species strains was “M. paraterreae” (GenBank accession number EU919229.1), with four base pair mismatches over the full gene. For this reason, the proposed type strain for the species was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) (DSM 45127) for confirmation and comparison. However, it was determined that the isolate submitted to DSMZ as the type strain of “M. paraterreae” has a strikingly different 16S rRNA gene sequence than that deposited in GenBank, differing from it by 47 bp (96.8%). This was also confirmed in the DSMZ (with a 100% sequence identity to that determined in our facility) and stated on their website along with strain
The complete 16S rRNA gene sequence determined for the strain of "M. paraterrae" presently in the DSMZ collection (DSM 45127) appears to represent a novel species closest to M. cookii ATCC 49103T (GenBank accession number AF480598) (9-bp difference [99.4% identity]). Interestingly, a BLAST analysis of the corresponding rpoB sequence of "M. paraterrae" previously deposited in GenBank (accession number EU919230) reveals a closest match (283/298 bp [95.0%]) with that of M. cookii CIP105396T (accession number AY544904). Similarly, BLAST analysis of the corresponding hsp65 sequence of "M. paraterrae" deposited in GenBank (accession number EU919230) reveals a close match only to a single entry (579/583 bp) described as M. cookii-like (accession number JX566891).

Of the five isolates from the 1991 publication by Ridderhof et al. (3) identified by nonmolecular methods as M. nonchromogenicum, two were identified by complete 16S rRNA gene sequencing as M. arupense, two were M. heraklionense, and one belonged to the proposed new species, "M. virginiense" (MO-233).

**rpoB partial gene sequencing region V.** Two of the five members of the proposed new species (MO-233 and MO-1300) had 100% sequence identity to each other by rpoB region V partial gene sequencing but differed by 21 bp (97.1%) from its closest species, M. nonchromogenicum. The sequence of the third member (MO-5116) differed by 5 bp (99.3%) from the first two isolates. The last two isolates (MO-3559 and MO-4693) differed from each other by 29 bp (96.0%) of the rpoB region V sequence and from the proposed type strain MO-233 by 28 bp (96.1%) and 18 bp (97.5%), respectively. Their relationship to other available members of the M. terrae complex is shown in Fig. 2.

Clinical isolate MO-2762, with a single deletion in the 3’ end of the 16S rRNA gene sequence in comparison with the type strain sequence of M. kumamotonense, corresponded also to M. kumamotonense NCTC 1342T (accession no. IN571251) by rpoB region V (3-bp difference [99.6%]) and by rpoB region III (see below). This high degree of similarity further confirms that MO-2762 is a strain of M. kumamotonense. To our knowledge, this is the first reported case of tenosynovitis due to this new species, first described in 2006 (36).
**rpoB** partial gene sequencing region III. A 315-bp fragment of rpoB region III was analyzed (analysis with a shorter fragment depending on available matches is otherwise indicated). Two of the five members of the new species (MO-233, MO-1300, and MO-5116) had 100% sequence identity to each other by rpoB region III sequencing but differed by 15/305 bp (95.1%) from the type strain of its closest established species, *M. arupense*. The sequence of the third member (MO-5116) differed from the other two by 15 bp (95.2%) and only by 7/312 bp (97.8%) from its closest species, *M. heraklionense*. This does not represent the type strain (no examples are available for this region); however, this GenBank entry is from the same reference (and authors) that described the species (1). The sequences of the last two (MO-3559 and MO-4693) differed from the “new species” proposed type strain MO-233 by 17 bp (94.6%) and 16 bp (95.0%), respectively. Their relationship to other members of the *M. terrae* complex is shown in Fig. 3.

The isolate with a 1-bp difference (a deletion) in complete 16S rRNA gene sequence from *M. kumamotonense* (MO-2762) had an rpoB gene region III sequence that differed by only 1 bp (99.7% identity) from *M. kumamotonense* NCTC 12342\(^1\). This does not represent the type strain (no examples are available for this region); however, this GenBank entry is from the same reference (and authors) that described the species (1). The sequences of the last two (MO-3559 and MO-4693) differed from the “new species” proposed type strain MO-233 by 17 bp (94.6%) and 16 bp (95.0%), respectively. Their relationship to other members of the *M. terrae* complex is shown in Fig. 3.

**hsp65** partial gene sequencing. hsp65 gene sequencing was done on isolates MO-233, MO-1300, MO-5116, MO-3559, and MO-4693 using the primers used by Telenti et al. (49). A 401-bp hsp65 sequence (within-primer region) was analyzed for all 5 strains. MO-1300 and MO-5116 differed from the proposed type strain, MO-233, by 1 bp and 3 bp, respectively, representing 99.8% and 99.3% similarities. The closest match for MO-233 to the type strain of an established species was with *M. engbaekii* (8 bp [98.0%]), followed by *M. arupense* (13 bp [96.8%]). Strain MO-4693 diverged from MO-233 by 10 bp (97.5%), and its closest established species was *M. engbaekii* (12 bp [97.0%]). Strain MO-3559 diverged by 20 bp (95.0%) from MO-233, and its closest established species was *M. heraklionense* (7 bp [98.3%]). Their relationship to other members of the *M. terrae* complex is shown in Fig. 4.

Sequence comparisons with non-type strains by BLAST analysis. To assess the presence of the novel species elsewhere, a BLAST analysis was also performed using only the first 500 bp of the 16S rRNA gene, allowing for comparison with GenBank sequences closer to 500 bp in length, as is performed in many clinical laboratories. Sequences of clinical isolates presenting with a 100% match were strains FI-10193 (accession number JN571170.1) and N177 (accession number AY215361.1), both indicated as members of the *M. terrae* complex. Upon BLAST analysis of the 16S rRNA gene (full) against the whole-genome shotgun contigs (wgs) database, a 99.9 to 100% match (0 to 1 bp) was achieved with 3 of 4 strains obtained from the trunk washes of captive elephants. These strains were described as new genomospecies within the *M. terrae* complex (56).

Further investigation against non-type strain sequences was also done using hsp65. With the large number of hsp65 sequences related to the MTC deposited in public sequence databases, many of which are either not identified or misidentified, comparison was restricted to only those with a 100% match. A BLAST analysis

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**FIG 2** rpoB partial gene sequence dendrogram of region V (47) for the three members of the proposed new species, “*M. virginiense*” (MO-233, MO-1300, and MO-5116), the unidentified isolates MO-3559 and MO-4693, and the isolate that by 16S rRNA complete gene sequencing differed by a single deletion from *M. kumamotonense* (MO-2762). There is no region V sequence for *M. arupense* ATCC BAA1242\(^2\) in GenBank, so the rpoB V region was sequenced and the sequence was used; the sequence was submitted to GenBank. The strain relationships are based on neighbor-joining and complete deletion analysis.
of 401 bp of the hsp65 of strain MO-233 against the nr (nucleotide collection) revealed a 100% match with 4 sequences in GenBank: (i) strain InDRE Chiapas1942, a clinical isolate from Mexico (accession number JX154109.1); (ii) strain IEC35, a pulmonary specimen from Brazil (accession number HM056146.1); (iii) strain P51, a clinical isolate from Brazil (accession number GQ478699.1); and (iv) strain FI-10193, a clinical specimen from Italy and described as an unassigned strain of the M. terrae complex (accession number JN571212.1) (1). Strain MO-4693 revealed a 100% match with “M. terrae” variant MS699 (accession number GQ478699.1).
number \textit{AY550212.1} (57) and FI-11039 (accession number \textit{JN571213.1}) (1). No identical matches were found for the remaining 3 strains, though close matches (1 to 3 bp) were found.

\textbf{Susceptibility testing.} Antimicrobial agents active against the six species of \textit{M. terrae} complex associated with tenosynovitis or osteomyelitis included clarithromycin (26/26, or 100%), ethambutol (24/26, or 92%), rifabutin (26/26, or 100%), and sulfamethoxazole (3/4) or trimethoprim-sulfamethoxazole (19/22, or 86%). The isolates were almost all resistant to rifampin (23/26) and the quinolones ciprofloxacin (26/26) and moxifloxacin (23/86%). The isolates were almost all resistant to rifampin (23/26) butol (24/26, or 92%), rifabutin (26/26, or 100%), and sulfamethoxazole. Previous reports have noted the benefits of a macrolide complex producing tenosynovitis or osteomyelitis. Previous reports have noted the benefits of a macrolide complex producing tenosynovitis or osteomyelitis.

\textbf{DISCUSSION.} This study clearly demonstrates the inability of phenotypic tests and mycolic acid analysis (HPLC) to recognize new mycobacterial species defined by DNA sequencing, including members of the \textit{M. terrae} complex. Tenosynovitis or osteomyelitis caused by members of the \textit{M. terrae} complex was believed on the basis of phenotypic testing by mycolic acid analysis (HPLC) to be due to \textit{M. nonchromogenicum} or \textit{M. terrae} for more than 30 years. The recognition of \textit{M. arupense} as a cause of tenosynovitis in 2006 (28) was the first indication that other species might be responsible. The current study suggests that neither \textit{M. terrae} nor \textit{M. nonchromogenicum} is a cause of tenosynovitis and that earlier isolates identified as these species by nonsequencing methods were misidentified (3). There is no treatment of choice for \textit{M. terrae} complex tenosynovitis. Previous reports have noted the benefits of a macrolide combined with one or more additional agents that included ethambutol, rifabutin, and/or a sulfonamide, including trimethoprim-sulfamethoxazole (TMP-SMX) (3, 31). The major pathogens defined in the current study are \textit{M. arupense} and \textit{M. heraklionense}. These species are generally susceptible to clarithromycin, ethambutol, rifabutin, and TMP-SMX. A recent report of susceptibilities of 40 isolates of \textit{M. arupense} by Beam et al. gave similar results to the current study (31) with 100% of tested isolates susceptible to clarithromycin, ethambutol, and rifabutin, and approximately 50% susceptible to TMP-SMX (31).

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|c|c|c|}
\hline
Organism and isolate no. & MO strain designation & AMK & RMP & RBT & EMB & CIP & MOX & CLA & DOX & LZD & MIN & SMX & TMP-SMX \\
\hline
\textit{M. arupense} & 1 & 49 & 16 & 8 & 1 & >8 & 2 & 32 & 2/38 & & & & \\
& 2 & 1082 & >64 & 16 & >0.25 & 0.5 & >16 & >8 & 2 & >16 & 16 & >32 & 2/38 \\
& 3 & 1791 & 4 & >0.25 & 1 & >16 & >32 & 2 & >16 & 16 & 64 & & \\
& 4 & 3556 & >64 & 2 & >0.25 & >0.5 & >16 & >8 & 0.25 & 16 & 16 & 2/38 & \\
& 5 & 3744 & >64 & 8 & >0.25 & >0.5 & >16 & >8 & 1 & >16 & 8 & 8/152 & \\
& 6 & 4448 & >64 & 2 & >0.25 & >0.5 & >16 & >8 & 0.25 & >16 & 8 & 0.25/4.25 & \\
& 7 & 2220 & 32 & 4 & 0.12 & >0.5 & 16 & >8 & 2 & >16 & 8 & 0.5/9.5 & \\
& 8 & 86 & 64 & 4 & >0.25 & >0.3 & >16 & >8 & 0.5 & 16 & 8 & 4/76 & \\
& 9 & 1089 & >64 & 4 & >0.25 & >0.5 & >16 & >8 & 0.12 & 16 & 16 & 1/19 & \\
& 10 & 4781 & 2 & 1 & >0.25 & >0.5 & 8 & >8 & 1 & 8 & 8 & 8/152 & \\
\hline
\textit{M. heraklionense} & 1 & 7 & 8 & 2 & 4 & 8 & 2 & 32 & 8 & & & & \\
& 2 & 51 & 8 & 8 & 16 & >8 & 1 & 32 & 16 & & & & \\
& 3 & 778 & 64 & 8 & >0.25 & 2 & >16 & >8 & 1,025 & >16 & 8 & 1/19 & \\
& 4 & 786 & 64 & >8 & 1,1 & 4 & >16 & >8 & 1 & >16 & 32 & 2/38 & \\
& 5 & 3474 & 16 & 4 & 0.5 & 2 & >16 & >8 & 2 & >16 & 64 & 1/19 & \\
& 6 & 4449 & 16 & 1 & >0.25 & 2 & >16 & >8 & 0.5 & >16 & 32 & 2/38 & \\
& 7 & 4967 & 64 & 0.5 & >0.25 & 8 & 16 & >8 & 0.5 & >16 & >64 & 0.12/2.38 & \\
& 8 & 5013 & >64 & 8 & 0.5 & 2 & >16 & >8 & 4 & >16 & 64 & 1/19 & \\
& 9 & 5024 & 16 & >8 & >0.25 & 2 & >16 & >8 & 0.5 & 8 & 16 & 1/19 & \\
& 10 & 5210 & 32 & >8 & 0.5 & 2 & >16 & >8 & 0.5 & >16 & 32 & 1/19 & \\
\hline
\textit{M. kumamotonense} & 1 & 2762 & 64 & >8 & 1 & >0.5 & 16 & 4 & 1 & 4 & 16 & 0.25/9.75 & \\
\hline
Newly proposed species & 1 & 233 & >8 & 1 & 1 & 4 & >16 & >8 & 1 & >16 & 16 & 1/19 & \\
& 2 & 1300 & >64 & 64 & >0.25 & 2 & >16 & >8 & 1 & 16 & 64 & 2/38 & \\
& 3 & 5116 & >64 & >8 & 1 & 2 & >16 & >8 & 1 & >16 & 32 & >8 & 2/38 & \\
\hline
Unique strains & 1 & 3559 & >64 & >8 & >0.25 & 2 & >16 & >8 & 0.5 & >16 & 32 & 2/38 & \\
& 2 & 4693 & >64 & 4 & >0.25 & 1 & 8 & 8 & 0.25 & 16 & 2 & 1/19 & \\
\hline
\end{tabular}
\caption{Antimicrobial susceptibilities of isolates within the \textit{M. terrae} complex producing tenosynovitis or osteomyelitis}
\end{table}

\footnotesize
\textsuperscript{a} Abbreviations: AMK, amikacin; RMP, rifampin; RBT, rifabutin; EMB, ethambutol; CIP, ciprofloxacin; MOX, moxifloxacin; CLA, clarithromycin; DOX, doxycycline; LZD, linezolid; MIN, minocycline; SMX, sulfamethoxazole; TMP-SMX, trimethoprim-sulfamethoxazole.

\footnotesize
\textsuperscript{b} Original MICs determined in 1988 on MO-233.

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Five of these 26 isolates (19%) belonged to a previously unrecognized species. Three of the five isolates had 100% sequence identity for their complete 16S rRNA gene and differed by 5 bp from its closest validated species. Three isolates, including the proposed type strain (MO-233, MO-1300, and MO-5116), exhibited >99% sequence identity for region V of the rpoB gene, the sequence of the hsp65 gene highlighted by Telenti et al., and two-thirds of region III of the rpoB gene. Strain MO-3559, however, exhibited <96.2% identity for these sequences, and MO-4693 exhibited from 97.9% to 95.3% identity for the same sequences. The high degree of variance of the strains MO-3559 and MO-4693 from each other and from the other three strains of the proposed new species possibly indicates emergence of new species that is beyond the scope of this study. The proposed name for the three isolates, "M. virginiense," refers to the geographic location of the first recognized case.

Like the other previous 11 members of the M. terrae group, this new species has a two-nucleotide insertion in helix 18 of the 16S rRNA gene (37, 58) characteristic of members of the M. terrae complex. It also shares other culture features of this group, including lack of pigmentation and growth rate of more than 7 days.

In the current study, one isolate of M. kumamotoense, whose complete 16S rRNA gene sequence differed by one deletion from the type strain, was identified. Given the likelihood of the presence of two ribosomal operons and the high degree of similarity of region V of the rpoB between the current strain and the type strain (99.6%), it is highly likely that the current isolate is M. kumamotoense. The extra base pair occurs within 20 bp of the 3’ end of the sequence in GenBank, while the current sequence with the gap is approximately 80 bp longer (1).

**ACKNOWLEDGMENTS**

We gratefully acknowledge the Amon G. Carter Foundation for support for the DNA sequencing.

We also acknowledge the authors of the initial study of five of these isolates in 1991 (3), especially John Ridderhof, and Joanne Woodring for her expert clerical assistance.

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