Utility of *rpoB* Gene Sequencing for Identification of Nontuberculous Mycobacteria in the Netherlands

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In the Netherlands, clinical isolation of nontuberculous mycobacteria (NTM) has increased over the past decade. Proper identification of isolates is important, as NTM species differ strongly in clinical relevance. Most of the currently applied identification methods cannot distinguish between all different *Mycobacterium* species and complexes within species. *rpoB* gene sequencing exhibits a promising level of discrimination among rapidly and slowly growing mycobacteria, including the *Mycobacterium avium* complex. In this study, we prospectively compared *rpoB* gene sequencing with our routine algorithm of reverse line blot identification combined with partial 16S rRNA gene sequencing of 455 NTM isolates. *rpoB* gene sequencing identified 403 isolates to species level as 45 different known species and identified 44 isolates to complex level, and eight isolates remained unidentifiable to species level. In contrast, our reference reverse line blot assay with adjunctive 16S rRNA gene sequencing identified 390 isolates to species level (30 distinct species) and identified 56 isolates to complex level, and nine isolates remained unidentified. The higher discriminatory power of *rpoB* gene sequencing results largely from the distinction of separate species within complexes and subspecies. Also, *Mycobacterium gordonae*, *Mycobacterium kansasii*, and *Mycobacterium interjectum* were separated into multiple groupings with relatively low sequence similarity (98 to 94%), suggesting that these are complexes of closely related species. We conclude that *rpoB* gene sequencing is a more discriminative identification technique than the combination of reverse line blot and 16S rRNA gene sequencing and could introduce a major improvement in clinical care of NTM disease and the research on the epidemiology and clinical relevance of NTM.

Nontuberculous mycobacteria (NTM) are environmental bacteria that incidentally cause opportunistic infections in humans. In the Netherlands, an aging population and increasing prevalence of chronic disorders like chronic obstructive pulmonary disease (COPD) have led to an increase in clinical NTM isolation, mainly from pulmonary specimens (1). To determine the clinical relevance of NTM, the Statement by the American Thoracic Society and the Infectious Disease Society of America offers an important support to guide clinicians (2). At present, more than 160 distinct *Mycobacterium* species have been validly published (<http://www.bacterio.net/mycobacterium.html>). The clinical relevance of isolated NTM differs strongly by species, with a spectrum ranging from species which should nearly always be considered pathogenic (e.g., *Mycobacterium kansasii* and *Mycobacterium malmoense*) to typical saprophytes, like *Mycobacterium gordonae* and *Mycobacterium phlei* (3). Optimal treatment regimens also differ by species, with major differences between slowly and rapidly growing NTM (4). Because of these differences in clinical relevance and specific treatment regimens, accurate species identification of isolated NTM is of the utmost importance.

Since the early 1990s, sequence analysis of the 16S rRNA gene has served as the gold standard in identification of mycobacteria (5). After the introduction of commercial line probe assays and other molecular probe assays, most laboratories implemented these assays as a rapid first identification to overcome the practical or financial inaccessibility of DNA sequencers and to reduce turnaround time. Although 16S rRNA gene sequencing offers much more resolution in identification of NTM than line blot assays, it still lacks discrimination for some groups of NTM, especially among the rapid growers in the *Mycobacterium fortuitum* and *Mycobacterium chelonae-abscessus* complexes (6, 7). Therefore, DNA sequence analysis of the *rpoB* gene was suggested in previous stud-

ies as a more suitable tool for identification of NTM than 16S rRNA genes (8), and promising results have been obtained both among rapidly growing mycobacteria (RGM) (9) and within the *M. avium* complex (8). It has also been observed that the *rpoB* sequence-based phylogenetic tree was similar to a concatenated tree on the basis of *rpoB*, *hsp65*, and 16S rRNA gene sequences (6).

In this study, we prospectively compared partial *rpoB* gene sequencing to our routine algorithm of reverse line blot identification supplemented with partial 16S rRNA gene sequencing, testing 455 clinical NTM isolates received at the *Mycobacterium* reference laboratory at the National Institute for Public Health and the Environment in the Netherlands (RIVM) during a 15-month period.

MATERIALS AND METHODS

Clinical NTM isolates submitted to the RIVM from January 2010 to March 2011 were subjected to reverse line blot identification (INNO-LiPA Mycobacteria v2 [Innogenetics, Ghent, Belgium]; referred to here as LiPA). Isolates identified as *Mycobacterium* species by this assay but not identifiable to species or complex level with the assay's probes were then identified by partial 16S rRNA gene sequence analysis starting from the position corresponding to *Escherichia coli* position 28 (479 bp, including the hypervariable regions A and B) (5, 10). Additionally, isolates were

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subjected to partial sequencing of the *rpoB* gene using previously published primers (9).

Both the 16S rRNA gene and *rpoB* gene sequences were analyzed using BioNumerics version 6.6 software (Applied Math, Belgium). The sequences were aligned with the GenBank database (NCBI), using the Basic Local Alignment Search Tool (BLAST). For 16S rRNA gene sequencing, identification was based on 100% matches with type strains or reference isolates of validly published species (<http://www.bacterio.net/mycobacterium.html>) (11). Isolates without a proper match were labeled “unidentified *Mycobacterium* species” (UMS) and numbered consecutively. The *rpoB* gene sequences were interpreted as previously suggested (8, 9); i.e., we tolerated 99.3% similarity to type strain sequences for slow growers and 98.3% for rapid growers. Additionally, SGM and RGM isolates with similarities between 94% and 99.3% or 98.3% to type strain sequences were considered identified to complex level only. Isolates showing less than 94% similarity to an accepted species were considered *rpoB* unidentified *Mycobacterium* species (rUMS). Additionally, matches with the *Mycobacterium terrae* complex strain sequences from the study of Tortoli et al. (12) and “*M. tuberculosis*” (13) were also accepted.

If *rpoB* sequencing results were not supported by GenBank results, the 16S rRNA gene was considered the gold standard. If *rpoB* sequencing results were supported by LiPA, the LiPA result was considered correct except when a match with another type strain in GenBank was found. In cases of a match with more than one species within the cutoff value, we selected the closest match.

Nucleotide sequence accession numbers. We have deposited the *Mycobacterium bohemicum* (KJ729110), *Mycobacterium heckeshornense* (KJ729109), and *Mycobacterium conspicuum* (HQ141573) sequences we obtained in GenBank.

RESULTS

Screening of 455 NTM isolates using the reverse line blot assay. Using the LiPA, 417 (92%) of the 455 isolates could be identified to species or complex level based on hybridization with one of the probes identifying a total of 17 NTM species and three NTM complexes (Table 1). Thirty-eight isolates (8%) could not be identified beyond the genus *Mycobacterium*, as hybridization with the *Mycobacterium* genus probe but not with a species- or complex-specific probe was observed in the reverse line assay.

16S rRNA gene versus *rpoB* gene sequencing. Thirty-eight isolates were classified as *Mycobacterium* species with the LiPA assay and underwent additional 16S rRNA gene sequencing. These 38 isolates were assigned to 26 different species (27 sequence types in 16S rRNA gene analysis versus 31 sequence types in *rpoB* gene sequencing), of which five were possibly novel species, and three different isolates could not be identified beyond the *M. terrae* complex level.

Eleven isolates had matching results in both 16S rRNA gene and *rpoB* gene sequencing and were identified as *M. phlei* ($n = 2$), *M. lentiflavum* ($n = 2$), *M. branderi* ($n = 1$), *M. florentinum* ($n = 1$), *M. shimoidei* ($n = 1$), *M. triviale* ($n = 1$), *M. goodii* ($n = 1$), *M. mucogenicum* ($n = 1$), or *M. iranica* ($n = 1$) (14). Two isolates showed more than 98.3% similarity with established species by *rpoB* gene sequencing but showed one base pair mismatch with 16S rRNA gene sequencing: *M. phlei* ($n = 1$) and *M. novocastrense* ($n = 1$). Three isolates could only be identified by 16S rRNA gene sequences, as no *rpoB* reference sequence was available for these species: *M. bohemicum* ($n = 1$), *M. heckeshornense* ($n = 1$), *M. conspicuum* ($n = 1$) (Fig. 1, 2, and 3).

Six isolates belonged to the *M. terrae* complex on the basis of both the 16S and *rpoB* analysis. Three isolates gave a sufficient match with 16S rRNA gene and *rpoB* gene sequences and were

TABLE 1 Reverse line blot assay results

Line	INNO-LiPA probe	Species	No. of samples	No. (%) with matching result by <i>rpoB</i> sequencing
2	MYC	<i>Mycobacterium</i> species	38 ^a	36 (94.7)
3	MTBC	<i>M. tuberculosis</i> complex	0	
4	MKA-1	<i>M. kansasii</i> I	33	35 (81.4)
5	MKA-2	<i>M. kansasii</i> II	7	
6	MKA-3	<i>M. kansasii</i> III (type III-V + <i>M. gastri</i>)	3	
7	MXE	<i>M. xenopi</i>	6	6 (100)
8	MGO	<i>M. goodii</i>	62	49 (79.0)
9	MGV	<i>M. genavense</i>	0	
10	MSI	<i>M. simiae</i>	5	4 (80)
11	MMU	<i>M. marinum</i> / <i>M. ulcerans</i>	16	16 (100)
12	MCE	<i>M. celatum</i>	0	
13	MAIS	<i>M. avium</i> complex (MAC)	26	11 (42.3)
14	MAV	<i>M. avium</i>	112	112 (100)
15	MIN-1	<i>M. intracellulare</i> (sequevar Min-A, -B, -C, and -D)	18	15 (83.3)
16	MIN-2	<i>M. chimaera</i> (formerly <i>M. intracellulare</i> sequevar MAC-A)	24	22 (91.7)
17	MSC	<i>M. scrofulaceum</i>	2	0 (0)
18	MML	<i>M. mageritense</i>	15	15 (100)
19	MHP	<i>M. haemophilum</i>	2	2 (100)
20	MCH-1	<i>M. chelonae</i>	21	20 (95.2)
21	MCH-2	<i>M. abscessus</i>	38	24 (63.2)
22	MCH-3	<i>M. chelonae</i> (group I)	0	
23	MFO	<i>M. fortuitum</i> - <i>M. peregrinum</i> complex	27	19 (70.4)
24	MSM	<i>M. smegmatis</i>	0	
	TOTAL		455	384 (84.4)

^a Additional partial 16S rRNA gene sequencing was performed.

identified as *Mycobacterium arupense*, *M. terrae*, and *Mycobacterium kumamotoense* (12). The other three isolates could not be assigned to a species but did belong to the *M. terrae* complex according to both methods, having an *rpoB* gene sequence similarity of >94% to validly published *M. terrae* complex species (10) (Fig. 1 and 2).

Two isolates matched *M. fortuitum* CIP104534^T 16S rRNA gene sequencing but did not hybridize with the *M. fortuitum*-*M. peregrinum* complex (MFO) probe of the LiPA assay. In *rpoB* gene sequencing, the two exhibited >99.7% similarity with *M. houstonense* ATCC 49403^T (AY147173) and a similarity of 98.8% with *M. fortuitum* CIP104534^T (AY147165) (Fig. 1 and 3).

Seven isolates identified as *Mycobacterium interjectum* on the basis of the 16S rRNA gene sequence were separated into three sequence types on the basis of the *rpoB*. Three isolates matched *M. interjectum* DSM44064 (HM022207); the other two sequence types, comprising of four isolates, revealed only 97.9% similarity to the type strain sequence.

For the remaining seven isolates, no good match could be found with either sequencing method. Three isolates were grouped together (rUMS_06) in both methods, but no sufficient match was found with any known *Mycobacterium* species with either method; the closest match (98.7%) on the basis of 16S rRNA genes was with *Mycobacterium agri* DSM44515, and in *rpoB* gene sequencing, the closest match, only 93%, was with *Mycobacterium elephantis* (Fig. 1 and 3).

For four unique isolates (rUMS_02, rUMS_03, rUMS_04, and rUMS_05), no match could be found in both sequencing methods. In 16S rRNA gene sequencing, rUMS_02 (isolate NLA001002310) (Fig. 1) was a 100% match for *Mycobacterium* sp. strain FI-06081 (DQ986506); the closest species match was *Mycobacterium marinum*. The other three had 99.0% similarity with

FIG 1 Similarity on the basis of 16S rRNA gene sequences ($n = 38$).

Mycobacterium brisbanense, 97.3% similarity with *M. triviale*, and 99.6% similarity with *Mycobacterium frederiksbergense* in 16S rRNA gene sequencing (Fig. 1). In *rpoB* gene sequencing, the nearest species matches for these three isolates had 90% to 97% similarities according to BLAST (Fig. 2 and 3).

INNO-LiPA assay versus *rpoB* gene sequencing. With the LiPA, 417 of the 455 isolates could be identified beyond the genus level. The majority of these isolates, 348/417 (83.5%), showed a similar result in *rpoB* sequencing (Table 1).

All isolates of *Mycobacterium xenopi* ($n = 6$), *M. marinum* ($n = 16$), and *M. haemophilum* ($n = 2$) showed more than 99.3% *rpoB* gene sequence similarity with one another; for *M. haemophilum* isolates, only a 97.9% similarity with the type strain was observed (Fig. 2).

Four (80%) of the five isolates identified as *Mycobacterium simiae* in the LiPA assay exhibited a similarity of more than 99.3% but only 98.3% similarity with the *M. simiae* type strain, ATCC 25275 (GQ153313). One isolate identified as *M. simiae* by the LiPA assay was identified as *M. tilburgii* on the basis of its *rpoB* gene sequence (Fig. 2).

The LiPA assay identified three *M. kansasii* subtypes (MKA1 to -3; *Mycobacterium gastri* is included in MKA-3). The 43 isolates identified as *M. kansasii* by the LiPA assay were separated into three discordant groupings by *rpoB* gene sequencing, with >99.7% sequence similarity within these three groups (Fig. 2 and Table 2).

According to the LiPA assay, all *M. avium* ($n = 112$) and *M. malmoense* ($n = 15$) isolates had an *rpoB* gene sequence similarity of more than 99.3% with the reference strain *Mycobacterium avium* subsp. *hominissuis* (IWGMT49; EF521911) or *M. malmoense* ATCC 29571^T (GQ153314). The other *M. avium*-*intracellulare*-*scrofulaceum* group (MAIS; according to LiPA) isolates showed more variation and could be assigned to seven published species, as depicted in Table 3 and Fig. 2.

Sixty-two isolates reacted with the *M. gordonae* probe of the LiPA, of which 49 (79%) showed >99.3% *rpoB* gene sequence similarity with *M. gordonae* ATCC 14770^T (JF346873). The remaining 13 *M. gordonae* isolates identified by the LiPA showed only 95.8% *rpoB* gene sequence similarity and were classified as *M. gordonae* complex (Fig. 2).

The LiPA separates the *M. chelonae*-*abscessus* complex into three different groups (MCH1 to -3). Isolates reacting only with MCH-1 were referred to as *M. chelonae*, whereas isolates that reacted with both MCH-1 and MCH-2 were referred to as *M. abscessus*. Reactions with the MCH-3 probe were not seen (Table 1). Twenty out of 21 (95.2%) isolates that were identified as *M. chelonae* by the LiPA were identified equally by *rpoB* gene sequencing (Fig. 3). One isolated showed 95.9% similarity with the *M. chelonae* ATCC 19237^T (EU109289) and was classified as *M. chelonae* complex.

Of the 38 isolates identified as *M. abscessus* by the LiPA, 24 (63.2%) were identified as *Mycobacterium abscessus* subsp. *abscessus* and 14 (36.8%) as *Mycobacterium abscessus* subsp. *bolletii* by *rpoB* gene sequencing. Within the *M. abscessus* subsp. *bolletii* group, a separate branch was revealed for the former *Mycobacte-*

FIG 2 Comparison of identification on the basis of the LiPA assay and phylogeny on the basis of partial *rpoB* sequencing for slow-growing nontuberculous mycobacteria ($n = 355$).

FIG 3 Comparison of identification on the basis of the LiPA assay and phylogeny on the basis of partial *rpoB* sequencing for rapidly growing nontuberculous mycobacteria ($n = 100$). “*M. abscessus* subsp. *bolletii*” refers to organisms previously known as *M. bolletii*; “*M. abscessus* subsp. *bolletii**” refers to organisms previously known as *M. massiliense*.

TABLE 2 Correlation between *rpoB* sequencing and LiPA identification of *M. kansasii* isolates

<i>rpoB</i> sequencing result	No. of samples with LiPA result			
	MKA1	MKA2	MKA3	Total
<i>M. kansasii</i> ST ^a	30	3		33
<i>M. kansasii</i> ST2	3	3	1	7
<i>M. kansasii</i> ST3		1	2	3
Total	33	7	3	43

^a More than 99.3% similarity with *M. kansasii* ATCC 12478^T (HQ880687). ST, sequence type.

rium massiliense, but it was within the cutoff value of 98.3% (Fig. 3).

Twenty-seven isolates were identified as *M. fortuitum*-*M. peregrinum* complex (MFO) by the LiPA. Nineteen (70.4%) of these isolates showed a high similarity with either the *M. fortuitum* ($n = 16$) or *M. peregrinum* ($n = 3$) type strain (Fig. 3); 16 isolates showed 99.7% similarity with *M. fortuitum* CIP104534^T (AY147165). Two isolates had >99% sequence similarity to *M. houstonense* ATCC 49403^T (AY147173) but also 98.8% sequence similarity with *M. fortuitum* CIP104534^T (AY147165), although these two isolates only reacted with the *Mycobacterium* genus probe (MYC) of the LiPA assay and not with the *M. fortuitum*-*M. peregrinum* complex (MFO) probe.

Three isolates matched *M. peregrinum* ATCC 14467^T (JF712876) and one isolate showed the highest similarities with *M. alvei* CIP103464^T (AY859697), but all four isolates showed >98.3% similarity with *M. peregrinum*, which is within the cutoff value of the RGM. Four other species were identified within the *M. fortuitum*-*M. peregrinum* complex as labeled by the LiPA assay; three isolates showed *rpoB* gene sequence similarity of 99.2% with *Mycobacterium conceptionense* CIP108544^T (AY859695), two showed similarity of 98.3% with *Mycobacterium septicum* ATCC 700731^T (AY147167), one matched *Mycobacterium porcinum* CIP105392^T (AY262737), and one showed a similarity of 99.1% with *M. setense* CIP109395^T (EU371506) (Fig. 3).

DISCUSSION

rpoB gene sequencing proved to be more discriminative than the algorithm of LiPA with adjunctive 16S rRNA gene sequencing. Combined, the LiPA assay and adjunctive 16S rRNA gene sequencing identified 390 of the 455 isolates (85.7%) as 30 validly published species and 56 isolates (12.3%) up to complex level, and nine isolates (2%) were assigned to five possibly novel species. By *rpoB* sequencing, 403 isolates (88.5%) were identified up to species level and assigned to 45 validly published species; 44 isolates (9.7%) were identified up to complex or group level, and eight isolates (1.8%) were assigned to six possibly novel species. In this set of isolates, *rpoB* gene sequencing identified 15 more known species than the LiPA with adjunctive 16S rRNA genes (*Mycobacterium vulneris*, *M. colombiense*, *M. mantenii*, *M. timonense*, *M. yongonense*, *M. heidelbergense*, *M. tilburgii*, *M. conceptionense*, *M. porcinum*, *M. houstonense*, *M. septicum*, *M. peregrinum*, *M. alvei*, *M. setense*, and *M. novocastrense*). The discriminatory power of the LiPA and 16S rRNA gene sequencing algorithm could have been higher if strains identified as *M. avium* complex or *M. fortuitum*-*M. peregrinum* had also been subjected to 16S rRNA gene sequencing. Nonetheless, the discriminatory power of partial 16S

TABLE 3 Correlation between *rpoB* sequencing and LiPA identification of *M. avium*-*intracellulare*-*scrofulaceum* group isolates

<i>rpoB</i> identification	No. of samples with LiPA result			
	MAIS	MIN-1	MIN-2	MSC
<i>M. intracellulare</i>	6	15	2	0
<i>M. chimaera</i> ^a	0	0	22	0
MAC ^b	11	2	0	0
<i>M. yongonense</i>	0	1	0	0
<i>M. colombiense</i>	2	0	0	0
<i>M. mantenii</i>	1	0	0	0
<i>M. timonense</i>	3	0	0	0
<i>M. vulneris</i>	1	0	0	0
<i>M. heidelbergense</i>	1	0	0	0
<i>M. parascrofulaceum</i>	0	0	0	2
rUMS01	1	0	0	0
Total	26	18	24	2

^a 100% match with *M. chimaera* CIP107892^T (GQ153309) and >99.3% sequence similarity with *M. intracellulare*.

^b More than 94% but less than 99.3% similarity with MAC species.

rRNA gene sequencing is limited among NTM, particularly among RGM (7, 11). This contributes to the value of the LiPA assay, in addition to its being a more rapid approach than sequencing. Also, for 16S rRNA genes, it is difficult to differentiate between species, because sequences can vary within species (7). Two isolates (NLA001002096 and NLA001100042) were considered UMS on the basis of a 1-bp mismatch in 16S rRNA gene sequences, whereas by *rpoB* sequencing, the isolates were identified to species level as *M. phlei* and *M. novocastrense*.

Additionally, *rpoB* gene sequencing showed diversity greater than the cutoff for separate species designation within known species, which could in time result in a more precise taxonomy of NTM.

rpoB gene sequencing has been applied in several studies, mostly concentrating on a certain group of NTM, like *M. avium* complex (MAC) or RGM (8, 9). Two studies tested a larger variation of NTM species, from both human and veterinary samples (15, 16). However, the general utility of *rpoB* sequencing for identification of NTM isolates received at a *Mycobacterium* reference laboratory remained unclear. Two studies on the usefulness of *rpoB* sequencing for identification reported a slightly lower percentage of isolates identified to species level: 84% in the study by Simmon et al. of 139 human clinical isolates (16) and 80% in the study by Higgins et al. of 386 animal isolates (15).

The largest difference between *rpoB* sequencing and the LiPA/16S rRNA gene approach appears to be due to species being grouped together by LiPA/16S rRNA gene but not by *rpoB* sequencing. Misidentification or oversimplified identification of species by the LiPA has already been reported for *M. heidelbergense*, *M. mantenii*, and *M. parascrofulaceum*; also, the *M. fortuitum*-*M. peregrinum* complex consists of more valid species than *M. fortuitum* and *M. peregrinum* (17, 18). We also noted that the LiPA did not identify two *M. houstonense* isolates as *M. fortuitum*-*M. peregrinum* complex members, though by 16S rRNA gene and *rpoB* gene sequencing, this species is part of the *M. fortuitum*-*M. peregrinum* complex.

Both the *M. haemophilum* and *M. simiae* isolates in our study group had a high degree of mutual similarity but did not show a high similarity with the type strain *rpoB* gene sequence present in

GenBank. The taxonomy in the *M. simiae* group has been updated frequently in the past few years and has been found to be more diverse than previously thought (19, 20). The variation could also be due to a poorly selected type strain, as was also noted for *M. terrae* by Tortoli et al. (12).

Our findings indicate that there is more genetic variation within a species than previously suspected, for example, in *M. interjectum*. Other NTM species have already been grouped into complexes which cluster closely together on the basis of at least 94% similarity, like the MAC, *M. chelonae-abscessus* complex, *M. fortuitum* complex, and *M. terrae* complex (12). But a higher degree of genetic divergence could be disclosed in groupings like *M. kansasii* (21) and *M. gordonae* (22). Perhaps these two should be regarded as complexes of closely related species, rather than single species.

Four groups of species had debatable mutual similarities: *M. intracellulare* and *M. chimaera* (formerly *M. intracellulare* sequence var Mac-A [23]), *M. fortuitum* and *M. houstonense*, *M. peregrinum* and *M. alvei*, and the extensively discussed *M. abscessus* subsp. *bolletii*, which was previously divided into *M. bolletii* and *M. massiliense* (24). The latter three showed more similarity with one another than the cutoff value for separate species status allows (8, 9), but *rpoB* gene sequencing does separate them into different branches, enabling discrimination of the different groupings. Most strains identified as *M. chimaera* by the LiPA had identical *rpoB* gene sequences but shared a very high degree of similarity with the more variable *M. intracellulare*. On the basis of *rpoB* gene sequencing, *M. chimaera* seems to be a subspecies of *M. intracellulare*.

The separate species status of *M. intracellulare*, *M. chimaera*, *M. fortuitum*, *M. houstonense*, *M. peregrinum*, *M. alvei*, *M. bolletii*, and *M. massiliense* remains questionable if the cutoffs for separate species status (8, 9) are strictly applied. It should be noted that Adékambi et al. (9) found a 98.2% similarity between *M. fortuitum* and *M. houstonense*, and Ben Salah et al. (8) found a 99.2% similarity between *M. intracellulare* and *M. chimaera*, which is enough variation to justify separation into different species according to the cutoff value (8, 9). However, Simmon et al. also noted a lack of interspecies variability for these four species (16). The general problem is that in mycobacteriology, no cutoffs for separate species based on any genetic targets have been set and agreed upon (7).

The high degree of similarity between the subspecies and the complicated interpretation of *rpoB* gene sequencing results have led to a change in status for *M. massiliense* and *M. bolletii* from separate species to subspecies, and they are now referred to as *M. abscessus* subsp. *bolletii* (24). However, separation of *M. massiliense* from *M. bolletii* and *M. abscessus* subsp. *abscessus* has clinical relevance, as *M. massiliense* is susceptible to macrolides; e.g., clarithromycin-based treatment regimens are more successful in treatment of *M. massiliense* disease than disease caused by *M. bolletii* and *M. abscessus* subsp. *abscessus* (25).

It has been noted that *rpoB* sequencing alone can, in rare instances, misidentify the *M. abscessus* subspecies, possibly due to horizontal gene transfer (26). However, horizontal gene transfer would also affect performance of other targets, such as the *hsp65* gene (11, 26). *rpoB* gene sequencing is still a reasonable first approach; for *M. abscessus* an interesting second step is *erm*(41) gene sequencing, which identifies the different subspecies and predicts macrolide susceptibility (25).

One limitation of *rpoB* sequencing is the absence of sequence data for type or reference strains of the less commonly encountered NTMs in the GenBank database, as we recorded for *M. bohemicum*, *M. heckeshornense*, and *M. conspicuum*. *rpoB* gene sequences identified beyond doubt using other targets should be added to the GenBank database. The other limitation, relevant to any genetic target, is the lack of quality assurance in public sequence databases, which may hamper comparisons and requires strict monitoring of results (7, 11). Public databases inherently suffer from data quality issues, whereas closed, quality-controlled database inherently suffer from data quantity issues, i.e., they are incomplete (7, 11).

Identifications of NTM based on one genetic target may always result in limited reliability, and sequencing of additional targets will increase the quality of identifications. To resolve issues related to taxonomic status, the definite separate species definition should be provided by whole-genome sequencing of a large collection of NTM isolates. A more exact identification will be most helpful for studying differences in clinical relevance, environmental sources, and epidemiology of human NTM disease (3).

In conclusion, partial *rpoB* gene sequencing has a higher discriminatory power than a LiPA with adjunctive partial 16S rRNA gene sequencing. Most clinically important species have *rpoB* gene sequences of type strains available through public databases. On the basis of the data presented here, we consider *rpoB* sequencing an appropriate first-line identification method for NTM isolated from human samples.

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