Received: 14 April 2016 | Accepted: 24 January 2017

DOI: 10.1002/jcla.22184

RESEARCH ARTICLE

WILEY

Identification of nontuberculous mycobacteria using multilocous sequence analysis of 16S rRNA, *hsp65*, and *rpoB*

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Funding information
National Research Foundation of Korea (NRF); Ministry of Science; ICT; Future Planning, Grant/Award Number: 2013R1A1A1007615

Background: The isolation of nontuberculous mycobacteria (NTM) from clinical specimens has increased, and they now are considered significant opportunistic pathogens. The aims of this study were to develop a database and interpretive criteria for identifying individual species. In addition, using clinical isolates, we evaluated the clinical usefulness of 16S rRNA, *hsp65*, and *rpoB* as target genes for this method.

Methods: The sequences of NTM for 16S rRNA, *hsp65*, and *rpoB* were collected from GenBank and checked by manual inspection. Clinical isolates collected between 2005 and 2010 were used for DNA extraction, polymerase chain reaction, and sequencing of these three genes. We constructed a database for the genes and evaluated the clinical utility of multilocus sequence analysis (MLSA) using 109 clinical isolates.

Results: A total 131, 130, and 122 sequences were collected from GenBank for 16S rRNA, *hsp65*, and *rpoB*, respectively. The percent similarities of the three genes ranged from 96.57% to 100% for the 16S rRNA gene, 89.27% to 100% for *hsp65*, and 92.71% to 100% for *rpoB*. When we compared the sequences of 109 clinical strains with those of the database, the rates of species-level identification were 71.3%, 86.79%, and 81.55% with 16S rRNA, *hsp65*, and *rpoB*, respectively. We could identify 97.25% of the isolates to the species level when we used MLSA.

Conclusion: There were significant differences among the utilities of the three genes for species identification. The MLSA technique would be helpful for identification of NTM.

KEYWORDS
16S rRNA gene, *hsp65*, multilocus sequence analysis, nontuberculous mycobacteria, *rpoB*

1 | INTRODUCTION

Nontuberculous mycobacteria (NTM) are *Mycobacterium* species other than *M. tuberculosis* (MTB) complex and *M. leprae*. Most NTM are present in the environment, such as in water, soil, milk, and animals, and are recognized as colonizers or contaminants in the respiratory tract without causing any symptom.[1–3] However, NTM infections are increasing in immunocompromised patients, so the organisms are now considered significant opportunistic pathogens.[4,5]

Like MTB complex, NTM affects the lungs, and the symptoms are similar to those of tuberculosis (TB). Pulmonary NTM infections are caused mainly by *Mycobacterium avium* complex (MAC), *M. kansasii*, *M. abscessus*, *M. chelonae*, and *M. fortuiutm*. Pulmonary infection caused by MAC is often refractory to rifampin and ethambutol because of innate drug resistance. Other NTM infections are resistant to many common antimicrobial agents, and there are differences in the appropriate antibiotics according to the particular NTM species causing the infection. Therefore, accurate identification of NTM to the species level is necessary for appropriate treatment of infections.

Traditionally, the identification of *Mycobacterium* was based on the phenotypic biochemical characteristics.[6] However, these tests are time-consuming and cumbersome and sometimes produce ambiguous

results. Many molecular methods have been developed for NTM identification to overcome the limitations of conventional methods.[7–13] Gene sequencing is considered the gold standard for identification of *Mycobacterium* species. The 16S rRNA gene is most commonly studied for bacterial identification and is the first choice for sequence analysis. However, this gene has highly similar sequences in different *Mycobacterium* species, so it may not distinguish closely related species.[8,9,14,15] To overcome these limitations, recent efforts have focused on the use of other genes such as *hsp65* and *rpoB* for accurate identification of NTM.

To analyze the sequence data, it is necessary to use public or private databases of bacterial nucleotides. GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) is the most commonly used public database.[16,17] However, it contains some unreliable sequence data because of the absence of a specific validation system. The size and location of sequence data seem to be different according to registered sequences. Therefore, it is necessary to select reliable sequences of type strains and to construct databases of 16S rRNA, *hsp65*, and *rpoB* for NTM identification. Interpretive criteria are required for the standardization of mycobacterial identification. The Clinical Laboratory Standards Institute (CLSI) Guideline suggests interpretive criteria for identity scores to analyze the sequence data for the 16S rRNA gene.[18] However, this guideline is not applicable to other genes.

The aims of this study were to construct a sequence database containing type strains that are most suitable for identification of NTM and to determine the appropriate interpretive criteria for sequence-based identification using 16S rRNA, *hsp65*, and *rpoB*. In addition, using clinical isolates, we evaluated the usefulness of the three genes as targets for identification of NTM.

2 | MATERIALS AND METHODS

2.1 | Sequence database for *Mycobacterium*

2.1.1 | Collection of three gene sequences

The sequences of 16S rRNA, *hsp65*, and *rpoB* from the type, reference, or published strains were collected from GenBank. All sequences were checked by manual inspection for the culture collection catalogue number and relevant publications. Preferentially, for the 16S rRNA gene, we selected the sequence of the type strain and the longer sequence. For *hsp65* and *rpoB*, we preferentially selected the sequences of the type strains and the particular gene positions that are most commonly used.[19,20] We aligned the sequences using the multiple sequence alignment tool CLUSTAL W to check the position of the sequences in each gene.[21]

2.1.2 | Multiple sequence alignment and phylogenetic analysis

The percentage of similarity and multisequence alignment were calculated using the CLUSTAL W program and MEGA software. Phylogenetic analysis was conducted by MEGA software version 4.0 using the neighbor-joining method with Kimura's two-parameter procedure.[22]

2.1.3 | Difference of sequences between NTM species

The percent identity score is the minimum percent similarity of the first-matched sequence, and the cutoff value is the percent difference in the sequences of the first-matched and second-matched strains. We investigated most suitable score and value using interspecies divergence rates of each gene for species identification of mycobacteria and determined the criteria on the basis of both the % identity score and the cutoff value that can identify at least 70% to 80% of mycobacteria to the species level.

2.2 | Sequence analysis of clinical isolates

2.2.1 | Clinical isolates

A total of 109 isolates were collected from our clinical microbiology laboratory from 2005 to 2010. These were identified as routine testing by PCR-RFLP [23] and the line probe assay.[24] We chose NTM isolates so as to include a large number of species.

2.2.2 | DNA extraction and sequencing

The colonies, grown on Ogawa medium, were scraped into a 1.5 mL microtube, and 50 μL of extraction buffer containing ion-exchange resin was added to the pellet. After heating at 100°C for 20 min and centrifugation at 10 000-20 000 *g* for 2-3 min, the supernatant liquid, containing the mycobacterial DNA, was stored at −20°C until use. The primer and PCR condition for each gene are described Table 1. A sequencing reaction was carried out on both strands using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) using the same primer employed for PCR and applied to run on the ABI PRISM 3130 genetic analyzer (Applied Biosystems).

2.2.3 | Interpretation of sequencing results

The sequences of each gene were compared with our collected database to get a % similarity. We investigated the differences in

TABLE 1 Primers used in this study

Gene	Direction[a]	Sequence (5′→3′)	Size (bp)
16S rRNA	F	AGA GTT TGA TYM TGG CTC AG	495
	R	TTA CCG CGG CKG CTG GCA C	
hsp65	F	ACC AAC GAT GGT GTC TCC A	439
	R	CTT GTC GAA CCG CAT ACC CT	
rpoB	F	CGA CCA CTT CGG CAA CCG	342
	R	TCG ATC GGG CAC ATC CGG	

[a]F and R represent forward and reverse, respectively.

TABLE 2 Characteristics of sequences of 16S rRNA, *hsp65*, and *rpoB* genes by type of strain

	16S rRNA gene		*hsp65*		*rpoB*	
Classification	**Length (bp)**	**Strain (N)**	**Length (bp)**	**Strain (N)**	**Length (bp)**	**Strain (N)**
Type strain	Full sequence	127	≥600	52	398	90
	555	1	<600	71	306-424	27
Reference strain			<600	2	398	1
			≥600	3	423, 306	2
Published strain	Full sequence	2	603	1	306	2
Strain[a]	446	1	401	1		
Absent[b]				1		9
Total	131	131	131			

[a]Strain is neither a type, reference, nor published.
[b]Not containing sequences.

inter-species and intra-species sequence similarity to determine the interpretive criteria for NTM. In addition, we evaluated the diagnostic utility of individual genes and the combination of the three genes for the identification of NTM in clinical isolates.

3 | RESULTS

3.1 | Characteristics of 16S rRNA, *hsp65*, and *rpoB* sequences

For 16S rRNA, we collected 128 type strain sequences of *Mycobacterium* species and subspecies among the 131 species and subspecies. We found two sequences published in a journal, *M. haemophilus* and *M. leprae*, and one GenBank strain, *M. lepraemurium*. For *hsp65*, five sequences, from *M. avium*, *M. genavense*, *M. boenickei*, *M. bohemicum*, and *M. gilvum*, were the reference strains; and *M. lepraemurium* and *M. leprae* were a published and a GenBank strain, respectively. One strain, *M. pinipedii*, had no data available on the sequence of *hsp65*. Three of the reference strains (*M. brisbanense*, *M. gilvum*, and *M. genavense*) and two published strains (*M. massiliense* and *M. leprae*) were included in *rpoB* instead of those of the type strain. We could not find *rpoB* sequences for nine species among the 131 *Mycobacterium* isolates. These were *M. pinipedii*, *M. boenickei*, *M. colombiense*, *M. houstonense*, *M. lepraemurium*, *M. pyrenivorans*, *M. salmoniphilum*, *M. setense*, and *M. tusciae* (Table 2).

3.2 | Details of 16S rRNA, *hsp65*, and *rpoB* sequences

3.2.1 | Sequence similarity of three genes

The first-matched percent similarities between two species ranged from 96.57% to 100% (median 99.32%), 89.27% to 100% (median 97.45%), and 92.71% to 100% (median 96.56%) for 16S rRNA, *hsp65*, and *rpoB*, respectively. This means that the interspecies divergence rates were from 0 to 3.43% (median 0.68%), 0 to 10.73% (median 2.55%), and 0 to 7.29% (median 3.45%) for 16S rRNA, *hsp65*, and *rpoB*, respectively. We determined % identity values of 99%, 96%, and 96% and cutoff values of 0.2%, 0.3%, and 0.3% for 16S rRNA, *hps65*, and *rpoB*, respectively. 16S rRNA sequence of 11 species was identical to one or more species (Table 3). Four and two species had 100% similarity in *hsp65* and *rpoB*, respectively. These isolates could not be distinguished to the species level by these genes.

3.2.2 | Phylogenetic relations of three genes

A phylogenetic tree was analyzed to check the grouping ability of similar species using the Clustal W and MEGA programs. Thirty-seven isolates could not be discriminated at the species level by the 16S rRNA gene because these strains have identical sequences. However, the cluster pattern is different when using *hsp65* and *rpoB*. The subspecies of *M. avium* and *M. fortuitum* could not be differentiated by any of the three genes.

3.3 | Identification results of clinical isolates

3.3.1 | 16S rRNA gene

A total of 97 from 109 clinical isolates were analyzed and compared with our database. The ranges of % similarity with the first-matched, closest strains were between 97.90% and 99.90% (median 99.69%). The % identity scores of 81 strains were higher than 99.0%, whereas 16 strains showed <99.0% similarity and could not be identified to the species level. Among these 81 strains, 59 satisfied the cutoff value for species identification, so 60.83% of the clinical isolates could be identified to the species level by 16S rRNA gene sequencing. To confirm the identification of the remaining 38 strains, we performed phylogenetic tree analysis (data not shown). Ten strains could be identified, being *M. gordonae* (N=5), *M. septicum* (N=2), *M. fortuitum* (N=1), and *M. cookie* (N=2). Therefore, the rate of species-level identification using the 16S rRNA gene was 71.13% (69/97).

TABLE 3 *Mycobacterium* species sharing high homology of gene sequences

Gene	Species	Interspecies divergence (%)
16S rRNA	*M. abscessus/M. bolletii/M. massiliense*	0
	M. austroafricanum/M. vanbaalenii	0
	M. kansasii/M. gastri	0
	M. senegalense/M. houstonense	0
	M. mucogenicum/M. phocaicum	0
	M. caprae/M. bovis/M. tuberculosis/M. africanum/M. microti/ M. pinnipedii	0-0.07
	M. boenickei/M. neworleansense	0.07
	M. chimaera/M. intracellulare	0.07
	M. conceptionense/M. farcinogenes	0.07
	M. murale/M. tokaiense	0.07
	M. neworleansense/M. boenickei/M. porcinum	0.07
	M. marinum/M. ulcerans	0.14
hsp65	*M. parafortuitum/M. neoaurum*	0
	M. simiae/M. genavense	0
	M. bovis/M. microti/M. caprae/M. tuberculosis/M. africanum	0-0.25
	M. houstonense/M. farcinogenes	0.26
rpoB	*M. africanum/M. caprae/M. microti/M. bovis*	0
	M. chimaera/M. intracellulare	0
	M. bolletii/M. phocaicum	0
	M. murale/M. tokaiense	0.25

3.3.2 | *hsp65*

A total 106 clinical isolates were analyzed. The ranges of % similarity with the first-matched, closest strains were between 93.15% and 100% (median 99.25%). Five strains showed <96.0% similarity, and these could not be identified to the species level. Among these 101 strains, 78 satisfied the cutoff value for species identification, so 73.85% of the clinical isolates could be identified to the species level by *hsp65* sequencing. To confirm the identification of the remaining 28 strains, we performed phylogenetic tree analysis. In this way, 14 strains could be identified, being *M. gordonae* (N=4), *M. peregrinum* (N=3), and one each of *M. nebraskense*, *M. terrae*, *M. massiliense*, *M. phocaicum*, *M. porcinum*, *M. septicum*, and *M. pulveris*. Therefore, the rate of species-level identification using *hsp65* was 86.79% (92/106).

3.3.3 | *rpoB*

A total of 103 clinical isolates were analyzed. The ranges of % similarity with the first-matched, closest strains were between 94.43% and 100% (median 98.76%). Eleven strains showed <96.0% similarity, and these could not be identified to the species level. Among the 92 identifiable strains, 75 satisfied the cutoff value for species identification, so 72.82% of the clinical isolates could be identified to the species level by *rpoB* sequencing. To confirm the identification of the remaining 28 strains, we performed phylogenetic tree analysis. Nine strains were identified as *M. arupense* (N=3), *M. cookie* (N=2), and one each of *M. nonchromogenicum M. senuense*, *M. interjectum*, and *M. parmense*. Therefore, the rate of species-level identification using *rpoB* was 81.55% (84/103).

We compared the identification results of the three genes. The rates of species-level identification using the 16S rRNA, *hsp65*, and *rpoB* sequences were 71.13%, 86.79%, and 81.55%, respectively. Thirty isolates were identified as concordant results for all three genes, and 57 isolates were identified by only one or two genes. The identification rate by combination analysis of the three genes was 97.25% (106/109). Of the 106 isolates, 19 could be identified to the clade level only: *M. fortuitum* (N=6), *M. terrae* (N=7), *M. simiae* (N=4), and *M. chelonae* clade (N=2) (Table 4). Three isolates showed different results for 16S rRNA, *hsp65*, and *rpoB*, so we could not identify these isolates (Table 5).

4 | DISCUSSION

Infections caused by NTM have been increasing worldwide.[25–28] In Korea, the prevalence of NTM increased from 22.2% in 2002 to 45.9% in 2008, and there was a similar trend in the numbers of patients with NTM lung diseases.[29] So, there is a significant need for accurate methods for the identification of NTM.

Traditionally, the identification of *Mycobacterium* species was based on phenotypic methods employing growth rate and pigmentation. However, these methods require several days to a few weeks because of the slow growth of these organisms. Many clinical researchers face a difficulty in identification owing to similar

TABLE 4 Final identification of species distributions of 106 *Mycobacterium* clinical isolates

Species	No.
M. avium	10
M. intracellulare	9
M. kansasii	9
M. gordonae	8
M. terrae clade	7
M. arupense	6
M. fortuitum	6
M. fortuitum clade	6
M. abscessus	5
M. chelonae	5
M. lentiflavum	4
M. simiae clade	4
M. mucogenicum	3
M. peregrinum	3
M. septicum	3
M. goodii	2
M. phocaicum	2
M. scrofulaceum	2
M. cookii	2
M. chelonae clade	2
Others[a]	8

[a]Others: *M. conceptionense*, *M. kumamotonense*, *M. obuense*, *M. gastri*, *M. parascrofulaceum*, *M. porcinum*, *M. interjectum*, and *M. senuense*.

biochemical profiles.[30] To overcome these limitations, many molecular techniques have been introduced. At present, PCR-RFLP and the line probe assay are most popular in clinical laboratories. Gene sequencing based on PCR is the gold standard for bacterial identification, and several specific genes are employed for NTM. However, the clinical performance and characteristics of each gene have been rarely reported.

Three are some difficulties in identifying NTMs using sequencing analysis. First, there is no standardized database for comparison of sequence results. We use a public database, such as GenBank, but this site contains too many incorrect sequences and changes daily, so it is not adequate for use in the clinical laboratory. Second, we are faced with problems in interpreting the results. Therefore, it is essential to have a standardized database and appropriate guidelines for the correct identification of NTM. So, we collected genetic sequences of type, reference, and other published strains from GenBank.

We could evaluate the ability of each gene to identify NTM among clinical isolates using established databases. In our study, 19 isolates were not identified by the 16S rRNA gene because of high similarity. Similar results have already been reported by Slany et al.[31] Eleven isolates were not identified to the species level by *hsp65* sequencing, and these strains usually were members of the *M. simiae* and *M. fortuitum* groups. For *rpoB*, we could not identify 13 isolates to the species level, and most of them were part of the *M. terrae* clade. Kim et al.[32] reported the clinical utility of *rpoB* sequencing for NTM identification, but their study did not include *M. terrae* clade strains. Therefore, we discovered a limitation of *rpoB* for the identification of members of the *M. terrae* clade. A similarity of 99% is believed to be a minimum requirement for bacterial identification using the 16S rRNA gene. In this study, the similarities of 16 isolates were <99.0%. When we compared these results with phylogenetic analysis, 7 of 16 strains could be identified to the species level. These organisms were *M. gordonae* in five cases and *M. cookii* in two. Therefore, we can presume high variation of the 16S rRNA gene in these species.

When comparing the three genes, *hsp65* and *rpoB* had better identification ability than 16S rRNA. For example, the *M. abscessus-chelonae* group and *M. intracellulare/M. chimaera* could not be differentiated to the species level by 16S rRNA analysis. This result is a well-known limitation of using 16S rRNA for NTM identification. In addition, three strains of *M. peregrinum* were misidentified as *M. septicum* by 16S rRNA sequencing. *M. kansasii* and *M. gastri* were not discriminated by 16S rRNA gene analysis, whereas it was possible to distinguish them using *hsp65* and *rpoB*. Four strains of *M. lentiflavum* were misidentified as *M. triplex/M. montefiorense* using *hsp65*. However, they were identified as *M. lentiflavum* by GenBank. So, we recognize the limitations of our database. For *rpoB*, *M. gordonae* and *M. asiaticum* were not differentiated. It is essential to know the limitations of each gene for sequencing analysis. Only <30 mycobacterial species were included in this study. This point is the major limitation of this study, and further evaluation is needed.

Finally, we were able to collect large numbers of type, reference, and published *Mycobacterium* sequences of 16S rRNA, *hsp65*, and *rpoB*. This database is valuable for the identification of NTM isolated from clinical specimens. There were significant differences in identification ability among the three genes; therefore, multilocus sequence analysis would be useful to identify NTM.

TABLE 5 Conflicting results in comparison of three genes

Strain No.	16S rRNA gene	*hps65*	*rpoB*
NTM08-015	*M. seooulense/M. nebraskense*	*M. nebraskense/M. triplex*	*M. parmense*
NTM09-055	*M. septicum*	*M. hiberniae*	*M. arupense/M. kumamotonense*
NTM09-230		*M. pulveris*	*M. elephantis*

ACKNOWLEDGMENTS

This research was supported by Basic Science Research program through National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, and Future Planning (2013R1A1A1007615).

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How to cite this article: Kim SH, Shin JH. Identification of nontuberculous mycobacteria using multilocous sequence analysis of 16S rRNA, *hsp65*, and *rpoB*. *J Clin Lab Anal*. 2018;32:e22184. https://doi.org/10.1002/jcla.22184