Discovery of a Novel hsp65 Genotype within Mycobacterium massiliense Associated with the Rough Colony Morphology

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Abstract

So far, genetic diversity among strains within Mycobacterium massiliense has rarely been studied. To investigate the genetic diversity among M. massiliense, we conducted phylogenetic analysis based on hsp65 (603-bp) and rpoB (711-bp) sequences from 65 M. massiliense Korean isolates. We found that hsp65 sequence analysis could clearly differentiate them into two distinct genotypes, Type I and Type II, which were isolated from 35 (53.8%) and 30 patients (46.2%), respectively. The rpoB sequence analysis revealed a total of four genotypes (R-I to R-IV) within M. massiliense strains, three of which (R-I, R-II and R-III) correlated with hsp65 Type I, and other (R-IV), which correlated with Type II. Interestingly, genotyping by the hsp65 method agreed well with colony morphology. Despite some exceptions, Type I and II correlated with smooth and rough colonies, respectively. Also, both types were completely different from one another in terms of MALDI-TOF mass spectrometry profiles of whole lipid. In addition, we developed PCR-restriction analysis (PRA) based on the Hinf I digestion of 644-bp hsp65 PCR amplicons, which enables the two genotypes within M. massiliense to be easily and reliably separated. In conclusion, two distinct hsp65 genotypes exist within M. massiliense strains, which differ from one another in terms of both morphology and lipid profile. Furthermore, our data indicates that Type II is a novel M. massiliense genotype being herein presented for the first time. The disparity in clinical traits between these two hsp65 genotypes needs to be exploited in the future study.

Introduction

Rapidly growing mycobacteria (RGM) are ubiquitous organisms increasingly emerging as important human pathogens. Recently, there have been more frequent reports of RGM infections in immunocompetent people as well as in people with predisposing factors or those who are immunosuppressed [1,2]. In particular, among RGMs, Mycobacterium abscessus is commonly associated with wound infection and abscess formation and is the RGM that most frequently causes chronic lung disease. M. abscessus is also notable for its resistance to treatment and the poor clinical outcome of infection with the organism [2]. In South Korea, in contrast to countries such as the United States and Japan [3–5], infection of M. abscessus is the most prevalent RGM infection, and second only to the M. avium complex for nontuberculous mycobacterium (NTM) [6,7].

Recent application of multilocus sequencing has broadened our knowledge about the diversity between the M. abscessus complex. Two new species of mycobacteria closely related to M. abscessus, M. massiliense and M. bolletii, have been described [8,9]. A recent molecular epidemiologic study using 144 RGM isolates from Korean patients showed that two M. abscessus related species, M. abscessus and M. massiliense, were responsible for the most of the infections [M. abscessus (65/144 isolates, 51.2%) and M. massiliense (39/144 patients, 46.5%)]. However, M. bolletii has rarely been isolated from Korean patients [2/144 patients, 1.6%] [10]. It should be noted that the disparity between M. abscessus and M. massiliense infections in terms of clinical significance was reported by a recent paper using Korean patients infected with either M. abscessus or M. massiliense, thereby putting the stress on the separation between the two related species via molecular - based methods in the clinical aspects [11].

As alternatives to 16 S rRNA gene sequencing, several other gene targets have been effectively used for the NTM differentiation, demonstrating the limited value in the separation between some closely related NTM strains [12–16]. Among these gene targets, partial sequencing that targeted the rpoB or hsp65 has
increasingly been used to differentiate between M. abscessus, M. massiliense, and M. bolletii [8,10,11,17,19].

Due to the lack of clinical or epidemiological information regarding M. massiliense, studies about genetic or phenotypic traits of M. massiliense have rarely, if ever, been introduced, as compared to M. abscessus. Thus, the present study aims to elucidate the genetic diversity between M. massiliense clinical isolates by two different chronometer molecules - hsp65 and rpoB - genes and to identify the relationships between the determined genotype and phenetic traits, particularly in terms of colony morphology.

Results

Identification by hsp65 Sequencing Analysis

First, 109 M. abscessus complex strains from Asan medical center (AMC), which had been identified by rpoB PRA, were further analyzed using the hsp65 sequencing method, showing the prevalence of 44 M. abscessus (40.4%) and 65 M. massiliense strains (59.6%) (Table 1). However, no M. bolletii strains were found in our cohort (0%). Phylogenetic analysis based on the partial hsp65 sequence (603 bp) showed that there were two phylogenetic groups (Type I and II) within the 65 M. massiliense strains, composed of three sequevars (Figure 1 and 2A). Type I isolated from 35 patients (53.8%) included a single sequevar having the same 603-bp hsp65 sequences as M. massiliense CIP 108297T. But Type II, also isolated from 30 patients (46.2%), included two different sequevars, Type II-1 and Type II-2, which showed 2-bp (T444A and C714A) and 1-bp different sequences (T444A) from M. massiliense CIP 108297T, respectively (Table 2, Figure 1). Type I, Type II-1, and Type II-2 were isolated from 35 (53.8%), 25 (38.5%) and 5 patients (7.7%), respectively (Figure 2A).

rpoB Sequence Analysis of M. massiliense Strains

The partial rpoB sequences (711-bp) from the 65 M. massiliense strains were compared with each other to check the genetic heterogeneity between them. The rpoB based sequence analysis revealed the presence of four groups within the 65 M. massiliense strains, suggesting more genetic diversity within M. massiliense strains in the 711-bp rpoB sequence than in the 603-bp hsp65 sequence. All of the isolates showed different rpoB sequences than the M. massiliense CIP 108297T with sequence divergence ranging from 2-bp (R-I, R-II, and R-IV) to 14-bp (R-III). These groups were clearly separated by phylogenetic analysis based on rpoB gene sequences of the 65 M. massiliense strains (Figure 2B). Despite some minor exceptions, the genotype R-I, R-II, R-III and R-IV of four rpoB genotypes were related to the hsp65 Type I and Type II, respectively. The R-I were found most frequently in M. massiliense Type I (68.6%, 24/35 strains). The R-II, which had an rpoB sequence that was 2-bp different rpoB sequence (T2760C and G2907A) from M. massiliense CIP 108297T were most frequently found in M. massiliense Type II (93.3%, 28/30 strains), but not in Type I (Table 3).

Relationships between hsp65 Genotypes and Colony Morphology

Sub-cultured colonies of the 65 M. massiliense strains identified by hsp65 sequence analysis and M. massiliense CIP 108297T were analyzed on 7H10 agar plates. Notably, substantial differences in colony morphology were found between two hsp65 genotypes, Type I and Type II (Figure 3). Interestingly, all the 30 of the isolates belonging to Type II (Type II-1 and Type II-2) showed rough colony without any exception. However, the Type I isolates showed both rough and smooth colony morphologies. In Type I, as shown in M. massiliense CIP 108297T, smooth morphology was more common than rough morphology [smooth vs. rough; 23/35 isolates (65.7%) vs. 12/35 isolates (34.3%)] (Table 2). In addition to the different colony morphologies in the agar plates, Type I smooth morphotype and Type II of rough morphotype also differed in terms of the growth characteristics of 7H9 broth cultures. While Type I strain and M. massiliense CIP 108297T with smooth morphotype showed a dispersed growth pattern, the Type II isolate showed a typical aggregative pellicle growth that is confined to the surface of the medium, as shown in the M. tuberculosis clinical isolates (Figure 3). But, the Type I strain with rough morphotype was quite similar to Type II in terms of the growth pattern of the broth culture (data not shown).

Determination between Two Genotypes of M. massiliense Strains by Hinfl PRA

To enable the simple separation of M. massiliense Type II strains from other related RGMs, we developed a novel Hinfl PRA algorithm. The results and the algorithm obtained by applying the Hinfl I PRA method to the 65 M. massiliense-related strains and five reference strains are summarized in Table 2 and Figure 4A, respectively. As predicted, the M. massiliense Type II strains were clearly distinguished from other related RGMs, including M. massiliense Type I strains, producing distinct PRA patterns (200, 278, and 86-bp), although it was not possible to separate between the upper two bands, 280 and 278-bp (Figure 4B). When the results of both the Hinfl I PRA and hsp65 sequencing methods were compared, the sensitivity and specificity of our Hinfl I PRA method for separating between the two genotypes of M. massiliense were 100% and 100%, respectively (Table 2). The informations about rpoB and hsp65 genotypes, Hinfl I PRA patterns and colony morphology of each isolate were shown in Table S1.

Biochemical Tests and Drug Susceptibility Tests

The phenotypic characteristics of three reference strains, four M. massiliense Type I strains, and four M. massiliense Type II strains were analyzed and compared. With the exception of the colony morphology, no characteristic traits were not found to differentiate between Type I and Type II groups (Table S2). The results of our drug susceptibility test likewise showed no significant differences between Type I and Type II groups (Table S3). In addition, no point mutations were found at the adenine position 2058 (A2056G) or 2059 (A2059G) in the pep pidyltransferase region of the 23 S rRNA gene in any of the 65 M. massiliense strains (data not shown).

Comparison of HPLC and MALDI-TOF Mass Spectrometry Profiles between Two Genotypes of M. massiliense

The profiles of HPLC and MALDI-TOF mass spectrometry of M. massiliense CIP 108297T, four M. massiliense Type I strains, and four M. massiliense Type II strains were analyzed and compared. Generally, the HPLC profiles of all the M. massiliense strains were virtually identical. However, both genotypes showed different profiles at the peak of about 1.91 retention time to be separated between the MALDI-TOF mass spectrometry profiles were found between the two genotypes. Usually, the MALDI-TOF mass spectrometry profiles have two distinct clusters of peaks ranging from m/z 1171 to m/z 1316 and from m/z 1360 to m/z 1464. The first cluster and the second cluster represent diglycosylated glycopeptidolipid (GPL) and triglycosylated GPL, respectively [19]. All four of the Type I strains and M. massiliense
Figure 1. Sequence polymorphisms between the three hsp65 sequevars of M. massiliense. Type I strains have the same sequence as the M. massiliense type strain; however, Type II-1 and Type II-2 strains differed from the M. massiliense type strain by 2-bp (T444A and C714A) and 1-bp (T444A), respectively. The nucleotide numbers correspond to those from the complete sequence of the heat shock protein 65 kDa (hsp65) gene of M. abscessus ATCC 19977 (GenBank no. CU458896). doi:10.1371/journal.pone.0038420.g001

Figure 2. Phylogenetic trees based on the hsp65 gene (603 bp) and rpoB gene (711 bp) sequences. Phylogenetic trees based on the (A) hsp65 gene (603 bp) and (B) rpoB gene (711 bp) sequences from M. massiliense clinical isolates, M. massiliense CIP 108297T, M. bolletii CIP 108541T, M. chelonae ATCC 19237, and M. abscessus ATCC 19977. These trees were constructed using the neighbor-joining method. The bootstrap values were calculated from 1,000 replications. Bootstrap values of <50% are not shown. The bars indicate numbers of substitutions per nucleotide position. doi:10.1371/journal.pone.0038420.g002
CIP 108297<sup>T</sup> showed the typical MALDI-TOF mass spectrometry profiles of two clusters, but all four of the Type II strains showed unusual profiles, with a significantly low intensity of the putative diglycosylated GPLs and diverse peaks of high intensity from m/z 1398 to m/z 2477, suggesting a disparity in the GPL nature between the two genotypes (Figure 5).

**Discussion**

Following the recent taxonomic separation of three very closely related RGMs - *M. abscessus, M. massiliense* and *M. bolletii* - reports regarding human infections of *M. massiliense* have been increasing [20–23]. Thus, it has become more important to study the diversity between their interspecies or intraspecies [10,24]. A recent report based on multilocus sequencing showed that *M. massiliense* is composed of strains with more diverse genetic heterogeneity than its closely related species, *M. abscessus* [24]. To investigate the intraspecies genetic diversity within *M. massiliense*, we studied 65 Korean strains by applying a gene-based sequencing approach consisting of two independent chronometers (*hsp65* and *rpoB* gene).

Rather than 16 S rRNA gene-based analysis, which has been shown to have limited effectiveness in discriminating between mycobacterial strains, a 603-bp *hsp65* sequencing analysis has been proven to be useful for identifying mycobacteria [25–27], particularly for the separating between the three related species of *M. abscessus, M. abscessus, M. massiliense*, and *M. bolletii* [11,28]. Our *hsp65*-based sequence analysis showed that *M. massiliense* is more prevalent than *M. abscessus* (59.6% vs. 40.4%), supporting the previous report that, in South Korea, *M. massiliense* isolated with a higher relative frequency than in other areas [10]. So, this epidemiologic feature raised the possibility of the presence of a relative higher frequency than in other areas [10]. So, this epidemiologic feature raised the possibility of the presence of a relative higher frequency than in other areas [10].

Table 1. Separation of 109 *M. abscessus* related Korean strains used in this study into species or genotype level by sequence analysis based on the partial *hsp65* gene sequence (603 bp).

| Species and group | No. (%) of strains |
|-------------------|--------------------|
| *M. abscessus*     | 44 (40.4)          |
| *M. massiliense*   | 65 (59.6)          |
| Type I            | 35 (32.1)          |
| Type II           | 30 (27.5)          |
| Total             | 109 (100.0)        |

Table 2. The frequency of the two *hsp65* genotypes (Type I and Type II) determined by *hsp65* sequence analysis and *Hinf* I PRA methods, and the two colony morphotypes (rough and smooth) among 65 *M. massiliense* clinical strains.

|              | *hsp65* genotype<sup>a</sup> | *Hinf* I PRA | Colony morphology |
|--------------|------------------------------|--------------|-------------------|
|              | No. (%)<sup>b</sup>          |              |                   |
| Type I       | 35 (30.1) 35 (100.0) 0 (0.0) | 12 (34.3) 23 (65.7) |               |
| Type II      | 30 (27.4) 0 (0.0) 30 (100.0) 0 (0.0) |               |               |

<sup>a</sup>The *hsp65* genotypes were determined by *hsp65* sequence analysis (603-bp).

<sup>b</sup>The percentage was calculated among *M. massiliense* strains.

Our *rpoB* sequence analysis was also able to separate all 65 of the *M. massiliense* strains into four more diverse genotypes (R-I to R-IV) than *hsp65* sequence analysis. However, while the previous report [10] had discordant results between *hsp65* and *rpoB*-based sequence analysis in species determination in some *M. massiliense* strains, all 65 of the isolates identified as *M. massiliense* by the *hsp65* method were also identified as *M. massiliense* by *rpoB* methods (Table 3, Figure 2B). Phylogenetic analysis based on the *rpoB* sequences showed that most *hsp65* Type I strains also formed a monophyletic clade in a *rpoB* tree (Figure 2B), suggesting that *hsp65* Type I may be composed of genetically identical strains. However, *hsp65* Type I strains were divided into the three different clades in a *rpoB* tree, suggesting that *hsp65* Type I may be composed of more diverse genetic groups. These results provide a
likely explanation for the presence of phenotype divergence (the coexistence of both morphotypes) in type I over type II strains.

Although PRA is a previous century technique, replaced by sequencing analysis producing more accurate results, the PRA method has still been used for routine screening purposes in clinical settings for differentiating the mycobacterial strains, due to its ease and rapidity [33–35]. So, we developed a novel PRA method using \textit{Hinf} \textsubscript{I} enzyme targeting a 644-bp \textit{hsp65} genes for selective identification of \textit{M. massiliense} Type II. A blind test for evaluating this PRA method proved that it can successfully identify all Type II strains from Type I, suggesting its feasibility for the diagnostic or epidemiologic purposes.

Our \textit{hsp65} and \textit{rpoB} based methods failed to differentiate rough and smooth strains within \textit{hsp65} Type I. To overcome this limitation, methods with more discriminatory power such as variable-number tandem repeat (VNTR) [36–38] or whole genome sequencing [39–41] should be applied into \textit{hsp65} Type I strains for the future study.

In summary, through \textit{hsp65} sequencing analysis of 65 Korean \textit{M. massiliense} strains, we found two \textit{hsp65} genotypes, Type I from 35 patients (53.8%) and Type II from 30 patients (46.2%), which were related to smooth and the rough colony phenotypes, respectively. Furthermore, the two \textit{hsp65} genotypes were also completely different in terms of their lipid profiles by MALDI-
A total of four genotypes (R-I to R-IV) were also found by rpoB sequencing analysis, three of which (R-I, R-II, and R-III) were related to hsp65 Type I and the other (R-IV) which was related to hsp65 Type II. Our data indicates that the Type II hsp65 genotype, which also shows the R-IV rpoB genotype, is a novel M. massiliense group introduced for the first time in this study. In addition we developed a novel PRA method for selectively separating hsp65 Type II from other RGMs. Our data suggests that M. massiliense strains may be composed of genetically distinct diverse groups, of which pathogenic potentials need to be evaluated for future study.

Materials and Methods

Bacterial Strains

A total of 109 strains from sputa samples of different patients were provided by Tae Sun Shim, Asan Medical Center, South Korea were collected from January 2004 to June 2011. All the experiments were performed just on the extracted DNA the isolated strains, not directly sputa DNA. Furthermore, all the samples were collected in an anonymized manner, and any information about the personal details of the patients and any details about their clinical history were not supplied. In this case, the study could be under the waiver of informed consent. With the documentation for waiver of informed consent, this work was approved by the institutional review board of Seoul National University Hospital (C-1202-057-398) and Asan Medical Center (2012-0170). These samples were proved to be M. abscessus complex strains by rpoB PRA method [34]. For the further separation at the species level, these isolates were used for re-identification by direct sequencing protocol targeting the partial hsp65 gene [14]. The type strains of M. abscessus (ATCC 19977), M. massiliense (CIP 108297), and M. bolletii (CIP 108541) were used for comparison.

Colony Morphology and Broth Culture

To observe the colony morphology, the isolates cultivated on Ogawa media were sub-cultured on 7H10 agar medium supplemented by OADC at 37°C for 3 to 5 days [42]. To examine growth patterns on the broth culture, the clinical isolates and M. massiliense type strain were inoculated into 7H9 broth supplemented with ADC at approximately 1×10^4 CFU/ml and cultured at 37°C for 5 to 7 days [30,42].

Biochemical Tests and Drug Susceptibility Tests

To determine their taxonomic relationships, M. massiliense CIP 108297^T, four M. massiliense Type I strains (50375, 51843, 52352, and 52444), and four M. massiliense Type II strains (50375, 50375, 51048, 52188, and 52265) were tested for biochemical and drug susceptibility profiles. These strains were cultured into Middlebrook 7H9 broth supplemented by ADC at 37°C. Colony morphology, pigment production in the dark condition, photo-induction, and the ability to grow at various temperatures (25, 37,
and 45°C) were analyzed during six-week incubation on Middlebrook 7H10 agar plates. Acid-alcohol fastness was determined by Ziehl-Neelsen and auramine O staining. Other tests measured: niacin accumulation, nitrate reductase, arylsulfatase on days 3 and 14, heat-stable catalase (pH 7, 68°C), tellurite reductase, Tween 80 hydrolysis, urease and pyrazinamidase (PZA) [43]. Additional biochemical tests, such as to detect the activity of alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, and cystine arylamidase were conducted with the API ZYM kit (bioMerieux) as recommended by the manufacturer.

Inhibition tests including tolerance to thiophene-2-carboxylic acid hydrazide (TCH), p-nitrobenzoate (PNB), 5% sodium chloride, ethambutol (EMB) and picric acid were carried out, and the ability to grow on MacConkey agar without crystal violet was examined. Also, antimicrobial susceptibility was determined by the agar proportion method on 7H10 medium [43].

High-performance Liquid Chromatography (HPLC) Analysis

HPLC was used to analyze mycolic acids from *M. massiliense* CIP 108297T, the four aforementioned *M. massiliense* Type I strains, and the four aforementioned *M. massiliense* Type II strains as previously described [44]. The Microbial Identification system (MIDI Inc.) and the HPLC mycobacterium library (available online at http://www.MycobacToscana.it) were used to identify mycolic acid patterns.

Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry Analysis

To analysis MALDI-TOF mass spectrometry, lipids were extracted with CHCl₃/CH₃OH (1:1 v/v, adding 0.5 μl of 2,5-dihydroxybenzoic acid) from 30 ml 7H9 broth cultures of *M. massiliense* CIP 108297T, the four *M. massiliense* Type I strains, and the four *M. massiliense* Type II strains. MALDI-TOF mass spectrometry was performed on the extracted samples with a Voyager DE-STR MALDI-TOF instrument (Perceptive Biosys-
systems equipped with a pulse nitrogen laser emitting at 337 nm as previously described [45].

DNA Extraction and PCR

Total DNAs were extracted from cultured colonies using the bead beater-phenol extraction method [14], and then used as templates for PCR. To study genetic variations of the *M. massiliense* related strains, two independent target genes - *hsp65* and *rpoB* - were amplified. To amplify partial gene (644 bp), the *hsp65* PCRs were applied to a total of 109 clinical isolates. A set of primers HspF3 (forward; 5'-ATC GCC AAG GAG ATC GAG CT-3') and HspR4 (reverse; 5'-AGG GTG CCG CGG ATC TTG TT-3') was used [14]. To amplify partial gene (1092 bp), the *rpoB* PCRs (with a slight modification of previous methods) [12], were applied to 65 clinical isolates, which had already been identified into *M. massiliense* strains by the *hsp65* sequencing protocol. Primer sets of MycoF2 (forward; 5'-ATC GCC GAG GGT CCC TGC-3') and MycoR2 (reverse; 5'-GAA CCG CTG GCC ACC GAA CT-3') were used for PCR. The template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer, Daejeon, South Korea) containing one unit of Taq DNA polymerase, 250 μM of deoxynucleotide triphosphate, 10 mM Tris-HCl (pH 8.3), 10 mM KCl, 1.5 mM MgCl2, and gel loading dye. The final volume was adjusted to 20 μl with distilled water, and the reaction mixture was then amplified as previously described [14,46] using a model 9700 Thermocycler (Perkin-Elmer Cetus).

Sequence Analysis

Determined partial *rpoB* (711-bp), and *hsp65* (603-bp) sequences were aligned using the ClustalW algorithm in MEGA4 [47]. A phylogenetic tree based on the *rpoB* and *hsp65* gene sequences was constructed by the neighbor-joining [48] and maximum-parsimony [49] methods within the MEGA 4 program [47]. The constructed neighbor-joining tree was evaluated by bootstrap method within the MEGA 4 program [47]. The phylogenetic tree based on the *rpoB* and *hsp65* gene sequences was analyzed to observe any point mutation at the adenine at position 2058 (A2058) or at A2059 in the peptidyltransferase region of the 23 S rRNA gene [50].

Nucleotide Sequence Accession Numbers

Determined *rpoB* and *hsp65* sequences that were different from reference strains were deposited in GenBank under accession no. JQ061974 to JQ062103.

Statistical Analyses

Results were expressed as percentages. The differences between categorical variables were analyzed using the Chi-square test. For continuous variables the Student's *t*-test was used when the data showed a normal distribution, or the Mann-Whitney *U* test was used when the data was not normally distributed. A *p*-value of <0.05 (two-tailed) was considered to be statistically significant.
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