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Intra- and Interpatient Variability of the *hsp65* and 16S-23S Intergenic Gene Region in *Mycobacterium abscessus* Strains from Patients with Cystic Fibrosis

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To establish the exact pathogenic role of *Mycobacterium abscessus* in cystic fibrosis (CF), molecular tests are required for accurate identification. Forty-eight *M. abscessus* isolates from seven patients with CF were analyzed by sequence analysis for sequence variants within the *hsp65* gene and the 16S-23S intergenic sequence (ITS). We detected two different *hsp65* genes and correspondingly two ITS sequevars belonging to *M. abscessus* type I and type II.

Nontuberculous mycobacteria (NTM), ubiquitous in the environment, have continued to emerge as important human pathogens. In the past years an increasing prevalence of nontuberculous mycobacteria infection among the cystic fibrosis (CF) population was observed (7, 8, 9, 10, 13, 26). Several case reports associate infection with *Mycobacterium abscessus* with poor clinical outcome (5, 17, 18, 21, 22). The literature as to the accurate identification and the clinical impact caused by *M. abscessus* is complicated by several name changes. Early reports refer to *Mycobacterium fortuitum* complex, which grouped together *M. fortuitum* with *Mycobacterium chelonae* and *M. abscessus*. Before 1992, *M. chelonae* consisted of two subspecies, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus*, which now have their own species designations (14). This distinction is relevant because antibiotic susceptibility can vary between these two species (24), and, depending upon the site of infection, these species vary in their clinical significance (17, 18, 29). Accurate identification of mycobacteria to the species level using morphological and biochemical characteristics is often not possible due to the increasing number of *Mycobacterium* species with overlapping phenotypic characteristics (15, 16, 23). The most widely used method for molecular identification of NTM is PCR-restriction fragment length pattern analysis (PRA) of the *hsp65* gene (3, 6, 16, 25). Tools proposed for the molecular identification of NTM clinical isolates include analysis of the 16S rRNA gene (4, 11, 12), the genes *sod*, *dnaJ*, *rpoB*, the 32-kDa protein-encoding gene *recA*, and the DNA gyrase genes as well as the internal transcribed spacer 16S-23S rRNA region (ITS) (20) and sequencing of the *hsp65* gene (19). A target region must be sufficiently conserved among the strains of the species for use in genotype identification. Furthermore, the data should be reliable and unambiguous and comparable among laboratories.

We analyzed the intra- and interpatient variability of *M. abscessus* isolates from patients with CF over time. For this purpose we analyzed a total of 48 *M. abscessus* isolates from different time points of isolation by sequence analysis for sequence variants and genetic variability within the *hsp65* gene and the ITS region.

All CF patients ($n = 61$) were routinely examined in the Department of Pediatrics every 3 months. Sputum samples were investigated every 3 months or more frequently in cases of acute exacerbation. The microbiological investigation included the screening for mycobacteria (2). In this regard, respiratory specimens were decontaminated with NALC-NaOH (0.25% N-acetyl-L-cysteine–1% sodium hydroxide). Acid-fast bacillus smears were stained with the BBL TB Kinyoun stain kit (Becton Dickinson, Heidelberg, Germany) and scored according to Deutsches Institut für Normung guidelines. For a positive result at least four bacteria in 100 fields of view are necessary. One Löwenstein-Jensen and one Stonebrink slant was inoculated for each specimen. The slants were examined twice weekly for 2 weeks and then weekly for another 10 weeks. The two reference strains, *M. chelonae* strain DSM 43804 and *M. abscessus* strain DSM 44196 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The respective clinical isolates had been submitted to conventional identification procedures. *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium gordonae* were excluded by using the Accu-Probe technique (Gen-Probe Inc., San Diego, CA). All isolates were submitted to 16S-rRNA, *hsp65*, and ITS sequencing (see below). For this purpose a loopful of a *Mycobacterium* mass from solid cultures was added to sterile NaCl (0.9%) and submitted to an inactivation procedure at 95°C for 1 h. Then the bacterial suspensions were submitted to three cycles of freezing-boiling (5 min in liquid nitrogen and 10 min at 100°C). DNA was extracted by using a blood DNA kit (QIAGEN, Hilden, Germany) according to the instructions of the manufacturer. PCR and sequencing of the 16S rRNA gene was performed as described previously (28). The sequences were aligned with the Sequencher 4.0 program for Microsoft Windows and compared to existing sequences by BLAST analysis

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of the National Center for Biotechnology Information (http://www.ncbi.nlm.gov) and the Ribosomal Differentiation of Microorganisms project (www.ridom.de) databases.

The sequence variants and genetic variability within the *hsp65* gene and the 16S-23S intergenic sequence were analyzed by *hsp65* PCR-restriction fragment length polymorphism (RFLP) and *hsp*65 and ITS sequencing. A 441-bp region of the 65-kDa *hsp* gene was amplified as described previously (19). After PCR, each 65-kDa *hsp* gene amplimer (10 μl) was separately digested with 10 U (each) of the restriction endonucleases HaeIII and BstEII as described by the manufacturer (Roche, Mannheim, Germany). The fragments were separated on a 3% agarose gel containing ethidium bromide. The fragment band sizes were estimated by a computerized BioImage system using a 50-bp DNA ladder as an external molecular size marker. Sequencing of the 65-kDa *hsp* gene amplimers of all strains was performed as described previously (22, 33). The sequences were aligned with the Sequencher 4.0 program for Microsoft Windows. For sizing of the respective restriction fragments (in silico digestion with BstEII and HaeIII), the restriction map of the same program was used. The ITS PCR was performed as described by Roth et al. (20). The amplified fragments were either digested with the restriction enzymes HaeIII, CfoI, and TacI or were sequenced using Sp1 and Sp2 as sequencing primers. The fragment band sizes were estimated by a computerized BioImage System using a 100-bp DNA ladder (Roche, Mannheim, Germany) as an external molecular size marker. The sequences were aligned with the Sequencher 4.0 program for Microsoft Windows.

We prospectively studied 61 patients with CF for NTM in respiratory samples from 1 January 1997 to September 2004 and screened approximately 2,000 sputum samples. NTM were obtained in 65 sputum samples from 10 children and young adults, respectively. Among them, one child harbored *Mycobacterium kansasii* and two children were infected with *M. avium*. In the remaining 48 sputum samples from seven children, the NTM were identified as *M. abscessus* by sequencing the whole 16S rRNA gene including the 500 bp of the 16S rRNA gene at the 5′ region. Four children had three or more positive samples, and *M. abscessus* infection persisted over at least 1 year. The patients' data are summarized in Table 1.

TABLE 1. Case patients with *M. abscessus* isolation

Case no.	Sex	Age of patient at positive culture	*M. abscessus* type identified
1	Male	12 yr 7 mo	Type I
		13 yr 1 mo	
		14 yr 7 mo	
		14 yr 7 mo	
		14 yr 11 mo	
		16 yr 1 mo	
		16 yr 2 mo	
		16 yr 3 mo	
2	Female	15 yr 1 mo	Type I
		15 yr 5 mo	
		17 yr 11 mo	
		18 yr 7 mo	
		19 yr 5 mo	
3	Female	16 yr 4 mo	Type I
		16 yr 4 mo	
		17 yr 5 mo	
4	Female	14 yr 8 mo	Type I
		14 yr 9 mo	
		14 yr 10 mo	
		15 yr 2 mo	
		15 yr 9 mo	
		16 yr 0 mo	
		16 yr 1 mo	
		16 yr 1 mo	
		16 yr 2 mo	
		16 yr 3 mo	
		16 yr 9 mo	
		16 yr 9 mo	
5	Male	13 yr 11 mo	Type II
		14 yr 1 mo	
		14 yr 2 mo	
		14 yr 3 mo	
		14 yr 3 mo	
		14 yr 5 mo	
		14 yr 8 mo	
		14 yr 11 mo	
		15 yr 3 mo	
		15 yr 4 mo	
		15 yr 8 mo	
		15 yr 8 mo	
		15 yr 9 mo	
		15 yr 9 mo	
6	Female	21 yr 4 mo	Type II
7	Male	17 yr 7 mo	Type I
		17 yr 9 mo	
		18 yr 1 mo	
		18 yr 5 mo	

All isolates of *M. abscessus* were submitted to PRA of the *hsp65* gene. Restriction analysis generated two different patterns among the 48 strains tested for the enzyme HaeIII (Fig. 1). The restriction profiles of 33 strains belonging to patients 1, 2, 3, 4, and 7 and the *M. abscessus* reference strain (DSM 44196) were identical (BstEII, 245/220 bp; HaeIII, 160/60 bp) (Fig. 1). The restriction profile of the strains ($n = 15$) from patients 5 and 6 were different and were in agreement with the algorithm for *M. abscessus* type II (BstEII, 245/220 bp; HaeIII, 210/60 bp) as described by Devoillas et al. (6) (Fig. 1). The *M. abscessus* type infecting each patient is summarized in Table 1, and both *M. abscessus* type I and II organisms were found. *hsp65* sequence variants among mycobacterial species has been reported in previous studies based on PRA and DNA sequencing (19). However, in most studies only one or two examples of most NTM, including *M. abscessus*, were included. Moreover, there is still subjectivity in interpreting results from PCR-RFLP of the *hsp65* gene. Every laboratory established its own logarithm with slightly different lengths of restriction fragments for the identification of NTM.

The precise detection of different sequence variants and the intraspecies variability of *M. abscessus* can help to trace the epidemiology of *M. abscessus*, in terms of both geographical distribution and pathogenicity. For *M. kansasii*, it was demonstrated that only distinct sequevars are associated with human infections (1, 27). We performed sequencing of the respective 441-bp fragment of the *hsp65* gene by an automated fluorescence-based method involving capillary electrophoresis. All 33 *M. abscessus* isolates from patients 1, 2, 3, 4, and 7 showed 100% sequence identity to the *M. abscessus* reference strain (*M. abscessus* type I). In comparison to the *M. abscessus* reference strain, all isolates from patients 5 and 6 ($n = 15$) showed sequence variations at positions 530 (T to G), 533 (T to

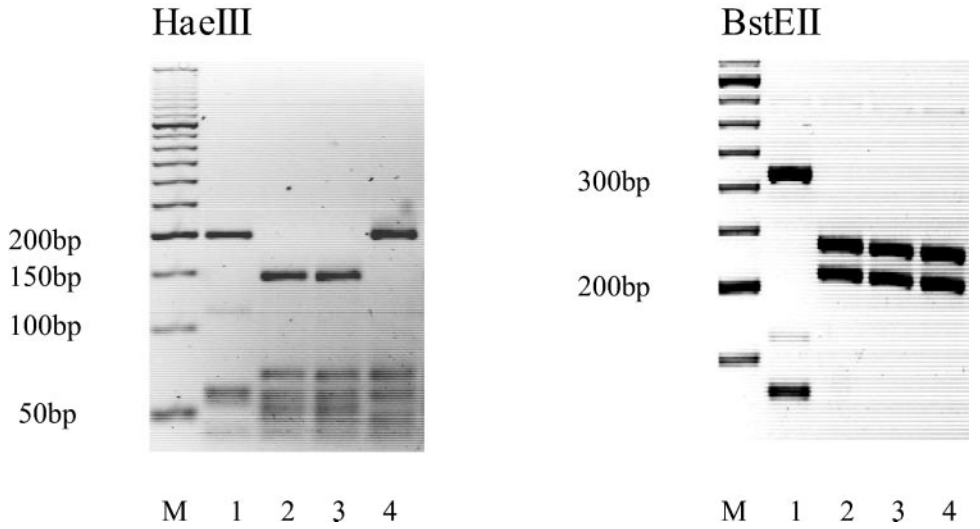

FIG. 1. PCR-RFLP of *hsp65*. The amplimers of the partially amplified *hsp65* gene were digested with BstEII and HaeIII and separated by 3% agarose gel electrophoresis. M, molecular size marker (50-bp ladder); lanes 1, *M. chelonae* reference strain (DSM 43804); lanes 2, *M. abscessus* reference strain (DSM 44196); lanes 3, one representative isolate from patient 1 (*M. abscessus* type I); lanes 4, one representative isolate from patient 5 (*M. abscessus* type II).

C), 542 (C to T), 605 (C to T), and 755 (C to T) in the *hsp65* gene (nucleotide positions from published *Mycobacterium tuberculosis* sequence). These base exchanges were also observed by Ringuet et al. (19) and are consistent with *M. abscessus* type II (6, 19). Thus, in our study two well-defined *M. abscessus* alleles were obtained.

Additionally, we analyzed the *M. abscessus* isolates for genetic variability within the 16S-23S ITS region. All strains produced an amplicon which possessed an identical TacI RFLP. Two sequence types were found that were consistent with type I and type II. In this regard, the 33 *M. abscessus* isolates from patients 1, 2, 3, 4, and 7 showed 100% sequence identity and one base substitution to the *M. abscessus* strains S322 (AJ291581) and C93328 (AJ314872) at position 240 (A to G) of the ITS amplicon. All *M. abscessus* isolates from patients 5 and 6 showed sequence variations at positions 71 (A to G), 109 (insertion of C), and 240 (A to G).

In summary, our data confirm previous reports of the sequence variants within the *M. abscessus hsp65* gene and extend these findings to the ITS region. In addition we report for the first time on the intra- and interpatient variability over time of *M. abscessus* isolates from patients with cystic fibrosis. Our results show that patients with CF may be chronically infected with *M. abscessus* type I and type II. Within each sequevar there was no further sequence variation detectable. Furthermore, we demonstrate that there were no intrapatient sequence variabilities within the individual sequevars of both sequence loci. These findings are important for the characterization of the species and for future analysis to understand the pathogenicity and epidemiology of this organism.

Nucleotide sequence accession numbers. The sequences of the *M. abscessus hsp65* genes were deposited in the GenBank database under accession numbers AY603553 (*M. abscessus* type I) and AY603554 (*M. abscessus* type II). The sequences of the *M. abscessus* ITS sequevars were deposited under accession numbers AY635142 (*M. abscessus* type I) and AY635143 (*M. abscessus* type II).

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