Identification of Mycobacteria of the MAIS Complex and *M. tuberculosis* by Restriction Fragment Length Polymorphism Analysis of hsp65 Gene

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Restriction fragment length polymorphism analysis of hsp65 gene was performed on museum strains of mycobacteria using Hin6I restrictase. Study of restriction profiles allowed us to distinguish mycobacterial species of the MAIS complex and several strains of nontuberculous mycobacteria.

Key Words: *nontuberculous mycobacteria; MAIS complex; species determination; restriction fragment length polymorphism; hsp65 gene*

There are about 80 species of the genus *Mycobacterium* not belonging to the tuberculosis complex and receiving the name nontuberculous (atypical) mycobacteria. They cause mycobacteriosis in humans, which manifests in local damage to lymph nodes, skin, joints, and lungs. Disseminated processes are mainly observed in patients with HIV infection, AIDS, or primary immunodeficiency [2, 3,7,9,11,12].

Species identification in mycobacteriological laboratories is based on the study of cultural characteristics of mycobacteria (pigment formation, growth rate, and growth capability of the culture at various temperatures). The use of biochemical methods depends on enzyme activity of the species [2]. Published data show that the average time for identification of nontuberculous mycobacteria is more than 6 months [1].

A highly specific method of gas-liquid chromatography allows us to perform rapid species determination of mycobacteria. Interspecies differences were revealed in the composition of methyl esters of fatty acids in the cell wall [6]. The method of high-performance liquid chromatography demonstrates differences in the composition of mycolic acids in the cell wall and provides more reliable results. Previous studies by this method identified more than 60 species of mycobacteria [7].

Molecular and genetic methods for species determination of mycobacteria are characterized by high sensitivity and specificity, do not require the use of expensive equipment and reagents, and rapidly give the result.

There are several molecular and biological approaches to perform species determination of mycobacteria. They are based on the determination of minute differences between genomic targets even at the level of 1 bp [4,5,8,10,13]. Sequencing of 16S rRNA gene is the most precise method. This method allows us not only to determine the species of mycobacteria, but also to identify new species of the genus *Mycobacterium* [10]. Polymerase chain reaction (PCR) of 16S rRNA gene followed by hybridization with species-specific probes is another method of identification [5]. Restriction fragment length polymorphism (RFLP) analysis proceeds in 2 stages (PCR of the gene fragment and restriction analysis of PCR products). The most reliable genomic target for species determination is hsp65 gene encoding heat shock protein with a molecular weight of 65 kDa. This gene is present in DNA of

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all mycobacteria and is characterized by considerable interspecies variability of nitrogen bases. Therefore, the hsp65 gene holds promise for identification of mycobacteria [4,13].

Here we studied whether RFLP analysis of the hsp65 gene can be used to identify the species of mycobacteria of the MAIS complex and distinguish them from *M. tuberculosis complex*.

MATERIALS AND METHODS

Experiments were performed with museum strains and clinical isolates of cultures belonging to the tuberculosis complex (*M. tuberculosis H37Rv* MNPTsBT, *M. bovis Ravenel* SPbNIIF, and *M. bovis BCG* SPbNIIF) and nontuberculous mycobacteria (*M. avium* GISK, *M. avium* ATSS35712 SPbNIIF, *M. intracellulare* Astrakhan' MNPTsBT, *M. intracellulare* GISK, *M. intracellulare* ATSS35761 SPbNIIF, *M. scrofulaceum* ATSS35787 SPbNIIF, *M. scrofulaceum* Astrakhan' MNPTsBT, *M. chelonae* GISK, *M. fortuitum* GISK, and *M. kansasii* GISK). All cultures were obtained from mycobacteriological laboratories (Moscow Scientific and Practical Center for Prevention of Tuberculosis), St. Petersburg Research Institute of Phthisiopulmonology (Russian Ministry of Health and Social Development), L. A. Tarasevich State Institute of Standardization and Control of Medical and Biological Preparations (Russian Ministry of Health and Social Development).

The cells were washed 2 times with Tris-EDTA buffer (TE, pH 8.0) and centrifuged at 11,000*g* for 10 min to obtain mycobacterial DNA from clinical isolates. The lysing buffer (TE and 1% Triton X-100, pH 8.8) was added to the pellet. The mixture was heated in a dry thermostat (95°C) at 15 min and cooled on ice for 5-10 min. The study was performed with the supernatant.

Primers of HSP-1 (5'GCCAAGAAGACCGA TGACGT3') and HSP-2 (5'GGTGATGACGCCCT CGTTGC3') were used for amplification.

The amplification profile and primers were selected by means of Oligo6 software [13]. Specificity of primers was estimated using Blast software [13]. Amplification profile consisted of cycle 1 (94°C, 2 min×1), cycle 2 (30 sec at 94°C, 40×30 sec at 62°C, 1 min at 72°C), and cycle 3 (72°C, 6 min×1).

Restriction endonucleases were selected using Primer Premiere 5 and BioEdit 7.0.1 softwares. Endonuclease should allow us to obtain the map of restriction profiles for DNA fragments identified with ethidium bromide. Experiments were performed with restrictase Hin6I 5"G^CG_C3'. Restriction conditions were selected experimentally by changing the ratio of components in the reaction mixture, restriction time, and restriction conditions. Clear restriction profiles were obtained after incubation of the medium containing 5 µl buffer Y+/Tango (Fermentas Inc.), 9.6 µl restrictase Hin6I (Fermentas Inc.), and 17 µl amplicons in a thermostat at 37°C for 70 min.

Products of amplification and restriction were detected by horizontal electrophoresis in 1.5 and 3% agarose gel, respectively. The length of restriction fragments was estimated visually using Gene Ruler 50 bp DNA Ladder (Fermentas Inc.).

RESULTS

Restriction profiles were analyzed at the first stage of the study. Figure 1 shows restriction profiles of some species of the MAIS complex and *M. tuberculosis* after treatment of amplicons synthesized from the hsp65 gene fragment with restrictase Cfr13I.

The lengths of restriction fragments after treatment with restrictase Cfr13I were 200 and 40 bp for *M. tuberculosis*, 200, 125, 100, and 70 bp for *M. avium* and *M. intracellulare*, and 200, 125, 100, and 40 bp for *M. scrofulaceum*. Restriction profiles of the MAIS complex (Fig. 1) differed from those of *M. tuberculosis* (Fig. 1, *4*). No differences were found between restriction profiles of *M. avium* (Fig. 1, *3*) and *M. intracellulare* (Fig. 1, *2*). Restrictase Cfr13I did not identify the species of the MAIS complex and, therefore, was not suitable for our study.

Further experiments were performed with restrictase Hin6I (CfoI isoschizomer), which allowed

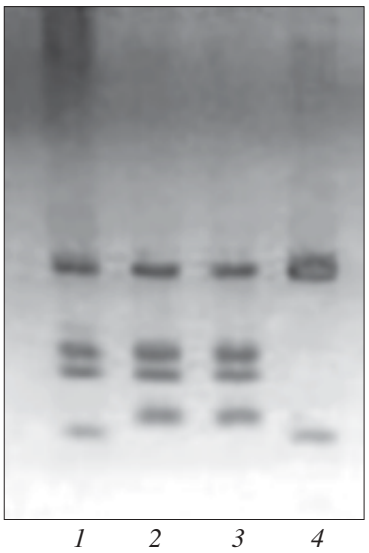

Fig. 1. Restriction profiles of the MASI complex after treatment with restrictase Cfr13I: *M. scrofulaceum* (ATSS35787, *1*), *M. intracellulare* (ATSS35761, *2*), *M. avium* (ATSS35712, *3*), and *M. tuberculosis* (*H37Rv*, *4*).

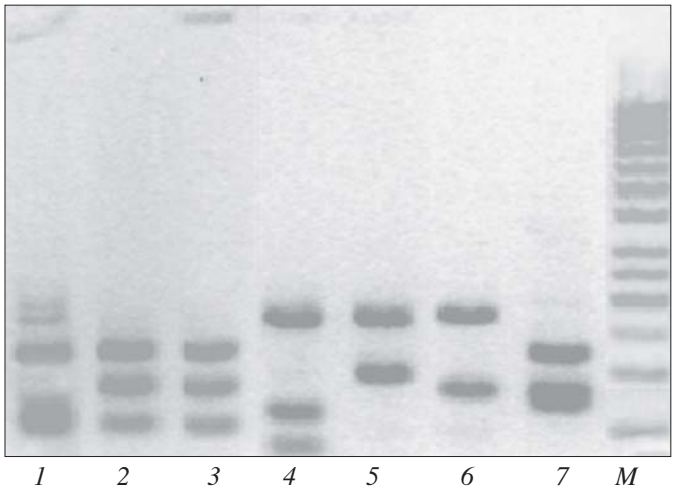

Fig. 2. Restriction profiles for various species of the genus *Mycobacterum* obtained after treatment with restrictase Hin6I: *M. kansasii* (*1*), *M. fortuitum* (*2*), *M. chelonae* (*3*), *M. scrofulaceum* (ATSS35787, *4*), *M. intracellulare* (ATSS35761, *5*), *M. avium* (ATSS35712, *6*), and *M. tuberculosis H37Rv* (*7*). Here and in Fig. 3: M, molecular weight marker (in bp).

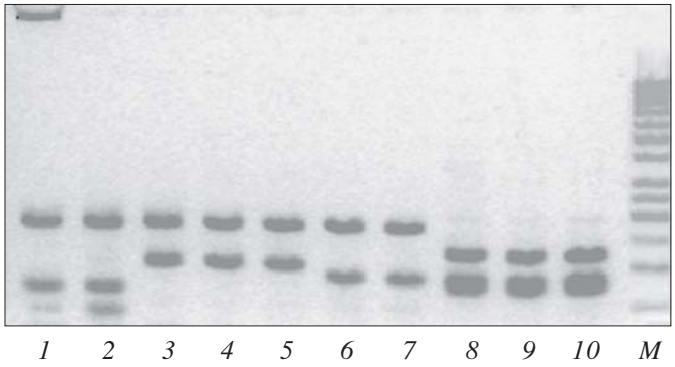

Fig. 3. Coincidence of restriction profiles obtained after treatment with restrictase Hin6I: *M. scrofulaceum* (Astrakhan', *1*), *M. scrofulaceum* (ATSS35787, *2*), *M. intracellulare* (ATSS35761, *3*), *M. intracellulare* (*4*), *M. intracellulare* (Astrakhan', *5*), *M. avium* (ATSS35787, *6*), *M. avium* (*7*), *M. bovis BCG* (*8*), *M. bovis Ravenel* (*9*), and *M. tuberculosis* H37Rv (*10*).

us to distinguish species of *M. avium* and *M. intracellulare* (Fig. 2).

Restriction profiles of the MAIS complex differed not only from each other (Fig. 2, *4-6*), but also from *M. tuberculosis* (Fig. 2, *7*). Restriction profiles of other nontuberculous mycobacteria differed from those of the MAIS complex and *M. tuberculosis* (Fig. 2, *1-3*). After treatment with restrictase Hin6I the lengths of restriction fragments were 125, 85, and 75 bp for *M. tuberculosis complex*, 185 and 85 bp for *M. avium*, 185 and 100 bp for *M. intracellulare*, 185, 75, and 40 bp for *M. scrofulaceum*, 125, 85, and 50 bp for *M. fortuitum*, 125, 85, and 50 bp for *M. chelonae*, and 125, 75, and 50 bp for *M. kansasii*.

Our results indicate that study with restriction endonuclease can differentiate mycobacterial species of the MAIS complex and distinguish them from *M. tuberculosis complex*. This approach simplifies the experiment and reduces the cost of study (as distinct from test systems with 2 or more restrictases) [4,13]. The results of the study with restriction profiles [13] indicate that changes in the composition of the reaction mixture and restriction conditions (volume of amplicons, restriction time, and incubation in a thermostat on a water bath) provides a clear restriction pattern after detection in 3% agarose gel [13].

We compared restriction profiles for various museum strains of mycobacterial species obtained after RFLP of the hsp65 gene (Fig. 3). Restriction was similar in various strains of the same species, including *M. avium* (Fig. 3, *6*, *7*), *M. intracellulare* (Fig. 3, *3-5*), and *M. scrofulaceum* (Fig. 3, *1*, *2*). It should be emphasized that restrictase Hin6I can be used for differention of *M. tuberculosis complex* from the MAIS complex. However, the study with this restrictase does not distinguish species of the tuberculosis complex.

Our results indicate that RFLP of the hsp65 gene holds promise for the use in mycobacteriological laboratories to identify mycobacteria of the MAIS complex and distinguish them from *M. tuberculosis complex*. This method allows us to perform rapid analysis. It is well reproducible, easily interpretable, highly sensitive, and specific. Study with RFLP of the hsp65 gene requires the equipment available at any diagnostic PCR laboratory. Hence, this method is simple and economically advantageous. RFLP of the hsp65 gene significantly shortens the time for identification of mycobacteria. Prescription of adequate etiotropic chemotherapy

during the earliest stage of the disease will improve the quality of life in patients with mycobacterioses.

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