COMPARISON OF DIFFERENT METHODS IN THE DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS AND ATYPICAL MYCOBACTERIA

IHSAN HAKKI CIFTCI, ENGİN KARAKEÇİ, SINEM HIZAL, YUSUF AYDEMIR, HUSEYİN AGAH TERZİ
‘Sakarya University, Training and Research Hospital, Department of Medical Microbiology, Sakarya - ‘Sakarya University, Training and Research Hospital, Department of Chest Diseases, Sakarya, Turkey

ABSTRACT

Introduction: We aimed to examine clinical specimens of Mycobacterium tuberculosis complex (MTC) strains and to identify the methods used to isolate MTC. In addition, we aimed to classify nontuberculous mycobacteria (NTM) strains and to analyze their hsp65 fragments.

Materials and methods: Polymerase chain reaction (PCR) was performed to amplify the hsp65 gene. PCR products were digested with the enzymes and agarose gel electrophoresis was performed for restriction fragment length polymorphism (RFLP) analysis.

Results: Based on MGIT 960 automated system analysis, 221 samples belonging to 81 patients were found positive and, of these patients, 71 (87.7%) were identified as MTC positive. The hsp65 gene was amplified in 10 (12.3%) samples that were identified as positive by MGIT 960 automated system but were not defined as MTC in the identification study. The following were identified upon analysis of the bands after RFLP: four M. intracellulare, M. gordonae I, M. gordonae I/M. xenopi, M. peregrinum, M. peregrinum/M. scrofulaceum, M. szulgai/Mycobacterium spp. and Mycobacterium spp.

Conclusion: The identification of atypical mycobacteria should be enlightening for the distribution rates in public and defining appropriate drug regimens. Therefore, a molecular approach is of fundamental importance for the correct diagnosis of these infections due to atypical mycobacteria which may be easily misinterpreted.

Key words: Atypical- typical mycobacteria, Molecular diagnosis, RFLP.

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Introduction

Clinical microbacteriology laboratories play important roles in quicker recognition, identification, and detection of drug resistance which aid in preventing the spread of tuberculosis. Thus, it is recommended that laboratories conduct consecutive or concurrent and differential diagnostic studies of laboratory samples, that the studies be completed in 28 to 30 days, and that laboratories provide the results of sensitivity studies of Mycobacterium tuberculosis complex (MTC)⁶.

In addition to MTC induced infections, the incidence of atypical or nontuberculous mycobacteria (NTM) tends to increase, especially in developed countries. NTM have been reported to be associated with the lungs, lymphatic system, skin and bone⁵,⁶,⁷. Correct identification of the agent provides an adequate and appropriate treatment strategy, and correct management of the diagnostic process is important in distinguishing between the MTC and NTM⁶. Moreover, accurate diagnosis increases the success of the treatment, decreases the hospital stay length and reduces unnecessary drug administration, minimizing economic losses⁶.

The hsp65 gene has been identified as an important target for sequence analysis in order to identify NTM. The hsp65 gene is 439 bp, has a
molecular weight of 65 kilodalton, is conserved in mycobacterial species, and has variable regions at positions 624, 664, 683 and 725\(^{(7)}\). Research has shown that analyses performed on amplified hsp65 fragments, after having been digested by restriction enzymes at variable regions, could be used to identify the mycobacteria\(^{(8)}\).

In this study, we examined methods for isolating, and identifying MTC strains from clinical samples. We also determined the sensitivity of isolated MTC strains to first-line anti-tuberculosis drugs, and classified NTM and analyzed their hsp65 fragments.

**Materials and methods**

Our study included 1975 clinical samples submitted to the medical microbiology laboratory between June 2012 and February 2013. Patients’ respiratory tract samples (sputum, bronchial aspirate, bronchoalveolar lavage) and extrapulmonary samples (urine, needle aspiration fluids) were homogenized, decontaminated, and concentrated on the same day using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method, and a Mycoprosafe (Salubris Inc., Turkey) kit in accordance with the manufacturer’s recommendations.

The resulting suspension preparations were stained using the Ehrlich-Zielh-Neelsen (EZN) technique and auromine-rhodamin (AUR) staining and were examined for the presence of acid-fast bacilli (AFB) using light and fluorescence microscopy. Then, all samples were inoculated into commercial MGIT tubes with Lowenstein Jensen (LJ) medium and a Middlebrook 7H9 broth with a silicon-containing fluorescent indicator. The LJ tubes were incubated at 37°C for up to eight weeks, while the MGIT tubes were incubated in a MGIT 960 (Becton Dickinson, USA) device for a maximum of six weeks. Growth in the LJ tubes was visually monitored on a weekly basis, while growth control in the MGIT 960 instrument was monitored automatically. The presence of AFB positivity in LJ and MGIT tube samples was investigated.

A TBc ID (Becton Dickinson, USA) identification kit was used according to the manufacturer's recommendations to classify the AFB-positive MGIT growth samples as either MTC or NTM. No additional tests were applied to determine LJ positivity. The sensitivity of the identified MTC strains to primary anti-tuberculosis drugs, such as streptomycin (STR), isoniazid (INH), rifampicin (RIF), and ethambutol (EMB), was investigated using the MGIT 960 system after antibiotics had been added to the MGIT tubes according to the manufacturer’s instructions.

Strains that were not identified as MTCs by the TBc ID test were described by analyzing restriction fragment length polymorphism (RFLP) at the hsp65 gene region. During this process, samples obtained from the MGIT tubes were inoculated into LJ media and were incubated at 37°C for four weeks.

For DNA synthesis, colonies obtained from the LJ media were transferred into a microcentrifuge tube with 750 µl of 1x TE and vortexed. The sample was then spun at 14,000 rpm for two minutes. The supernatant was discarded. The pellet was resuspended in 300 µl of 1x Tris-EDTA (TE) by vortexing and incubated in a 95°C water bath for 30 minutes. The sample was centrifuged again at 14,000 rpm for three minutes, and the supernatant was transferred into a sterile microcentrifuge tube. This isolated DNA was used as a template in molecular studies.

The total volume of the polymerase chain reaction (PCR) was 25 µl (3 µl template DNA, 1 µl of each primer, 0.125 µl of DreamTaq DNA polymerase, 2.5 µl of PCR buffer, and 2.5 µl dNTP). The PCR cycle was set at 95°C for 5 minutes for denaturation, followed by 45 cycles of 94°C for 30 seconds, 58°C for 45 seconds, and 72°C for 30 seconds. The amplicons were stored at 4°C. The primers, Tb11 [5’-ACCAACGATGGTGTGCAT] and Tb12 [5’CTTGTCGAACCCTACCT], have been used in previous molecular studies of hsp65\(^{(9)}\).

PCR products were incubated with BsuRI and Eco911 restriction enzymes (Fermentas, USA) at 37°C for 16 hours. After digestion, 10 µl of the product were run on a 2% agarose gel (ORTE, Salubris, Turkey) for RFLP analysis and were analyzed according to band sizes. The M. tuberculosis H37Ra strain was used as a control during RFLP analysis. The Statistical Package for the Social Sciences (SPSS 16.0) packaged software was used to analyze the data obtained in this study, and the directions and intensities of the relationships between variables were evaluated using Pearson’s correlation test. A p value <0.05 was considered significant in statistical analysis.
Results

In our study, we investigated a total of 1975 samples: 1866 (95.5%) sputum, 29 (1.5%) aspiration fluids, 36 (1.8%) bronchoalveolar lavage, and 24 (1.2%) urine, from 685 patients. Sample positivity rates for AFB, AUR, LJ, and MGIT samples were 7.1% (141), 8.2% (162), 9.6% (190), and 11.2% (221), respectively. LJ and/or MGIT positivity were observed in 11.8% (234) of the samples.

Sensitivity and specificity were calculated with total culture positivity as a reference. AFB, AUR, LJ, and MGIT sensitivity values were 0.5043, 0.6311, 0.8120, and 0.9444, respectively, while specificity values were 0.9868, 0.9954, 1.0000, and 1.0000, respectively. All methods were significantly correlated with total culture positivity (p < 0.05). Other data are summarized in Table 1.

MGIT 960 automated system analysis identified 221 samples belonging to 81 patients as positive and, of these patients, 71 (87.7%) were identified as MTC positive. The antibiotic susceptibility studies, performed on one strain isolated from each patient, showed that INH, RIF, EMB, and STR resistance rates were 23.9% (17/71 strains), 16.9% (12/71 strains), 11.3% (8/71 strains), and 9.8% (7/71 strains), respectively. In our study, 16.9% (12/71) of strains were multi-drug resistant, and 2.8% (2/71) were resistant to all first-line drugs. Resistance profiles for the MTC strains are summarized in Table 2.

Table 1: The results of sensitivity and specificity calculations based on total positivity.

* Calculated based on total positivity.
**Variables obtained using Pearson’s correlation analysis

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>-</th>
<th>Sensitivity*</th>
<th>Specificity*</th>
<th>PPV**</th>
<th>NPV**</th>
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<th>p**</th>
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<td>1834</td>
<td>92.9</td>
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<td>0.9688</td>
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<td>AUR</td>
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<td>8.2</td>
<td>1813</td>
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<td>0.6311</td>
<td>0.9954</td>
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<td>LJ</td>
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<td>9.6</td>
<td>1785</td>
<td>90.4</td>
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<td>1</td>
<td>0.9754</td>
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<td>1754</td>
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<td>1</td>
<td>1</td>
<td>0.9926</td>
</tr>
<tr>
<td>TOTAL</td>
<td>234</td>
<td>11.8</td>
<td>1741</td>
<td>88.2</td>
<td>-</td>
<td>-</td>
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</tr>
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</table>

Table 2: Resistance profiles of strains isolated from the MTC to first choice drugs.

Table 3: The products obtained after digestion with Eco91I and RsuRI restriction enzymes.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Restriction products</th>
<th>Description</th>
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<tr>
<td>Eco91I</td>
<td>RsuRI</td>
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<tr>
<td>1</td>
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<td>M. gordonae I</td>
</tr>
<tr>
<td>2</td>
<td>235,120.100</td>
<td>M. intracellular</td>
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<tr>
<td>3</td>
<td>225,205</td>
<td>M. peregrinum/M. scrofulaceum</td>
</tr>
<tr>
<td>4</td>
<td>440</td>
<td>M. tuberculosis</td>
</tr>
<tr>
<td>5</td>
<td>235,120.100</td>
<td>M. intracellular</td>
</tr>
<tr>
<td>6</td>
<td>440</td>
<td>M. gordonae I/M. xenopi</td>
</tr>
<tr>
<td>7</td>
<td>235,110.80</td>
<td>M. gordonae I/M. xenopi</td>
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<td>8</td>
<td>235,120.100</td>
<td>M. intracellular</td>
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<td>9</td>
<td>245,215</td>
<td>M. peregrinum</td>
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<td>10</td>
<td>235,120.100</td>
<td>M. intracellular</td>
</tr>
<tr>
<td>H37Ra</td>
<td>225,105.85</td>
<td>M. tuberculosis</td>
</tr>
</tbody>
</table>

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During the identification study, 10 (12.3%) cultures determined positive by MGIT 960 auto-
mated analysis were not identified as MTC, and the hsp65 gene region in those samples was amplified. The isolates of the amplicons were digested with EcoR11, and BsuRI restriction enzymes, and the bands and descriptions are summarized in Table 3. Figure 1 shows the results of gel electrophoresis, performed after restriction enzyme treatment.

**Discussion**

Globally, tuberculosis remains an important issue and causes bacterial infections that lead to serious morbidity and mortality associated with resistant strains\(^{10}\). Individuals who have contact with a tuberculosis patient prior to the start of their treatment, who live in areas with low socio-economic status, and high drug resistance, and whose immune systems are compromised are at higher risk for resistance\(^{11}\). NTM infections as a cause of mortality and morbidity have increased in parallel with the increase in immunosuppressant administration, and NTM distribution is diverse between countries and regions\(^{6}\). In our study, we investigated MTC strains isolated from Sakarya, one of the top three regions with the highest incidences of tuberculosis, 38 per 100,000. We investigated the resistance states of the isolated MTC strains, as well as, the NTM strains that were identified in the cultures.

Although low rates of AFB positivity, such as 46.8%\(^{12}\) and 58.2%\(^{13}\), have been reported in culture-positive samples, microscopic examination of culture-positive samples in Turkey revealed an AFB positivity rate of 70%\(^{14}\). In a study with clinical samples, the AFB and AUR staining methods were reported to have sensitivities of 0.571 and 0.736, respectively, and specificities of 0.996 and 0.973\(^{15}\). In our study, culture-positive samples had an AFB positivity rate of 50.4% and an AUR positivity rate of 65.1%. In addition, AFB and AUR sensitivities were 0.5043 and 0.6311, respectively, and specificities were 0.9868 and 0.9954, respectively. The ratios we obtained were below the country average, potentially due to the quality of the samples, and so, we planned to inform the patients more effectively during the process of obtaining the samples.

In studies of clinical samples, LJ culture positivity rates between 4.0%\(^{16}\) and 27.1%\(^{17}\) have been reported. In culture-positive samples, LJ positivity rates between 49.0%\(^{18}\) and 92.7%\(^{19}\) have been reported. In our study, 81.2% of culture-positive samples were positive on LJ medium, a rate consistent with the literature.

Studies using automated liquid culture systems have reported positivity rates of 3.0%\(^{18}\) and 67.0%\(^{20}\). In our study, we used a MGIT automated system, which is widely used in Turkey, and we determined a positivity rate of 11.2%. In addition, using the MGIT system, 13 (0.7%) samples were determined to be negative. Of the studied diagnostic methods, the MGIT system offered diagnostic advantages with high sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) in our study.

Increased resistance to anti-tuberculosis drugs makes it necessary to perform sensitivity tests to achieve effective treatment. Saygan et al.\(^{21}\) studied drug sensitivity using 505 MTC strains and reported the following resistance rates to primary choice anti-tuberculosis drugs: 9.1% STR, 13.2% INH, 13.2% RIF, and 3.3% EMB. The 2011 Report on Turkey’s Fight against Tuberculosis reported that, in 2009, patient resistance rates to STR, INH, RIF, and EMB were 8.5%, 13.1%, 6.5%, and 4.7%, respectively (14). In our study, resistance rates to the first choice drugs STR, INH, RIF, and EMB were determined to be 14.1%, 23.9%, 16.9%, and 5.6%, respectively. In addition, two strains (2.8%) isolated from the clinical samples of the first patients were resistant to all first-choice drugs. The high resistance rates observed in our study were thought to be associated with the chronic patients followed in the Tuberculosis Dispensary of Sakarya.

According to data obtained in various studies, infections created by NTM species account for 1-35% of all mycobacterial infections. Such a wide range may be due to the difficulty in diagnosing NTM thus far\(^{22}\). In our study, the NTM detection rate was 12.3% (10/81), which is consistent with the literature. In addition, in the present study, M. intracellulare, M. gordonea, M. peregrinum, and M. parafortuitum were detected in our region for the first time.

In conclusion, the identification of atypical mycobacteria should be enlightening the distribution rates in public, defining appropriate drug regimens and increasing the quality of tuberculosis control programs. Therefore, a molecular approach is of fundamental importance for the correct diagnosis of these infections due to atypical mycobacteria which may be easily misinterpreted.
Additionally, to reduce the incidence of tuberculosis in our region to an acceptable level, public health units, family physicians, state hospitals, and university hospitals must successfully coordinate with each other.

References


Corresponding author
Dr. HUSEYIN AGAH TERZI
Sakarya University Training and Research Hospital, Department of Medical Microbiology, Sakarya (Turkey)