The Use of High Performance Liquid Chromatography to Speciate and Characterize the Epidemiology of Mycobacteria

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Objective: We evaluated high-performance liquid chromatography (HPLC) for species identification of mycobacteria from various clinical specimens in an urban hospital in South Korea between January 2005 and December 2009.

Methods: In the study period 24,774 cultures were completed, yielding the 3215 clinical isolates cultivated for mycobacteria and positive cultures that had mycolic acid investigated by HPLC. For species identification, we compared HPLC patterns of clinical isolates with 33 standard Mycobacterium species.

Results: There were 3 different HPLC groups with single, double, and triple-cluster patterns representing 9, 20, and 4 mycobacterial species, respectively. Species identification rates of HPLC for Mycobacterium tuberculosis and nontuberculous mycobacteria (NTM) were found to be 100% and 95.6%, respectively. Among mycobacterial isolates, 12.1% were NTM-positive. There were 20 different NTM species with frequencies of 0.3%-15.5%.

Conclusion: The HPLC method was highly sensitive identifying NTM isolated from clinical specimens.

Keywords: mycobacteria, Mycobacterium tuberculosis, nontuberculous mycobacteria, HPLC

Over the past 2 decades, the incidence of mycobacterial infections has been notably increasing, caused mostly by higher rates of immunosuppression by HIV infection and treatments for transplantation and rheumatologic diseases.7-9,32,34,37,38 However, immunocompetent individuals are also infected by mycobacteria and can pose a diagnostic and treatment challenge.11-14,29,31,33 The diagnosis and treatment of mycobacterial infections can be difficult, as nontuberculous mycobacteria (NTM) is widespread in the environment, and treatments must often be tailored to the infecting mycobacterial species.1-2,21,26,28,30,36

For the identification of mycobacterial species, the Korean National Tuberculosis Association and most medical institutions in South Korea use conventional biochemical methods, polymerase chain reaction (PCR), and gene probe assays,16,22,23,37,34 however, these methods are time consuming, vulnerable to contamination, difficult to evaluate for various mycobacterial species, and often require multiple steps to identify species level.2,22-24,27,33 High-performance liquid chromatography (HPLC) is 1 of the methods used for mycobacterial identification. This method analyzes an organism’s mycolic acids (fatty acids found in the cell walls of mycobacteria) and is highly species specific.3-6,15 This study aimed to use HPLC to distinguish mycobacterial species and to characterize the distribution rate of these species in Ulsan, South Korea.

Methods

Study Population and Specimens

All 24,775 clinical specimens were obtained from the Department of Laboratory Medicine, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan, South Korea. During the period of January 2005 to December 2009, specimens were processed for acid-fast bacilli (AFB) culture. For AFB culture, specimens were inoculated in 3% Ogawa solid egg-based medium (Eiken Chemical, Tokyo, Japan) and Mycobacteria Growth Indicator Tube liquid medium (Becton Dickinson, Le Pont de Claix-Cedex, France) at the same time, following the Clinical and Laboratory Standards Institute (CLSI) protocol.

Identification of Mycobacteria

For identification, HPLC patterns were compared with patterns from standard Mycobacterium species,3-6,15 and these standards were obtained from 28 American Type
Culture Collection (ATCC) standard *Mycobacterium* species and 5 Korean Type Culture Collection (KTCC) standard *Mycobacterium* species (Table 1). The HPLC instrument was equipped with Waters 2690 separation module (Milford, MA), reverse phase analytical cartridge column, 3.9 × 75 mm, packed with 3 μm silica (Nova-Pak C18), and photodiode array detector (Waters Corporation, Milford, MA). For internal standards for low- and high-molecular weight, 6,7-dimethoxy-4-coumarinylmethyl ester and p-bromophenyl ester were used, respectively.

The HPLC samples were prepared by cell harvesting, saponification, extraction, and a clarification to isolate mycolic acids. The procedure involved a whole cell saponification, acidification, and methanolic extraction of all cellular fatty acids, including the mycolic acids. Mycobacteria were removed from a 3% Ogawa slant or Mycobacteria Growth Indicator Tube with a sterile polyester swab and placed in 2 mL of saponification reagent composed of 25% potassium hydroxide in a water methanol (1:1) mixture. The suspension was vigorously mixed and autoclaved for 1 hour at 121°C. After the mixture was cooled to room temperature, 2 mL of chloroform was added followed by 1.5 mL acidification reagent and a mixture of water and concentrated hydrochloric acid (1:1). This solution was mixed and the organic/aqueous layers were separated. The lower, organic layer was transferred with a Pasteur pipette to a new tube and evaporated in a heat block at 85°C-105°C with a stream of air. The commercial derivatization reagent was suspended in acetonitrile and consisted of p-bromophenacyl bromide p-bromophenacyl ester (PBPA) and a crown ether catalyst. A mycolic acid sample was derivatized to PBPA esters by adding 20 mg of potassium hydroxide in a water methanol (1:1) mixture. The suspension was cooled and then filtered through a 0.45-micron-size nylon 66 membrane filter.

The prepared samples were subjected to HPLC analysis using UV detector. The HPLC peaks, which were shown between low- and high-molecular weight standards, were first separated into single, double, triple, and multiple cluster patterns. Each cluster group was identified by its number of peaks, retention times, and relative peak heights.

From the AFB HPLC pattern analysis, single cluster included 9 species: *M. asiaticum*, *M. bovis*, *M. ganti*, *M. gordonae*, *M. kansasii*, *M. marinum*, *M. szulgai*, *M. triviale*, and *M. tuberculosis*; double cluster included 20 species: *M. abscessus*, *M. acapulcensis*, *M. agri*, *M. avium*, *M. celatum*, *M. chelonae*, *M. diernhoferi*, *M. flavescens*, *M. fortuitum*, *M. gilvum*, *M. goronae*, *M. intracellulare*, *M. kansasii*, *M. kansasii*, *M. phlei*, *M. porcinum*, *M. scrofulaceum*, *M. smegmatis*, *M. terrae*, and *M. xenopi*; and triple cluster group included 4 species: *M. australficanum*, *M. pulveinum*, *M. simiae*, and *M. vaccae* (Figures 1, 2, and 3). Multiple-cluster groups showing more than 4 peak clusters were not included in this study since they are yet to be confirmed as infectious. High-performance liquid chromatography patterns, which did not match with any of 33 standard *Mycobacterium* species, were included as ‘unclassified’ NTM.

### Results

**Identification of Mycobacteria Species Using HPLC**

During the study period, a total of 24,778 clinical specimens of various types from 13,241 patients were collected.

![Figure 1](https://example.com/figure1.png) **Figure 1.** Examples of a HPLC chromatogram of standard *Mycobacterium* species with single-cluster peak patterns. A) *M. gordonae*, B) *M. kansasii*, and C) *M. tuberculosis*.

<table>
<thead>
<tr>
<th>Mycobacterium Species</th>
<th>Description</th>
<th>Mycobacterium Species</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td><em>M. abscessus</em> ATCC 19977</td>
<td>ATCC 927</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. acapulcensis</em> KTCC 9501</td>
<td>M. marinum ATCC 49650</td>
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<tr>
<td><em>M. agri</em> ATCC 27406</td>
<td>M. nonchromogenicum ATCC 19530</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. asiaticum</em> ATCC 25276</td>
<td>M. peregrinum ATCC 14467</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. australficanum</em> KTCC 33464</td>
<td>M. phlei ATCC 354</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. avium</em> ATCC 25291</td>
<td>M. porcinum KTCC 9517</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. bovis</em> ATCC 19210</td>
<td>M. pulveinum KTCC 9518</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. celatum</em> ATCC 51131</td>
<td>M. scrofulaceum ATCC 19981</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. chelonae</em> ATCC 35752</td>
<td>M. simiae ATCC 25275</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. diernhoferi</em> KTCC 9506</td>
<td>M. smegmatis ATCC 21701</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. flavescens</em> ATCC 14474</td>
<td>M. szulgai ATCC 35799</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. fortuitum</em> ATCC 6841</td>
<td>M. terrae ATCC 15755</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gastri</em> ATCC 15754</td>
<td>M. triviale ATCC 22392</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gilvum</em> KTCC 9512</td>
<td>M. tuberculosis H37R</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. gordonae</em> ATCC 14470</td>
<td>M. vaccae ATCC 15483</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. intracellulare</em> ATCC 13950</td>
<td>M. xenopi ATCC 19250</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. kansasii</em> ATCC 12478</td>
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<td></td>
<td></td>
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</tbody>
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Among the specimens, 3212 isolates (13.0%) from 1421 patients (10.7%) were positive by AFB culture. Mycobacteria from these cultures were selected for further study via HPLC traces of mycolic acids. The vast majority of HPLC patterns (3195 out of 3212, 99.5%) matched to those of the 33 standard Mycobacterium species. The other 17 isolates (0.5%) did not match any of the standards and hence were grouped into “unclassified” NTM.

Excluding M. tuberculosis, 388 isolates were identified as 19 NTM species and unclassified NTM. In the single-cluster group, 6 species: M. asiaticum, M. gordonae, M. marinum, M. szulgai, and M. triviale were identified (Figure 1). In the double-cluster group, 12 species: M. abscessus, M. acapulcensis, M. avium, M. chelonae, M. fortuitum, M. intracellularare, M. mucogenicum, M. nonchromogenicum, M. peregrinum, M. scrofulaceum, M. terrae, and M. xenopi (Figure 2) were identified, whereas only 1 species, M. simiae was found in the multi-cluster group and no species were found in the triple-cluster group (Figure 3). Additionally, identical species were identified from all patients with multiple specimens who were culture positive for AFB.

Distribution of NTM

Among 3212 isolates from 1421 patients, 2824 isolates (87.9%) from 1218 patients (85.7%) were identified as M. tuberculosis and the rest were classified as NTM. Among the 388 NTM isolates, the prevalence of the various species ranged between 15.5% to <1% (Table 2).

Consistent with the distribution of types of clinical specimens, most NTM were isolated from sputa, pleural fluid, and bronchial aspirates (74.7%), but NTM were identified in other specimen types (25.3%): urine (7.0%), skin (5.9%), lymph node (5.7%), pus (2.6%), cerebrospinal fluid (CSF) (1.6%), gastric aspirates (1.3%), bone marrow (0.8%), and joint fluid (0.5%).

When we investigated trends, NTM species detected in >5% of specimens (M. kansasii, M. intracellularare, M. gordonae, M. avium, M. fortuitum, M. abscessus, M. peregrinum, and M. szulgai) demonstrated no significant changes during the study period (2005-2009) (Table 3). However, when compared to the 1981-1994 study period, there was a decrease in M. avium-intracellularare, M. fortuitum, and M. chelonae but an increase in M. kansasii and M. gordonae.

A national survey of mycobacterial disease other than tuberculosis in Korea conducted by the Scientific Committee in the Korean Academy of Tuberculosis and Respiratory Disease for 14 years between 1981 and 1994 reported that 158 isolates out of 159 isolates, which were admitted to the Korean National Tuberculosis Association for infection examination, were identified as NTM by biochemical methods. In this report, NTM species distribution was: 104 cases in M. avium-intracellularare (65.2%); 20 in M. fortuitum (12.7%); 15 in M. chelonae (9.5%); 7 cases in M. gordonae.
evaluation for various mycobacterial species, and often require they have a high degree of false-positive results, are difficult to methods include some chances for contamination. Therefore, fast and accurate in their species level detection, but these methods using PCR and gene probe assay are known to be difficulty in discriminating species level. Also, identification identification are limited in their sensitivity, time needed, and

In 1960, the occurrence rate of NTM infections by AFB


time, needed, and difficulty in discriminating species level. Also, identification methods using PCR and gene probe assay are known to be fast and accurate in their species level detection, but these methods include some chances for contamination. Therefore, they have a high degree of false-positive results, are difficult to evaluate for various mycobacterial species, and often require multiple steps to identify species level. However, HPLC methods, employed in this study and reported by others, used species-specific mycolic acid for analysis, and these can overcome the drawbacks mentioned above. We used the 33 standard Mycobacterium species for HPLC comparison and found that 3195 of our isolates (99.5%) had matching HPLC patterns. When excluding tuberculosis isolates, 371 NTM isolates (95.6%) were speciated.

In our hospital a HPLC machine is available, and the cost per sample is $4 United States dollars. This would equate to $12,848 spent to screen the 3212 samples in this study. This would compare favorably to PCR-based methods that would cost $40 per sample.

In 1960, the occurrence rate of NTM infections by AFB culture in South Korea was less than 1%, but more than 30% of NTM species, detected from water, soil, air, and humans, were estimated to exist but have yet to be reported to cause human disease.

The widely used biochemical methods for mycobacterial identification are limited in their sensitivity, time needed, and difficulty in discriminating species level. Also, identification methods using PCR and gene probe assay are known to be fast and accurate in their species level detection, but these methods include some chances for contamination. Therefore, they have a high degree of false-positive results, are difficult to evaluate for various mycobacterial species, and often require

Discussion

Species-specific mycobacterial identification in clinical specimens is an essential step for the diagnosis of mycobacterial infection. Until now, more than 91 NTM species were reported, but more than 30% of NTM species, detected from water, soil, air, and humans, were estimated to exist but have yet to be reported to cause human disease. The present study showed that between 2005 and 2009, NTM infections reported in Ulsan, South Korea, were 2.5 times higher than the rate between 1981 and 1994.

Table 2. Annual Distribution of Species Over 5% of Nontuberculous Mycobacteria

<table>
<thead>
<tr>
<th>Years</th>
<th>2005 (n=57)</th>
<th>2006 (n=58)</th>
<th>2007 (n=57)</th>
<th>2008 (n=59)</th>
<th>2009 (n=62)</th>
<th>Total (n=293)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. kansasi</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>13</td>
<td>60</td>
</tr>
<tr>
<td>M. intracellular</td>
<td>12</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>10</td>
<td>52</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>10</td>
<td>9</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>M. avium</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>5</td>
<td>6</td>
<td>8</td>
<td>5</td>
<td>7</td>
<td>31</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>M. peregrinum</td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>M. szulgai</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>20</td>
</tr>
</tbody>
</table>

(4.4%); 5 in M. terrae (3.2%); 3 in M. scrofulaceum (1.9%); 2 in M. kansasi (1.3%); 2 in M. szulgai (1.3%), and 1 in M. avium-intracellularum and M. terrae (0.6%). The present study found that between 2005 and 2009, NTM infections reported in Ulsan, South Korea, were 2.5 times higher than the rate between 1981 and 1994.
Although there are only a few reports of NTM species rate and distribution, a national survey of mycobacterial diseases other than tuberculosis in Korea conducted by the Scientific Committee in the Korean Academy of Tuberculosis and Respiratory Disease for 14 years between 1981 and 1994 reported that 158 isolates out of the 159 isolates admitted to the Korean National Tuberculosis Association for infection examination were identified as NTM by biochemical methods. Notably, the present study indicated that between 2005 and 2009, NTM infections reported in Ulsan, South Korea, were 2.5 times higher than the rate between 1981 and 1995. This difference in occurrence may in part be due to the fact that this study used HPLC, while the Scientific Committee in Korean Academy of Tuberculosis and Respiratory Disease used biochemical tests for the identification of mycobacteria. The increase may indicate that the NTM infection rate is escalating and also that the HPLC method is more precise than standard biochemical methods in discriminating species. However, based on the fact that the species found in more than 5% in this study (M. kansasi, M. intracellulare, M. gordonae, M. avium, M. fortuitum, M. abscessus, M. peregrinum, and M. szulgai) showed no increase for 5 years, we conclude that the increased number of species found in this study is most likely the result of using the more sensitive HPLC method.

In species distribution, M. avium complexes including M. avium and M. intracellulare were found the most (24.7%) in this study, but when compared to the national survey of mycobacterial diseases other than tuberculosis in Korea (65.2%), these mycobacteria species decreased by 40.5% when compared to the national survey of mycobacterial diseases other than tuberculosis in Korea (65.2%), this study, but when compared to the national survey of mycobacterial diseases other than tuberculosis in Korea (65.2%), M. intracellulare were found the most (24.7%) in respiratory systems including sputum, bronchial aspirates, and pleural fluid (290 cases, 29.6%), and also that the HPLC method is more precise than standard biochemical methods in discriminating species. Among the inspected mycobacteria, 20 species were identified NTM (12.1%). Based on this study, the epidemiology of NTM seems to be changing in South Korea, showing that M. avium-intracellulare, M. fortuitum, and M. chelonae have been decreasing in prevalence, while M. kansasi and M. gordonae have been relatively increasing. This study also validated HPLC as a useful method for speciating AFB-positive cultures, which is practical for diagnosis and proper treatment thereafter. This study also establishes a benchmark in South Korea for mycobacterial distribution, which will be useful for future mycobacteria investigations.

**Conclusion**

In conclusion, HPLC methods/speciated 99.5% of mycobacteria cultured from clinical specimens and 95.6% of NTM species. Among the inspected mycobacteria, 20 species were identified NTM (12.1%). Based on this study, the epidemiology of NTM seems to be changing in South Korea, showing that M. avium-intracellulare, M. fortuitum, and M. chelonae have been decreasing in prevalence, while M. kansasi and M. gordonae have been relatively increasing. This study also validated HPLC as a useful method for speciating AFB-positive cultures, which is practical for diagnosis and proper treatment thereafter. This study also establishes a benchmark in South Korea for mycobacterial distribution, which will be useful for future mycobacteria investigations.

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