RESEARCH ARTICLE

Determination of antibiotic resistance and high-performance liquid chromatography profiles for *Mycobacterium* species

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Background: Incidence of mycobacterial infections has been increasing. However, diagnosis and treatment of mycobacterial infections can be difficult. The aim of this study was to investigate high-performance liquid chromatography (HPLC) analysis of the mycolic acids for rapid identification and dendrogram cluster analysis of mycobacterium species.

Methods: Clinical specimens received for mycobacterial culture and antimicrobial susceptibility test were processed by standard laboratory protocols. Positive cultures were analyzed with HPLC method. Mycolic acid analysis with HPLC was used for diagnosis of tuberculosis and other mycobacterial infections. These reports were compared with Sherlock Library mycobacterial species, and the similarity index was analyzed. This value was formed by a software in multidimensional space that was the calculation of the average distance between the nearest library profile and unknown profile.

Results: The ninety-two samples were identified as *M. tuberculosis*. (similarity index between 0.593 and 0.994). One of the other strains was identified as *M. avium intracellulare* (strain No. 82) (SI = 0.906); one of them was identified as *M. interjectum* (strain no. 89) (SI = 0.644). Total 94 samples were identified, and dendrogram was applied to these samples. Profile A (10.6%), profile B (59.6%), profile C (11.7%), profile D (3.2%), and other profiles as single different profiles were identified. Rates for each as 1% (89, 94, 1, 82, 26, 42, 32, 41, 100, 43, 47, 44, 40, 35).

Conclusion: High-performance liquid chromatography is a useful, rapid, reliable, and practical method for diagnosis of mycobacterium species.

KEYWORDS

antibiotic susceptibility, dendrogram analysis, HPLC, mycobacteria, *Mycobacterium tuberculosis*

1 | INTRODUCTION

Tuberculosis (TB) always has had an important place in infectious diseases throughout human history. The number of new TB cases in recent years is rapidly increasing despite growing global effort. About one-third of the world's population are infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). The World Health Organization (WHO) estimates that each year, 8.6 million people are infected with TB and resulting in 2 million deaths.¹⁻³ TB incidence in our country is

reported as 0.00022 in 2012.⁴ Great difficulties are experienced in the treatment of TB today due to the improper treatment regimens and drug usage without surveillance with result of the inadequate TB control programs in the past. TB infection is still first cause of death in preventable infectious diseases of the world. The prevention of TB disease depends on medical treatment. In addition, there is a continuous transmission of infection due to huge global reservoir. TB is a chronic granulomatous infectious disease that is created by particularly *Mycobacterium tuberculosis*, *Mycobacterium africanum*.

M. tuberculosis which are responsible for most of the cases. TB disease can be seen in all of the organs but most commonly affected organ is lung.⁵ TB is diagnosed by clinical signs and symptoms, radiographic appearance, the evaluation of the tuberculin skin test results, ARB, and culture.⁶⁻⁸ The search for a fast, reliable, and cheap method for TB has been going on for centuries. ARB is most widely used, quick, and simple method which is the direct microscopic examination of clinical samples. It must be confirmed with culture, but there are some disadvantages of culture method which are the difficulties of material procurement especially in children, and impossibility of taking samples from healthy people in latent infection period.⁹ Speed and specificity of ARB microscopy are higher than culture but sensitivity is lower than molecular test method. The method commonly used in molecular diagnosis of tuberculosis is based on the target nucleic acid amplification.¹⁰ The major disadvantage of this method is DNA contamination. In studies, when direct PCR applications used, high rate of false negatives and positives was revealed.¹¹ Real-time PCR seems to be quite useful in the solution of problems. But, there are disadvantages of the new system which are dependent mostly equipment and supplies, and not yet fully standardized, are multistep process during the assessment of the challenges and various mycobacterial species.¹⁰⁻¹² High-performance liquid chromatography (HPLC) for the diagnosis of tuberculosis in clinical samples is recommended in conjunction with ARB microscopy and culture.¹³ Many new techniques have been developed to determine some specific compounds in patients with tuberculosis. Mycobacteria contain large amounts of lipids such as mycolic acid. HPLC is an alternative identification method, but is generally performed from solid media and requires manual inspection of mycolic acid chromatographic profiles, as well as extensive expertise. The identification of mycobacterial species by HPLC is also possible. Indeed, differences in the types of mycolic acid patterns, such as the *M. tuberculosis*, *M. kansasii*, *M. avium*, and *M. intracellulare*, allow this distinction. Identification of mycobacteria from culture can be completed in a very short time. However, these methods have a requirement for the presence of mycobacterial cells 10^7 - 10^8 to be effective that makes this process necessitates the 14-21 culture day. HPLC is quick and repeatable method based on chromatographic method to determine mycolic acid esters present in the cell wall of mycobacteria.¹³⁻¹⁵ There were a limited number of study for HPLC analysis for mycobacterium species. Also in our country, no data were found in the literature.

The purpose of this study was to investigate the use of a commercially available HPLC system with pattern recognition software for the identification of mycobacteria. In this report, we will examine HPLC analysis of the mycolic acids for rapid identification and dendrogram cluster analysis of mycobacterium species.

2 | MATERIALS AND METHODS

This study is a prospective study. All *M. tuberculosis* strains (n = 98 clinical strains, n = 2 *atypical mycobacterium spp.*) were identified and

antibiotic susceptibility tests (for streptomycin, isoniazid, rifampin, and ethambutol) performed by TK Rapid Mycobacterial Culture System (Salubris Technica, İstanbul, Turkey). A total of 100 clinical isolates which were all derived from 78 patients. More specifically, isolates 42, 79, 86 from one patient; isolates 19, 45, 88 from one patient; isolates 10, 21, 25, 29 from one patient; isolates 4, 6 from one patient; isolates 23, 24 from one patient; isolates 22, 63 from one patient; isolates 15, 27 from one patient; isolates 1, 8, 74 from one patient; isolates 26, 30, 33 from one patient; isolates 20, 44, 48, 69 from one patient; isolates 71, 73 from one patient; isolates 68, 72 from one patient; isolates 65, 66 from one patient; isolates 89, 97 from one patient; and isolates 92, 100 obtained at different times.

In this study, identification and dendrogram of the strains were determined with Sherlock® Mycobacteria ID System (MYCO-LCS) (USA). Cellular mycolic acid analysis of the strains was determined with HPLC method.¹⁶ Clinical specimens received for mycobacterial culture were processed by standard laboratory protocols and inoculated on to Lowenstein-Jensen agar. Sherlock requires pure cultures. Mycolic acid analysis with HPLC is being used by laboratories for diagnosis of tuberculosis and other mycobacterial infections. MYCO-LCS is used for identification of *M. tuberculosis* and differentiation from other mycobacteria and related species through the analysis of mycolic acids derived from cultured bacterial samples. Sherlock utilizes a sample preparation method and HPLC to obtain qualitatively and quantitatively reproducible mycolic acid composition profiles. Automated pattern recognition software matches the sample's composition with stored patterns of mycobacteria species/groups and related species. This system is also be calibrated and monitored by the software to provide appropriate functioning. The Sherlock Mycobacteria Library includes over 25 species/groups, created from well-characterized mycobacterial strains. After the samples are separated to mycolic acids, time of the response from the device for each sample takes approximately 14 minutes. Reports include an identification for each isolate, along with a similarity index (SI). An acceptable identification requires an SI of ≥ 0.5 , with a minimum of 0.2 SI separation from the next closest species identification.

Dendrogram cluster analysis technique produces unweighted pair matchings based on fatty acid compositions. The results are demonstrated graphically to show the relationship between organisms. Euclidian distance (ED) is the distance in multidimensional space between two strains when their fatty acid contents are compared. The "unweighted pair-grouping" method used in the Sherlock software is effective at comparisons at taxonomic levels of species and lower, but should not be used for genus comparisons or above. Guidelines for the Euclidian distance in dendrogram: If samples linked at about 10 or less are typically the same species. If samples linked at about 6 or less are typically the same subspecies or biotype. If the samples linked at about 2.5 or less are typically two different runs of the same strain. These rules for dendrogram do not apply in reverse that a linkage of 10 or above does not necessarily mean two microorganisms are different species. From the dendrogram, conclusions about the relatedness or similarity between sample runs can be made. In addition, dendrogram can be used to determine

FIGURE 1 Antibiotic resistance patterns and high-performance liquid chromatography profiles of mycobacterium species

whether a group of samples can be reliably separated into two or more subgroups.

3 | RESULTS

A total of 100 mycobacterium species: 98 strains of *M. tuberculosis* and 2 atypical mycobacterium species were included in the study. Six samples (69, 75, 81, 84, 88, 96) could not be identified and dendrogram applied. This result was evaluated as that samples may be old samples and lost their viability. Ninety-two samples were identified as *M. tuberculosis*. (similarity index between 0.593 and 0.994). One of remaining strains was identified as *M. avium intracellulare* (strain No. 82) (SI = 0.906); one of them was identified as *M. interjectum* (strain no. 89) (SI = 0.644). A total of 94 samples were identified and dendrogram applied. Atypical detected isolates in TK Rapid Mycobacterial Culture System was identified as *M. tuberculosis* in HPLC. Majority of isolates were found to be susceptible to streptomycin (S), isoniazid (I), rifampicin (R), and ethambutol (E). More specifically, resistance (R) rates were determined of 98 samples as 2% R for streptomycin, 4% R for isoniazid, 6% R for rifampicin, and 0% R for ethambutol.

Dendrogram cluster analysis of the Sherlock System for the 94 isolates was shown on Figure 1. based on fatty acid compositions analysis criteria proposed by Sherlock, Euclidian distance, major four profiles were found to be epidemiologically different from each other: Profile A is one of the commonly identified profile (10.6%), profile B is most commonly identified profile (59.6%), profile C is another commonly identified profile (11.7%), profile D that is another least commonly identified profile (3.2%), and other profiles are identified as single different profiles. Rates for each profile as 1% (89, 94, 1, 82, 26, 42, 32, 41, 100, 43, 47, 44, 40, 35). Profile B was found to

possess four subprofiles: profiles B1 (9.6%), B2 (29.8%), B3 (17%), and B4 (3.2%) on Figure 1.

4 | DISCUSSION

Tuberculosis is a common infectious disease caused by mycobacteria, usually *Mycobacterium tuberculosis*. It is as old as humanity. Tuberculosis typically infects the lungs, but can also spread to other parts of the body. *Mycobacterium tuberculosis* is found in almost every part of nature, such as waters, soil, and air. Cell wall of *Mycobacterium tuberculosis* bacilli is thicker than other bacteria and includes a high-lipid component as mycolic acid.¹⁷ Treatment of TB takes long and expensive courses. Much of the drug resistance worldwide is the result of inadequate or inappropriate therapy, which has toxic side effects.¹⁸

TK Rapid Mycobacterial Culture System has been developed recently. TK media is used for rapid mycobacterial culture. TK media is incubated and read by Mycolor TK automated incubator reader. There are a limited number of studies on the performance and advantages of TK tuberculosis culture system. Culture method is very important in the diagnosis of tuberculosis, but detection of growth takes a long time in a classical culture system.¹⁹⁻²¹

Today, HPLC analysis of mycolic acids for species identification is suggested instead of the traditional time-consuming processes. An isolate submitted on culture could be analyzed in hours with HPLC, compared to weeks or longer for conventional methods. But, suitability of the method should be considered before inclusion in the laboratory. HPLC method is considered advanced compared with other laboratory methods and a special, highly trained staff is necessary. Instrumentation is expensive compared with traditional methods.²² The use of continuously monitored liquid-based culture systems

for mycobacteria has resulted in decreased times for detection of positive cultures. Molecular analysis methods do not need standardized growth conditions, however, often need more hands-on time for sample processing. HPLC requires more viable cells than the molecular methods. Molecular methods have a greater detection sensitivity than the HPLC method. Both HPLC and automated sequencing methods require expensive and sophisticated equipment. Although automated sequencing equipment is as expensive as chromatographic instruments, a single sample can be processed in about 15 minutes with HPLC, but automated sequencing takes hours.¹³

The sensitivity of automated identification of *M. tuberculosis* complex and MAC from BACTEC 12B cultures and from fluorochrome-stained smear-positive sputum specimens has been shown. Jost et al²³ reported that rapid, sensitive, and specific identification of MTB and MAC from young BACTEC 12B cultures was possible with HPLC-FL. We did not find any study about TK medium and HPLC method like article of Jost et al by searching the available literature.

In this study, identification and dendrogram cluster analysis of strains were determined with MYCO-LCS. Reports of MYCO-LCS are provided in two forms: First one is the chromatographic report which was formed by fluorescent detectors obtained from electronic signal, and second one is the composition report indicating the mycolic acid composition of organism. These reports compared with Sherlock Library mycobacterial species and the similarity index will be analyzed. This value formed by a software in multidimensional space is the calculation of the average distance between the nearest library profile and unknown profile. This value is the expression of a relative from the average.

Sherlock dendrogram analysis determined for mycobacterium isolates, shown on Figure 1, revealed that profiles A, B, C, and D are unrelated (acceptable identification required an SI of ≥ 0.5). Majority of the isolates were grouped into profile B family with four closely related subprofiles. In regard to resistance to antimycobacterials in profile A, isolates had the same antibiotics profile and susceptible to streptomycin, isoniazid, rifampin, and ethambutol while one of the isolates in profile B2 possessed streptomycin resistance. In profile B3, rifampicin resistance was found in four isolates. There was no resistance in profile B4. It was found that all subprofiles of profile B were epidemiologically closely related to each other with an SI of ≥ 0.5 . Here, profile B2 was most commonly identified subprofile (28 specimens of 56, 29.8%).

Isolates 42, 79, 86 from one patient: 79 and 86 were found in the same profile (profile B) while 42 was found in another profile. Isolates 19, 45, 88 from one patient: 19 was found in profile B2, 45 was found in profile C but 88 was not identified and grouped. Isolates 10, 21, 25, 29 from one patient: 21 was found in profile A, 10 was found in profile B, 25, 29 were found in profile C. Isolates 4, 6 from one patient: isolates 23, 24; isolates 22, 63 from one patient were in profile B. Isolates 15, 27 from one patient: 15 in profile B, 27 in profile C; isolates 1, 8, 74 in profile B from one patient; isolates 26, 30, 33 from one patient: 30 in profile D, 26, 33 in profile C; isolates 20, 48, 44, 69 from one patient: 20, 48 in profile B, 44 in different single profile, 69 (not identified); isolates

71, 73 in profile B from one patient; isolates 68, 72 from one patient: 68 in profile B, 72 in profile A; isolates 65, 66 in profile B from one patient; isolates 89, 92, 97, 100 from one patient: 89 is *Mycobacterium interjectum*, 92 in profile B, 97 in profile A, 100 in different single profile obtained at different times. We have seen that some profiles obtained from the same patient in the study have not the same profile in the dendrogram. We interpreted this result as reinfection, but we have seen the strains also in the same profile in dendrogram. We considered this result as reactivation. These different profiles in the same patients suggested that both of the reactivation and reinfection in the same patient at different times. One of the reasons might be self-mistreatment of antibiotics or taking antimycobacterial drugs.

This study showed that HPLC is a useful, rapid, reliable, and practical method for diagnosis and proper treatment thereafter. This study also establishes a benchmark in Turkey for mycobacterial distribution that will be useful for future mycobacteria investigations.

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