Short Communication

Are we overlooking infections owing to non-tuberculous mycobacteria during routine conventional laboratory investigations?

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ABSTRACT

A large number of potentially pathogenic non-tuberculous mycobacteria (NTM) encountered in the clinical laboratory makes it necessary to identify their species to ensure appropriate treatment. However, labor-intensive conventional methods of speciation are not used in every laboratory, and hence NTM infections are often ignored. Polymerase chain reaction (PCR) restriction analysis (PRA) was applied in this study for early identification and speciation of mycobacterial species on 306 cultures of acid-fast bacilli isolated from patients suspected of suffering from tuberculosis. Mycobacterium tuberculosis was identified in 85.6% of the isolates. The NTM isolated most commonly was Mycobacterium kansasii/gastri group (3.5%), followed by Mycobacterium fortuitum (3.2%). Four of the M. fortuitum were grown from cultures obtained on the same day, but from samples from different patients and were probably laboratory contaminants. Mycobacterium intracellulare and Mycobacterium avium were identified in 2.94% and 2.28% of the isolates, respectively. Three isolates of M. avium and two isolates of M. intracellulare were obtained in repeated cultures from sputum samples of the same patients and were thus pathogenic. A single isolate of Mycobacterium abscessus was obtained from a breast abscess. A rare pathogen Mycobacterium phocaicum was isolated from one patient with epididymitis. However, whether it was the causative agent of epididymitis in this patient remains doubtful. The results of this study highlight the importance of speciation of mycobacteria for appropriate diagnosis and the importance of including molecular assays to augment conventional methods of diagnosis of mycobacterial diseases for rapid identification of NTM so that these potential pathogens are not overlooked in routine diagnostic procedures.

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Introduction

NTM are widely distributed in nature and have been isolated from soil, natural water, tap water, water used in showers and surgical solutions [1]. Although Mycobacterium tuberculosis is by far the most important mycobacterial species from a public health perspective, other species of non-tuberculous mycobacteria (NTM) are being encountered with increasing frequency and new species are being identified. While not all NTMs are pathogenic for humans, many are. Pathogenic NTMs are usually less virulent than M. tuberculosis, and potential pathogens may be isolated without obvious association with a disease. On the contrary, species usually considered benign may produce disease, especially in immunocompromised individuals [2].

The prevalence of NTMs among world populations has significant geographic variations that might occur even within the same country [3]. Because of the large number of potentially pathogenic NTMs that can be encountered in the clinical laboratory, diagnosis of these organisms is required to ensure appropriate treatment. However, diagnosis of NTM infection is often difficult. Bacteriological, radiological and clinical criteria to diagnose a disease are often not able to discriminate between M. tuberculosis and NTM infection [4]. Moreover, a positive tuberculin skin test does not allow for a distinction between NTM and M. tuberculosis infection [4]. Since labor-intensive conventional methods of speciation are not used in every laboratory, NTM infections go unreported and are thus ignored [5,6]. Hence, there is a need for an assay which would overcome the disadvantages of biochemical tests and would rapidly detect these organisms in clinical specimens.

PCR restriction analysis (PRA) – which is simple and easy to perform – was used in this study in order to get a preliminary assessment on the prevalence of NTM in the clinical specimens received in the laboratory from non-HIV patients and to understand the importance of including assays to identify NTM in a routine clinical microbiology laboratory in North India.

Materials and methods

Clinical isolates

A total of 306 clinical isolates were obtained from clinically suspected patients of pulmonary tuberculosis (n = 304) and extrapulmonary tuberculosis (n = 2) admitted at Rajan Babu Institute of Respiratory Medicine and Tuberculosis, Delhi and Vallabhbhai Patel Chest Institute (VPCI) during the time period 2007–2010. Rajan Babu Institute of Respiratory Medicine and Tuberculosis serves as a referral center for patients with tuberculosis in North India and VPCI serves as a referral hospital in North India for chest diseases. The patients were adults ≥ 18 years old and were not co-infected with HIV. The study was approved by the Institutional ethical committee, and written and informed consent was taken from the patients.

Identification of cultures

The isolates were subjected to biochemical identification using niacin, nitrate reduction and semi-quantitative catalase tests by the standard procedure [7].

DNA extraction for PCR

DNA was extracted from the reference strain H37Rv and the clinical isolates by boiling. Briefly, a loopful of mycobacterial growth was transferred to a micro-centrifuge tube containing 100 μl of 1% TritonX-100 and 50 μl of sterile double distilled water. The suspension was vortexed and boiled at 100 °C for 30 min. The suspension was centrifuged at 8000 rpm for 10 min and the clear supernatant containing mycobacterial DNA was taken for PCR.

Polymerase chain reaction (PCR)

PCR of the test samples was performed to amplify a 294 bp region of the hsp65 gene. The primers used in the study have been described previously [8]. H37Rv DNA and double distilled water were used as positive and negative controls, respectively. Briefly, mycobacterial DNA was amplified in a 50 μl reaction mixture containing 240 μM deoxynucleoside triphosphates (Biotools B & M Labs, Madrid, Spain), 5 μl of 10x Buffer, 2.5 mM of MgCl₂ and 1.5 U of Taq DNA polymerase [8].

Restriction analysis

Restriction digestion of the 294 bp product was carried out with 1 U each of Sau96I and CfoI (Boehringer Mannheim Biochemicals, Mannheim, Germany) in separate reactions as described earlier, with slight modifications [8]. The restriction digestion was carried out with 10 μl of the amplicon at 37 °C for 2 h. The digests were then electrophoresed in 10% non-denaturing polyacrylamide gel with a 50 bp molecular weight marker (Fermentas Life Sciences, Lithuania).

Sequencing

PCR products of 294 bp hsp65 gene of a subset of clinical isolates were subjected to sequencing by Automated Sequencer (Ocimum Biosolutions, Bangalore, India). Sequences were identified by similarity using Blastn available at NCBI (www.blast.ncbi.nlm.nih.gov/blast.cgi). Species identification was confirmed if a 97% match was achieved with any sequence deposited in the database, according to the criteria proposed by McNabb et al. [9].

Quality control

Quality laboratory practices were strictly followed at every step. Sterile distilled water was used for reagent preparation. Positive and negative controls were included with every new batch of media and reagents.
Results and discussion

NTM or atypical mycobacteria are environmental organisms that are normally found in soil and water. The incidence of NTM infection has increased in recent years [10–13], which could partly be due to a changing epidemiology, an increase in incidence of HIV co-infection or due to increased awareness. However, resource deficient countries often overlook infections owing to NTM because of a lack of extensive identification schemes that can be used in peripheral laboratories. Moreover, phenotypic tests are often difficult to interpret, cumbersome and have low specificity [14]. Hence, the importance of molecular assays to identify NTM cannot be underestimated.

NTM have been reported from patients with HIV in North India [15]. Therefore, PCR restriction analysis (PRA) was applied for early identification and speciation of mycobacterial isolates in the laboratory in patients suspected to be suffering from tuberculosis without HIV co-infection. Hsp65 PRA was applied to the DNA extracted from cultures of acid-fast bacilli (n = 306) obtained from patients suspected of suffering from tuberculosis to get a preliminary assessment on the prevalence of NTM in the clinical specimens received in the laboratory. The maximum numbers of specimens were obtained from patients with pulmonary tuberculosis; however, two samples were from extrapulmonary sites viz. pus from a breast abscess and a urine specimen. The isolates were obtained during the time period 2007–2010 and were subjected to biochemical identification using niacin, nitrate reduction and semi-quantitative catalase tests [7]. PCR was performed using published primers to obtain a 294 bp amplicon of the hsp65 gene [8] and restriction digestion carried out with Sau96I and CfoI (Fig. 1). The PCR amplicons of 51 clinical isolates were confirmed by sequencing and the sequences identified by similarity using NCBI Blastn. Sequencing confirmed 39 of the 51 isolates to be M. tuberculosis complex and 12 isolates as NTM in concordance with hsp65 PRA results.

Hsp65 PRA identified M. tuberculosis in 85.6% (n = 262) of the 306 isolates. Of the 44 (14.7%) NTM isolated in the laboratory, 2 were obtained from patients of extrapulmonary tuberculosis. Mycobacterium kansasi/gastri group was the most common NTM isolated and constituted 25% (n = 11) of all NTM. Mycobacterium fortuitum constituted 22.7% (n = 10) of the NTM isolated in our laboratory. Of these, 6 strains of M. fortuitum were isolated from samples cultured the same day and were not obtained on repeat cultures of the same patients. These isolates were thus considered to be laboratory contaminants. However, samples taken from various sites in the laboratory did not yield any NTM. The other 4 M. fortuitum were obtained in repeat cultures and were considered pathogenic. Another laboratory contaminant Mycobacterium gordonae was identified in 4 samples (Table 1).

Mycobacterium intracellulare and Mycobacterium avium constituted 20.45% (n = 9) and 15.9% (n = 7) of the NTM, respectively. The clinical relevance of three pulmonary M. avium isolates was established by the fact that they were isolated from sputum samples obtained from the same patient on three consecutive days, in pure culture. Similarly, two of the M. intracellulare isolates were obtained from sputum samples of the same patient on two different days and were considered to be clinically relevant. Mycobacterium abscessus was isolated from pus sample obtained from a patient presenting with a breast abscess. An emerging mycobacterial species, Mycobacterium phocaicum was isolated and were considered to be clinically relevant.

<table>
<thead>
<tr>
<th>Mycobacterium species</th>
<th>Total no. identified (%)</th>
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<tbody>
<tr>
<td>M. tuberculosis complex</td>
<td>262 (85.6%)</td>
</tr>
<tr>
<td>M. kansasi/gastri</td>
<td>11 (3.5%)</td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>10 (3.2%)</td>
</tr>
<tr>
<td>M. scrofulaceum</td>
<td>1 (0.32%)</td>
</tr>
<tr>
<td>M. avium</td>
<td>7 (2.28%)</td>
</tr>
<tr>
<td>M. intracellulare</td>
<td>9 (2.94%)</td>
</tr>
<tr>
<td>M. abscessus</td>
<td>1 (0.32%)</td>
</tr>
<tr>
<td>M. gordonae</td>
<td>4 (1.3%)</td>
</tr>
<tr>
<td>M. phocaicum</td>
<td>1 (0.32%)</td>
</tr>
<tr>
<td>Total</td>
<td>306</td>
</tr>
</tbody>
</table>

Fig. 1 – (A and B) PRA profiles for mycobacterial species detected in clinical isolates. (A) CfoI digests. Lane 1 is 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 10 is the digested positive control H37Rv. Lanes 3–9 are clinical isolates. Lanes 3, 6–8 are M. intracellulare. Lane 4 is M. fortuitum. Lanes 5 and 9 are clinical isolates of M. tuberculosis. (B) Sau96I digests. Lane 1 is 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 10 is the digested positive control H37Rv. Lanes 3–9 are clinical isolates. Lanes 3, 6–8 are M. intracellulare. Lane 4 is M. fortuitum. Lanes 5 and 9 are clinical isolates of M. tuberculosis.

Table 1 – Isolation of non-tuberculous mycobacteria from patients in North India.
from the urine of a patient who had previously been treated for tuberculous epididymitis. Curiously, this 82-year-old patient returned 6 years later with complaints of severe dysuria, frequency and urethral irritation, leading to a suspicion of reactivation of his earlier condition. The isolate obtained was identified as Mycobacterium mucogenicum on sequencing. Lane 1 is a 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 3 is the CfoI digest of the positive control H37Rv [Band sizes (bp): 122, 83, 72]. Lane 4 is the CfoI digest of the clinical isolate M. phocaicum [Band sizes (bp): 172, 83]. Lane 5 is the Sau96I digest of H37Rv [Band sizes (bp): 219, 54]. Lane 6 is the Sau96I digest of the clinical isolate M. phocaicum [Band sizes (bp): 138, 102, 54].

Fig. 2 – PRA profiles for M. mucogenicum/M. phocaicum. The clinical isolate was identified as M. phocaicum on sequencing. Lane 1 is a 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 3 is the CfoI digest of the positive control H37Rv [Band sizes (bp): 122, 83, 72]. Lane 4 is the CfoI digest of the clinical isolate M. phocaicum [Band sizes (bp): 172, 83]. Lane 5 is the Sau96I digest of H37Rv [Band sizes (bp): 219, 54]. Lane 6 is the Sau96I digest of the clinical isolate M. phocaicum [Band sizes (bp): 138, 102, 54].

Conclusions

A large number of NTMs were identified in patients with the initial presumptive diagnosis of tuberculosis in the laboratory. The application of rapid molecular assays would facilitate improved diagnosis of NTMs by obviating the use of the more cumbersome conventional diagnostic methods. In addition, PRA or PCR-RFLP can identify a vast range of mycobacterial species in a single experiment and it is also cost-effective [8,18]. The results of this study reinforce the observation that health centres and laboratories should take action to increase the awareness of appropriate diagnosis of NTM. Commercialization of NTM identification through quality-controlled kits and inter-laboratory linkages shall provide the epidemiological data to assess the importance and relevance of these isolates collectively dismissed as environmental contaminants.

Acknowledgment

This work was supported by funds from the Indian Council of Medical Research.

REFERENCES


