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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 $\geqslant$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 10× Buffer, 2.5 mM of $MgCl_2$ 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 *Sau*96I and *Cfo*I (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 under estimated.

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 *Sau*96I and *Cfo*I (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 kansasii/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

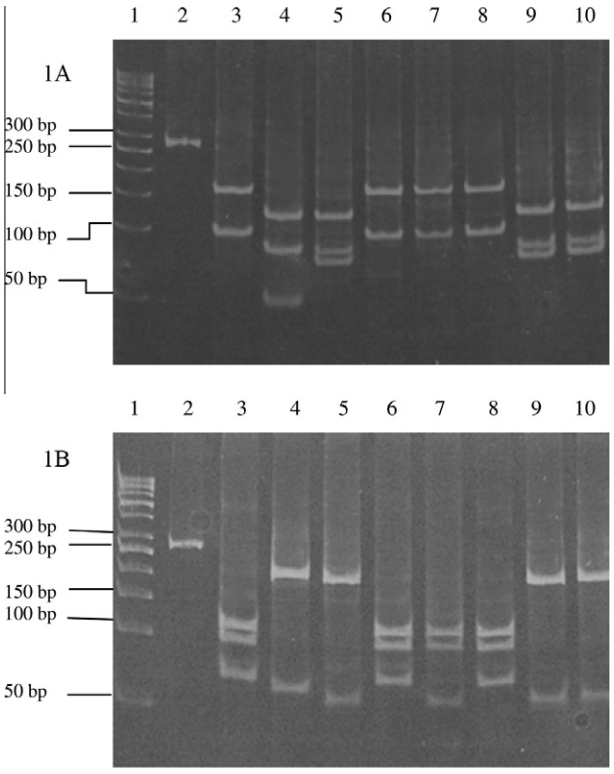

Fig. 1 – (A and B) PRA profiles for mycobacterial species detected in clinical isolates. (A) *Cfo*I digests. Lane 1 is 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 10 is the digested positive control H37Rv. Lanes 3–9 are clinical isolates. Lanes 3, 6–8 are *M. intracellulare*. Lane 4 is *M. fortuitum*. Lanes 5 and 9 are clinical isolates of *M. tuberculosis*. (B) *Sau*96I 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*.

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

Table 1 – Isolation of non-tuberculous mycobacteria from patients in North India.

Mycobacterium species	Total no. identified (%)
M. tuberculosis complex	262 (85.6%)
M. kansasii/gastri	11 (3.5%)
M. fortuitum	10 (3.2%)
M. scrofulaceum	1 (0.32%)
M. avium	7 (2.28%)
M. intracellulare	9 (2.94%)
M. abscessus	1 (0.32%)
M .gordonae	4 (1.3%)
M. phocaicum	1 (0.32%)
Total	306

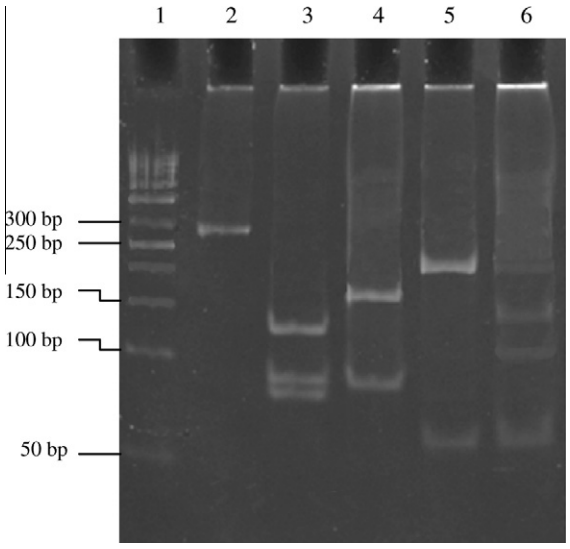

Fig. 2 – PRA profiles for *M. mucogenicum/M. phocaicum*. The clinical isolate was identified as *M. phocaicum* on sequencing. Lane 1 is a 50 bp DNA marker. Lane 2 is the undigested positive control H37Rv. Lane 3 is the *Cfo*I digest of the positive control H37Rv [Band sizes (bp): 122, 83, 72]. Lane 4 is the *Cfo*I digest of the clinical isolate *M. phocaicum* [Band sizes (bp): 172, 83]. Lane 5 is the *Sau*96I digest of H37Rv [Band sizes (bp): 219, 54]. Lane 6 is the *Sau*96I digest of the clinical isolate *M. phocaicum* [Band sizes (bp): 138, 102, 54].

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 *hsp65* PRA (Fig. 2). The restriction bands obtained with *Cfo*I were 172 bp and 83 bp. Restriction bands obtained with *Sau*96I were 138, 102 and 54 bp. An additional band was also visible at 219 bp in the *Sau*96I restrict. The organism was tentatively identified as *M. mucogenicum* on the basis of the algorithm published by Wong et al. [8] and the PCR amplicon was sequenced to confirm the results of PRA. The strain was identified as *M. phocaicum* on sequencing of the *hsp65* gene. Although *M. mucogenicum* and *M. phocaicum* are phylogenetically close and share sequence similarities [16], the algorithm created by Wong et al. [8] does not mention the NTM *M. phocaicum*. Since, the patient improved with conservative management, the pathogenic potential of *M. phocaicum* in the present case of epididymitis is doubtful. Very few cases of *M. phocaicum* have been reported worldwide. This pathogen has been isolated previously from blood stream infections in Texas [17] and respiratory specimens from France [16]. This is the first report of it being isolated from the urine specimen of a patient. One of the isolates obtained in this study was identified as *Mycobacterium scrofulaceum*. Since no clinical details were available for this patient, it could not be determined whether or not this strain was pathogenic. Several patients may not be able to get adequate treatment for NTM owing to a lack of identification or owing to delayed diagnosis. In addition, it is important for the laboratory personnel to be aware of the complete clinical details of patients prior to processing the samples in order to assess the importance of isolating a particular organism and to rule out laboratory contamination.

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.

Contribution of authors

Conceived the study: M.B., M.V.B.; *Experimental design:* M.V.B., M.B., S.K., K.G.; *Carried out experiments:* K.G., S.K., R.P., A.N., K.S.R; *Analyzed data:* M.V.B., K.G.; *Sample collection:* K.G., R.P., A.N., K.S.R.; *Wrote the paper:* M.V.B., K.G.; *Sample collection supervision:* A.C., D.N., V.G.R.

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