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Original Article

Nontuberculous Mycobacterial Infections in Indian AIDS Patients Detected by a Novel Set of ESAT-6 Polymerase Chain Reaction Primers

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SUMMARY: Nontuberculous mycobacteria are often underdiagnosed due to lack of proper diagnostic facilities. To overcome this, we created a rapid PCR method for the species-specific diagnosis of *Mycobacterium tuberculosis* and its differentiation from other mycobacteria. A set of PCR primers targeting the gene encoding for early-secreted antigen-6 (ESAT-6) of the *M. tuberculosis* complex was designed and standardized on mycobacterial standard strains and on 75 recent isolates from AIDS patients and 70 isolates from HIV-negative patients seen at the hospital of the All India Institute of Medical Sciences, New Delhi, India. All 145 fresh mycobacterial isolates were identified using phenotypic methods and 16S rRNA PCR followed by sequencing of hypervariable region A. The ESAT-6 PCR detected all of the *M. tuberculosis* strains correctly (100% sensitivity), but none of the nontuberculous *Mycobacterium* spp. gave positive results (100% specific). Most nontuberculous mycobacteria were identified in patients with AIDS (24%) followed by those with tuberculous lymphadenitis (12.5%) and those with pulmonary tuberculosis whose treatment had failed (4.3%). The most common nontuberculous mycobacterial species isolated from AIDS patients was *M. avium* (6.6%), followed by *M. fortuitum* (5.7%), *M. intracellulare* and *M. terrae* (2.6% each). *M. celatum*, *M. duvalii*, *M. austroafricanum*, *M. phlei* and *M. flavescence* were also isolated from one patient each. The combination of genus-specific PCR primers with the novel ESAT-6 primer set could provide accurate and rapid diagnosis of mycobacteriosis.

INTRODUCTION

According to the World Health Organization, tuberculosis still kills 3 million individuals per year, making it the leading infectious cause of death. It is believed that one person dies of tuberculosis every minute and that one in every three individuals on the planet harbors the causative microorganism belonging to the genus *Mycobacterium* (1,2). This genus represents a complex phenotypic and genotypic diversity among its more than 100 odd species (3). Although the most important human pathogenic species is *Mycobacterium tuberculosis*, the significance of nontuberculous mycobacteria (NTM), also known as mycobacteria other than tuberculosis, increased after the onset of the AIDS epidemic (1,2,4-12). Most of these infections do not respond to conventional antituberculosis treatment and are misdiagnosed as infection with multidrug-resistant strains of *M. tuberculosis* due to lack of species identification, particularly in the developing world (13,14).

In countries with limited resources, the diagnosis of mycobacterial infections is established empirically on a clinicoradiological basis or only by sputum smear examination (1,2). Due to a lack of diagnostic facilities, most laboratories in these countries do not identify the mycobacteria by culture, and the infections caused by NTM are underdiagnosed or misdiagnosed. Even if speciation is attempted using the conventional methods, the process is very slow, labor-intensive, hazardous and not always reproducible (14), hence not many laboratories attempt it (13). To overcome these shortcomings of conventional methods, in recent years molecular techniques have grown increasingly popular, as they are rapid, highly sensitive, specific and can be used on a large number of samples (3,15-17). In this trend 16S rRNA gene analysis is the most promising molecular method. The 16S rRNA gene is conserved in all species of the *Mycobacterium* genus (3,15-18). Therefore, this gene can be amplified by polymerase chain reaction (PCR) in all species of *Mycobacterium* and the species can be identified by species-specific probes, by PCR primers, or by sequencing the hypervariable region of the 16S rRNA amplicon (3,19,20). However, identification of all NTM species may require several species-specific primers and repeated experimentation. Therefore, we set out to separate the *Mycobacterium tuberculosis* (MTB) complex from NTM by using a rapid molecular method. Toward this end we developed a novel method for differentiating the MTB complex from NTM. This method uses a pair of PCR primers targeting 16S rRNA and *esat*-6 genes. The latter belongs to a cluster of five genes in the region of differentiation-1 (RD_1) of *M. tuberculosis*, which includes the genes of 6 kDa early-secreted antigen target (*esat*-6). This gene is conserved only in MTB complex species, *M. kansasii* and *M. marinum*, and is deleted from most NTM, including *M. bovis* (BCG) strains. The gene codes for the early-secreted antigen (3, 21,22). This antigen has been used for serological diagnosis and for evaluating the cell-mediated immune response against *M. tuberculosis* (21,22). It is also being studied as a vaccine candidate against tuberculosis (23), but its gene has never been used as a target for the molecular diagnosis of tubercu-

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losis. The present work shows its utility in enabling for the first time the species-specific and rapid molecular diagnosis of tuberculosis.

MATERIALS AND METHODS

Standard strains and clinical isolates: The protocol was standardized on 14 mycobacterial standard strains, including 2 *M. tuberculosis* and 12 NTM. The NTM strains were a kind gift from the Central JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. The BCG (*M. bovis*) strain (Chennai), used for routine vaccination at the institute, was also included as a negative control besides the other NTM.

Our laboratory routinely performs Ziehl-Neelson (ZN) and Auramine-O (A-O) acid fast staining. The samples are also subjected to culture isolation in Lowenstein-Jensen (LJ) medium and the MGIT-960 automated culture system. For this study, fresh mycobacterial strains were culture-isolated on LJ medium and Bactec MGIT-960® (Becton-Dickinson, Sparks, Md., USA) from patients seen at the hospital of the All India Institute of Medical Sciences, New Delhi, during a period of 2 years (2003 - 2004). A total of 145 clinical isolates were included in this study. All species were identified using the standard conventional methods including niacin, heat-stable catalase, arylsulphatase, and Tween-80 hydrolysis (24,25). To verify the biochemical results, all standard strains and fresh isolates were also subjected to PCR analysis using the newly designed ESAT-6 primer sets and 16SrRNA PCR sequencing simultaneously.

Extraction of genomic DNA for PCR: DNA was isolated directly from the mycobacterial growth by a procedure we have been using in our laboratory for several years. Briefly, 2 - 3 loopfulls (approximately 100 mg) of mycobacterial growth were transferred to an Eppendorf tube containing 200 μl of sterile distilled water. The suspension was boiled in a water bath for 20 min. To the suspension was added 200 μl chloroform, followed by vortexing. The suspension was again incubated at 80°C for 10 min and centrifuged at 9,000 rpm for 2 min. The clear supernatant containing mycobacterial DNA was taken for PCR.

Genus-specific PCR primers: Primers for the amplification of a 1,030-bp 16S rRNA gene target were used, as described elsewhere (15,17,19). The sequence and orientation of these primers were as follows: primer 16S rRNA 285, 5′ gag agt ttg atc ctg gct cag 3′; and primer 16S rRNA 264, 5′ tgc aca aca ggc cac aag gga 3′.

***M. tuberculosis*-specific primers**: All samples were also subjected to *M. tuberculosis*-specific PCR, using primers designed to amplify the complete ORF of *esat*-6 (Rv3875 gene) of RD_1. These novel primers were designed for the first time for the species-specific PCR-based diagnosis of *M. tuberculosis* and are patented (Singh, S., Sharma, P. Methods of amplification and detection of *Mycobacterium tuberculosis*; WO 2005/06130A1 [07-07-2005]). The primers also contained restriction sites for cloning in an expression vector in a related study. The primers were as follows: ESAT-6F, 5′ gcg gat ccc atg aca gag cag cag tgg a 3′ (*Bam*HI site underlined); and ESAT-6R, 5′ ccc aag ctt cct atg cga aca tcc cag tga cg 3′ (*Hin*DIII site underlined).

The PCR fragment obtained with these primers from genomic DNA of a local clinical isolate of *M. tuberculosis* was cloned into an intermediate vector pGEM-T Easy® (Promega, Madison, Wis., USA) and its nucleotide sequence was ascertained. The obtained sequence was identical to that published by Cole et al. (26). The sequence has been deposited with GenBank under accession no. AF420491.

PCR: Mycobacterial DNA (50 ng) was amplified in a 50-μl reaction mixture for the amplification of the 16S rRNA gene. The reaction mixture contained 200 μM deoxynucleoside triphosphates (Bangalore Genei, Bangalore, India), 5 μl of 10 × buffer (100 mM TAPS [pH 8.8], 15 mM $MgCl_2$, 500 mM KCl and 0.1% gelatin), and 1.5 units of *Taq* DNA polymerase. The working concentration of each primer was 0.5 μM. The temperature cycles used were: 94°C for 5 min; 94°C for 1 min, 62°C for 2 min, and 72°C for 2 min. A total of 40 amplification cycles were carried out, followed by final extension at 72°C for 10 min. The PCR products were resolved on 0.9% agarose gel after ethidium bromide staining. The amplicons of 1,030 bp size indicated a positive result (Figure 1A) and were subjected to sequencing.

The constituents for species-specific ESAT-6 PCR mixtures were the same as for the genus-specific 16S rRNA PCR, except that the reaction was carried out for 30 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, followed by final extension at 72°C for 10 min. The products were electrophoresed in 1.5% agarose gel to resolve the PCR product of 320 bp (Figure 1B).

Sequencing of the hypervariable region of 16S rRNA gene: The PCR-amplified products were separated on 0.9% agarose and purified using Centricon-100® devices. The purified product was sequenced using 2 pmol sequencing primer 244 (5′ ccc act gct gcc tcc cgt ag 3′) per reaction to

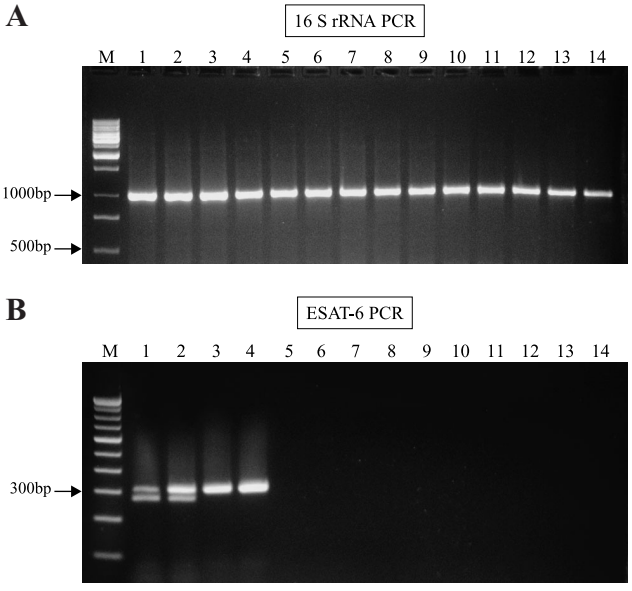

Fig. 1. Agarose gel electrophoresis of the PCR products (ethidium bromide stained) of 14 mycobacterial standard strains used to standardize the new set of *M. tuberculosis* specific PCR primers. (A) The PCR was carried out as per the detailed protocol mention in Materials and Methods for genus specific molecular identification of the growth, targeting 16s rRNA gene. The PCR product of 1,030 bp size was resolved on 0.9% agarose gel. (B) An aliquote of the extracted DNA from all the above standard strains were subjected to *M. tuberculosis* spp. specific ESAT-6 PCR (see Materials and Methods). The PCR product of 320 bp was resolved on 1.5% agarose gel. Lane M, 100-bp ladder (1 kb) molecular weight marker. Lanes 1-2, *M. kansasii*; Lane 3, *M. tuberculosis* (H37Rv); Lane 4, *M. tuberculosis* (clinical isolate); Lane 5, *M. bovis* (BCG); Lane 6, *M. avium*; Lane 7, *M. smegmatis*; Lane 8, *M. intracellulare*; Lane 9, *M. fortuitum*; Lane 10, *M. phlei*; Lane 11, *M. austroafricanum*; Lane 12, *M. scrofulaceum*; Lane 13, *M. celatum*; Lane 14, *M. terrae*.

determine the species-specific nucleic acid sequence of hypervariable region A of the 16S rRNA gene. Sequencing primer 159 (5′ ttt cac gaa caa cgc gac aa 3′) was used to determine the *Mycobacterium* genus-specific nucleic acid sequence of hypervariable region B (15,18).

RESULTS

The mycobacterial isolates were made from three patient groups: AIDS patients (n = 75), HIV-negative adult cases of tuberculous lymphadenitis (n = 24), and patients with pulmonary tuberculosis (n = 46) not responding to first-line anti-tuberculosis drugs (SHRE) after 6 months. AIDS patients had vague presentations, but the most common clinical manifestation was pulmonary (80%) followed by disseminated tuberculosis involving two or more organs. Intestinal tuberculosis was the most common extra-pulmonary manifestation; accordingly, out of 75 culture isolates from AIDS patients, 60 (80%) were from sputum, 10 (13.3%) were from fecal samples, and 5 (6.6%) were from lymph-node aspirates.

The overall sensitivity rates of ZN and A-O acid fast stainings in our laboratory were 19.6 and 22.3%, respectively. However, the sensitivity rates of A-O and ZN stains on culture-proven cases were 75 and 66.6% for multidrug-resistant cases, 66.6 and 58.3% for tuberculous lymphadenitis and 60 and 46.6% in AIDS cases, respectively. Hence A-O stain had a clear edge over ZN staining in all the patient groups. The overall detection rate of the LJ culture method from AIDS patients in our laboratory was 17% (75/441), as compared to 53% (24/45) from HIV-negative lymph-node aspirates and 27% (46/170) from HIV-negative pulmonary tuberculosis patients who had not responded to first-line anti-tuberculosis treatment (details not shown). Overall, the LJ culture showed an isolation rate of 22% (145 isolates from 656 samples). The comparative isolation in MGIT 960 vis-à-vis LJ and other methods is being analyzed and will be published elsewhere.

The PCR method showed excellent sensitivity and specificity. In all, 145 newly isolated mycobacterial strains of the 16S rRNA gene were PCR amplified, along with the 14 standard strains. The simultaneously run ESAT-6 gene amplification was accomplished in 122 (84.2%) strains (Table 1). In other words, 15.8% isolates were found to be NTM. All of these 23 strains were confirmed by sequencing. After 16S rRNA gene sequencing, all the ESAT-6-positive isolates were finally identified as *M. tuberculosis* (sensitivity of ESAT-6 PCR, 100%) while all ESAT-6 PCR-negative isolates were found to be NTM, indicating the 100% specificity of this PCR protocol for *M. tuberculosis*. *M. kansasii* was the only NTM species to have PCR amplification, but showed two bands, one of 285 bp and the other of 320 bp.

The phenotypic methods were poorly correlated with ESAT-6 and final sequence-based species identification (Table 1). The methods were also nonreproducible and lacked discriminatory power for mycobacterial species in a high percentage (11.2%) of isolates.

With regard to the isolation rate of NTM in various patient groups, we observed the highest incidence of NTM in HIV-infected patients (24%) followed by patients with lymphadenitis (12.5%), and the lowest incidence was found in patients unresponsive to anti-tuberculosis treatment (4.3%), indicating that most of the nonresponders were infected with multidrug-resistant *M. tuberculosis* only. The NTM isolation rates differed significantly among all three patient groups, but most significantly between AIDS patients and HIV-negative patients not responding to treatment ($P < 0.001$).

DISCUSSION

The All India Institute of Medical Sciences (AIIMS), New Delhi, is a tertiary care hospital to which patients from all parts of the country and neighboring countries come for medical advice, although approximately two-thirds are from northern India. In the last 5 years AIIMS has seen a steep rise in AIDS cases. This trend is in concordance with the national figures indicating widespread of HIV infection in the general public and particularly in the rural areas, where tuberculosis is also highly prevalent. However, there is no documentation of NTM among the population of northern India. The present study showed that the prevalence of NTM in these patients was significantly ($P < 0.001$) higher in AIDS patients than in HIV-negative patients (24 versus 4.3%). In a study using conventional methods carried out in South India about 2 decades ago, before HIV reached India, NTM were reported in 8.6% of patients (27). Since then, however, no other study in India has been carried out even though the HIV epidemic has engulfed the whole nation. A recent study (13), again from South India, on HIV-tuberculosis co-infection showed that in AIDS-associated tuberculosis the primary unresponsiveness to anti-tuberculosis treatment was as high as 33.9%, but the workers did not identify the species in order to find out whether all unresponsive cases were due to *M. tuberculosis* only or to NTM. Our findings clearly emphasize the need for speciating the mycobacterial isolates. Since most NTM are not susceptible to conventional anti-tuberculosis treatment, it becomes desirable to identify these isolates up to the species level, particularly from AIDS and drug-resistant cases.

The literature on the prevalence of NTM is very thin, but most of the early reports from the Western world reveal that in AIDS patients *M. avium* is the most common NTM species (4,6). In a recent study in southern Asia, *M. avium* was cultured from 17.4% of Thai AIDS patients. In Brazil, Ferreira et al. (9) isolated NTM from 15% AIDS patients; *M. avium* was found in more than half (57.8%) of the 15% cases. Ergin

Table 1. Detection rate of mycobacterial etiology in 3 patient groups using various conventional and molecular methods

Patient group	Culture isolates	Biochemical identification		PCR identification				Sequence identification	
				16s rRNA		ESAT-6			
		M. tb	NTM	+	−	+	− (%)	M. tb	NTM
AIDS	75	63	12	75	0	57	18 (24.0)	57	18
Lymphadenitis	24	19	5	24	0	21	3 (12.5)	21	3
Pulmonary TB	46	39	7	46	0	44	2 (4.3)	44	2
Total	145	121	24	145	0	122	23 (15.8)	122	23

M. tb, *Mycobacterium tuberculosis*; NTM, nontuberculous mycobacteria; TB, tuberculosis.

Table 2. Prevalence of nontuberculous mycobacteria isolated from Indian AIDS patients

Mycobacterial species	No. (%)
M. tuberculosis	57 (76.0)
M. avium	5 (6.6)
M. fortuitum	4 (5.7)
M. intracellulare	2 (2.6)
M. celatum	1 (1.3)
M. phlei	1 (1.3)
M. austroafricanum	1 (1.3)
M. flavescence	1 (1.3)
M. terrae	2 (2.6)
M. duvalii	1 (1.3)
Total	75 (100.0)

et al. (10) recently reported NTM in 20.8% of Turkish patients, and the prevalence rate in our patients was similar to theirs. In our study also, *M. avium* was the most common NTM species (27%, 5/18), followed by *M. fortuitum* (22.2%, 4/18). Other species were identified less frequently (Table 2). Other researchers have isolated *M. intracellulare*, *M. fortuitum*, *M. duvalii*, *M. terrae*, and *M. phlei* from human beings too (5-12). However, *M. celatum* was reported by us (28) for the first time from outside the US and Europe. There are only 19 reported cases of *M. celatum* infection, and 15 of these were in AIDS patients (11). The remaining 4 were also immunocompromised patients. All these cases were from either from the US or Europe. So far we have not found any record of *M. austroafricanum* or *M. flavescence* isolated from humans. Our isolates could be the first in the world. However, it is also possible that these were contaminants. By now we have isolated 4 strains of *M. kansasii*, but this was done after the present study was completed, hence the details of these cases are not included in this paper.

In our patients with lymphadenitis, *M. tuberculosis* was the leading cause of infection (87.5%). However, for the first time we have isolated NTM in 12.5% of cases, the highest rate reported so far from India. Also, most (80%, 4/5) of the NTM species were identified as *M. avium* complex. *M. tuberculosis* is reportedly a leading cause of lymphadenitis in the developing world (14,15), but recently in the US and Europe *M. avium* has overtaken *M. tuberculosis* in this regard, particularly in children (12). The data from India are scarce, and only a few case reports are available based on speciation using phenotypic features, which have several limitations. The high rate (12.5%) of NTM causing lymphadenitis in the present study could, therefore, be explained by the highly sensitive culture and *M. tuberculosis*-specific molecular methods used in this study.

The prevalence of NTM was significantly low in pulmonary tuberculosis HIV-negative patients, and *M. tuberculosis* was isolated from 95.5% of cases. The lone case from whom *M. duvalli* was isolated was a 25-year-old female who had not responded to first- and second-line treatments taken for 4 years intermittently.

The conventional phenotypic methods of speciation were not found to be reproducible or sensitive. About one-fourth of the finally identified NTM isolates gave inconclusive or erroneous results on phenotypic characterization. The *M. austroafricanum* was phenotypically identified as *M. phlei*, one *M. duvalii* as *M. vaccae*, and another as *M. fortuitum* and *M. celatum* as *M. avium* complex. Many *M. tuberculosis* isolates were not identifiable by these methods. Our experience with phenotypic methods was similar to those of others. Cook et al. (20) in a very comprehensive study found that the 16S rRNA PCR sequencing is not only more specific and reproducible but also highly cost-effective. The new set of PCR primers used here for amplifying the *esat*-6 gene is a unique and novel molecular method for rapid differentiation of NTM from *M. tuberculosis*. All *M. tuberculosis* isolates showed a single band of 320 bp, while none of the NTM species except *M. kansasii* showed any amplification (Fig. 1B). *M. kansasii* showed two bands of 320 and 285 bp, indicating that, unlike *M. tuberculosis*, the *esat*-6 gene in *M. kansaii* probably resides in different segments (29) and that these repeats are probably of different sizes or have a variation in sequences. Though several researchers have used this gene for antigen expression (3,21,22) we have used it for the first time for species-specific identification of mycobacterial isolates. Combining this target with a commonly used genus-specific target 16S rRNA, we could accomplish rapid and specific laboratory results to facilitate the correct and timely management of patients with mycobacterial infections.

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