

DNA fingerprinting and drug resistance profiles of *Mycobacterium tuberculosis* strains isolated from HIV-infected pulmonary tuberculosis patients

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Abstract

Introduction: Tuberculosis (TB) is a leading cause of morbidity and mortality among HIV/AIDS patients. These patients are more likely to develop resistance to anti-tuberculosis drugs as well. Identifying open cases of TB, studying their drug susceptibility pattern and knowledge of genetic diversity to know transmission dynamics among HIV-TB patients is therefore important to control TB.

Aims: 1) To determine the prevalence of HIV, among sputum positive pulmonary tuberculosis (PTB) patients. 2) To study drug resistance pattern of *Mycobacterium tuberculosis* isolates. 3) To evaluate genotypic diversity of *M. tuberculosis* isolates using random amplified polymorphic DNA (RAPD) technique.

Settings and Design: Hospital based cross sectional study

Materials and Method: *M. tuberculosis* strains were isolated from HIV seropositive PTB patients with irrespective of their anti-TB treatment. These isolates were processed for drug sensitivity testing (DST) by Proportion method and subsequently genotyped by RAPD technique.

Results: Prevalence of HIV-TB co-infection was 22.2%. Total 52.1% *M. tuberculosis* isolates were resistant to one or more number of drugs. We report 17.4% MDR-TB among study population. RAPD-PCR showed excellent discrimination with high degree of polymorphism among *M. tuberculosis* isolates. Transmission and diversity of tuberculosis was 10% and 90% respectively.

Conclusions: 17.4% of MDR-TB among studied patients indicated worrisome trend that calls for continuous monitoring and DST. RAPD analysis is quick, simple and has potential for molecular epidemiology of TB. RAPD-PCR showed notable genetic polymorphism, suggesting that source and type of infections within the population were varied and no any particular clone of *M. tuberculosis* is spreading among study population.

Keywords: HIV-TB, RAPD-PCR, Pulmonary tuberculosis, DNA fingerprinting

Introduction

HIV is the most important risk factor that promotes progression to active tuberculosis (TB) in people with *Mycobacterium tuberculosis* infection.⁽¹⁾ The clinical presentation of pulmonary TB depends upon degree of immunosuppression in the patients.⁽¹⁾ The presentation of TB in early stages of HIV infection is similar to that in HIV negative TB patients, resembling post primary pulmonary TB.⁽²⁾ Sputum smear results are often positive in this group of patients.⁽²⁾ These patients are epidemiologically important threat as it is known to increase TB transmission rates at the community level, further threatening the health and survival of HIV seronegative individual as well.^(1,3) HIV infected TB patients are significantly more likely to develop resistance to anti tuberculosis drugs than those without HIV infection.^(3,4) Determination of drug resistance pattern among this population is therefore very important in TB control.

TB develops in HIV infected hosts by any of the two pathogenic mechanism, either endogenous reactivation or exogenous reinfection. Reactivation is responsible for the major part of HIV associated TB, especially in countries with high level of TB

transmission.⁽⁵⁾ However, DNA fingerprinting of *M. tuberculosis* from Acquired Immunodeficiency Syndrome (AIDS) patients showed that reinfection and new infection also occurs.⁽⁵⁾ Increased risk of transmission of MDR-TB strains amongst HIV patients has also been confirmed by DNA fingerprinting.⁽⁶⁾ Also high HIV seroprevalence among newly diagnosed TB patients has been reported in India.^(7,8) Understanding of genetic diversity is therefore needed to study epidemiology of HIV-TB co-infection. Molecular typing methods are very helpful to identify individual strains of *M. tuberculosis* and transmission of a strain throughout community can thus be monitored.⁽⁹⁾ Random amplified polymorphic DNA (RAPD) technique is one of the versatile molecular techniques, have been used several times as suitable tool for fingerprinting of many medically important microorganisms including *M. tuberculosis*.^(10,11)

RAPD-Polymerase Chain Reaction (RAPD-PCR) allows better detection of polymorphism without prior knowledge of the nucleotide sequence.⁽¹⁰⁾ Data generated from this can be used to study genetic relatedness and to construct genetic maps as well.⁽¹⁰⁾ This method utilizes short (10-20) oligonucleotide primers of arbitrary sequences that are annealed in the

first few cycle of the PCR at low stringency.⁽¹²⁾ RAPD-PCR is technically less demanding and easy to perform.

Therefore, the present study was planned to determine drug resistance profile and to evaluate the genotypic diversity of *M. tuberculosis* strains isolated from HIV seropositive pulmonary tuberculosis patients using RAPD technique.

Materials and Method

After obtaining Institutional ethical Committee clearance, total 838 clinically suspected pulmonary tuberculosis patients, who gave written consent to participate in the study were enrolled consecutively from Revised National Tuberculosis Control Programme (RNTCP) center of our hospital over a period of one year. Patients belonging to all the ages and both the sexes irrespective of their history of anti-tuberculosis treatment were included in the study. Three sputum samples were collected in sterile container from each patient for sputum microscopy. Total 135(16.1%) were found to be sputum smear positive. All these patients were screened for HIV antibodies at Integrated Counselling and Testing Center (ICTC) of our hospital as per National AIDS Control Organization (NACO) guidelines.⁽¹³⁾ Of which, 30 (22.2%) were found to be HIV seropositive. From this HIV-TB co-infected patients, one of the three sputum samples showing maximum numbers of acid fast bacilli (AFB) was decontaminated by Kudoh and Kudoh method and inoculated on two Lowenstein Jensen (LJ) media.⁽¹⁴⁾ Culture bottles were incubated at 37°C and read weekly for eight weeks. No growth after eight weeks of incubation was treated as culture negative. Growth of *Mycobacterium* was typed by colony morphology, niacin test, catalase activity at 68°C and susceptibility to p-Nitrobenzoic acid (PNB), etc. as per standard recommended procedure.⁽¹⁵⁾ The isolates were identified up to species level and stored at -70°C and revived prior to drug susceptibility testing (DST) and genotyping.

DST was performed by conventional 1% proportion method (PM) against first line of anti-tuberculosis drugs such as Isoniazid (0.2 mg/l), Ethambutol (2 mg/l) Streptomycin (4 mg/l) Rifampicin (40 mg/l).⁽¹⁶⁾ All the drugs and chemicals were procured from Hi-Media, India. *M. tuberculosis* H37Rv strain was used as control.

Genomic DNA was extracted from mycobacterial culture by the method described earlier.^(11,12,17) Briefly, loopful colonies of *M. tuberculosis* were mixed with 1000 µl of saline. Bacterial cells were washed twice in saline and in Tris EDTA (TE) buffer (10mm Tris, 1mm EDTA, pH 8.0) by vortexing and centrifugation at 12000 rpm for 3-5 minutes. Lysozyme 40 µl (20mg/ml) was added to the pellet obtained. The suspension was vortexed and incubated at 37°C for two hours. The bacterial cells were disrupted by increasing the temperature to 65°C and adding proteinase K and

Sodium dodecyl sulphate(SDS) to a final concentration of 250 µg/ml and 1% respectively for 30 minutes with shaking. A mixture of Cetyltrimethyl ammonium bromide (CETAB) and NaCl (CETAB 10%, NaCl) was added to final concentration of 1%. Finally, the suspension was incubated 65°C for 30 minutes. DNA was extracted with chloroform and isoamyl alcohol (24:1) preparation and at the end extracted DNA was precipitated out in chilled ethanol.

DNA fingerprinting was done by RAPD method as described earlier.^(12,18,19) Two 20mer Oligonucleotideprimers namely INS-1(CGTGAGGGCATCGAGGTGGC) and INS-2 (GCGTAGGCGTCGGTGACAAA) were used for RAPD-PCR. Amplification of Mycobacterial DNA uses total volume of 25 µl reaction mixture. The reaction mixture contained 0.5 µl of Taq DNA polymerase, 2.5 µl of assay buffer (10 mMTrisHCl (pH 9.0), 1.5mM MgCl₂, 50 mMKCl, 0.1% (V/V) Triton X-100, 0.01% gelatin) 1 µl of dNTP mix, 0.5 µl primers and 1 µl of template DNA in 19.5 µl of sterile ultrapure deionized water. Amplification was carried out in a thermal cycler (Bio-Rad i-cycler). The cycling conditions consisted of an initial denaturation step for 5 minutes at 95°C, followed by 38 cycles at 95°C for one minute denaturation step, an annealing step for one minute at 55°C and extension step for one minute at 72°C followed by final extension at 70°C for 10 seconds The product obtained from RAPD-PCR was analyzed on a 0.8% agarose gel stained with gel red dye. Subsequently, the gel was visualized and photographed using a gel documentation system (AflaImager).

DNA fingerprinting pattern of individual strain were studied on the basis of presence or absence of bands. The banding patterns were analyzed visually as well as by using Gene directory (Band Match Software, Syngene, version 2.01). The data obtained were recorded and entered in the software, where a dendrogram was produced for further analysis. Clustering was based on Unweighted Pair-Group Method with Arithmetic mean (UPGMA).

Results

Of the 135 (16.1%) AFB positive on smear microscopy (even if one of three sputum samples was positive, patient was considered as AFB positive) 30 were co-infected with HIV-1. Thus prevalence of HIV and pulmonary tuberculosis co-infection in this area was 22.2%. Out of 30 sputum positive cases, 23 (76.6%) were culture positive. All the isolates were characterized as *M. tuberculosis* by conventional biochemical reactions and were used for further study. Of the 23 culture positive HIV-TB co-infected patients 15 were males and 8 were females. Male to female ratio was 1.9:1. Average age was around 41.6 years. Total 17/23 (74%) were new cases and 6/23 (26%) were having history of previous treatment. On drug sensitivity testing of all the isolates, 11/23 (47.9%)

were found to be sensitive to all frontline anti-tuberculosis drugs. Whereas 12/23(52.1%) were resistant to one or more number of drugs. Mono drug resistance to Rifampicin and Ethambutol was not seen among these isolates. Whereas three strains from new cases showed resistance to Isoniazid alone and two strains each from new and treated cases showed resistance to Streptomycin. One strain from new case was resistant to Isoniazid and Streptomycin (Table 1). In the present study, 4/23 (17.4%) MDR-TB strains were identified. Of which 01/17 (5.8%) was from new case and 03/06 (50%) were from treated group of patients (Table 2).

Table 1: Drug resistant profile of non-MDR isolates from HIV-TB co-infected patients

| Drug | New cases | Treated patients | Total |
|----------|-----------|------------------|-------|
| RMP | - | - | - |
| INH | 03 | - | 03 |
| EMB | - | - | - |
| SM | 02 | 02 | 04 |
| INH + SM | 01 | - | 01 |
| Total | 06 | 02 | 08 |

Table 2: Drug resistant profile of MDR isolates from HIV-TB co-infected patients

| Drug Resistance pattern | New | Treated | Total |
|-------------------------|-----|---------|-------|
| RMP + INH | 01 | - | 01 |
| RMP + INH + SM | - | 02 | 02 |
| RMP + INH + EMB | - | 01 | 01 |
| Total | 01 | 03 | 04 |

All the isolates were subjected for RAPD-PCR for molecular typing. To show the reproducibility of the RAPD-PCR, two different experiments were performed using two different primers. Each of which was carried out twice on two different days. By comparing banding pattern visually, slight difference in the band intensity and thickness between the strains could be revealed. Out of 23 isolates processed for RAPD-PCR, three isolates were recorded as non-typable, because the RAPD profile consisted of a single very bright band on the gel by both the primers. RAPD-PCR of remaining all *M. tuberculosis* strains showed variety of sizes of amplicons. Several fragments were amplified in each sample by both the primers. Most of these fragments were observed to be unique to certain strains. However, there were some fragments observed to be common to different strains (Fig. 1). All the *M. tuberculosis* strains showed a high degree of polymorphism with RAPD-PCR.

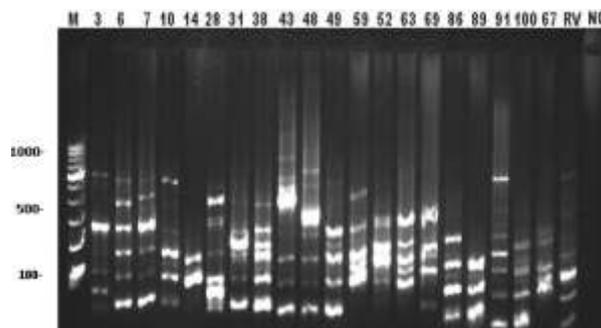


Fig. 1: RAPD profile generated from genomic DNA of *M. tuberculosis* strains (denoted with numbers) isolated from HIV-Tb coinfected patients by using primers INS 1 Lanes:-M- Marker (100 base pair DNA ladder [Merck, India]), NC- negative control, RV- *M. tuberculosis* H37Rv

Both the primers used for RAPD-PCR revealed distinguishing patterns. Among the two primers INS-1 showed maximum discrimination (Fig. 1). Upon dendrogram analysis, with primer INS-1, notable genetic diversity was observed (Fig. 2). The results showed that maximum *M. tuberculosis* strains within this population were highly diverse. Among the 23 DNA fingerprinting patterns obtained by RAPD-PCR, strain number 6 (lane 3) and 7 (lane 4) showed similar RAPD profiles (Fig. 1). Both were male patients with similar drug sensitivity profiles, indicating that both the strains were identical. However, infection with identical strains among these patients could not be explained by analysis of data such as family relations, address or coincidence of residence at hospital. However, there was history of exposure from unknown commercial sex worker somewhere in the same city. Thus, transmission and diversity of tuberculosis in the studied population was 10% and 90% respectively.

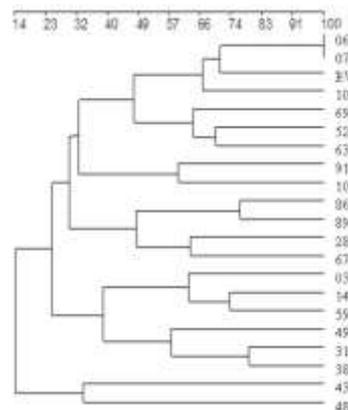


Fig. 2: Dendrogram showing genetic relatedness among *M. tuberculosis* strains isolated from HIV-TB co-infected patients with primer INS-1

Discussion

Considering the high rates of HIV-TB co-infection and morbidity and mortality associated with it, early

detection and management of HIV as well as tuberculosis gain importance as significant public health intervention in the national AIDS control program (NACO). The close association between TB-HIV, each potentiating the impact of the other, has been well described and is presently more pronounced particularly in India.⁽⁸⁾ In these patients the T-lymphocytes that normally mount a response to *M. tuberculosis* are being destroyed by viruses and patients cannot respond to therapy.⁽²⁰⁾ Analysis and timely updates of *M. tuberculosis* distribution in a given area among HIV seropositive people provides insights into transmission mechanism, emergence of drug resistance and information regarding virulent strains in this population.⁽²¹⁾ This study has been undertaken in HIV seropositive pulmonary tuberculosis (PTB) patients in order to gain a better understanding into the population structure of *M. tuberculosis* in this area.

In the present study prevalence of HIV-TB coinfection was high (23%). There is no official data on magnitude of TB/HIV infection from this area, but our results are in agreement with previous reports, where rate of TB in HIV seropositive patients was ranging from 0.4% to 28.75%.⁽²⁾ Mohite et al reported proportions of HIV reactive TB cases increased from 3.11% to 25.3% since year 2009 to 2011 in Western Maharashtra.⁽²²⁾ Our results also corroborate the facts that TB is more common in the young adults with age group ranging from 21-46 years and most commonly seen in male patients with male to female ratio 1:1.9.^(2,7)

The study reports a high level multi drug resistance among newly diagnosed 1/17 (5.9 %) as well as treated group of patients 3/6 (50%). Such high levels of resistance have also been reported from other part of our country.^(23,24) Expectedly the rate of resistance was high for treated group of patients. Sunil Jadhav et al reported similar findings from western part of Maharashtra where prevalence of MDR-TB in new cases was 11.36%.⁽²⁵⁾ High level of drug resistance can develop during the initial infection or it may occur as results of reinfection with resistant strains.⁽²⁴⁾ The drug susceptibility profile obtained from the present study showed no significant difference, when compared with previously published data of HIV sero-negative population in India.^(23,26) We report 4/23 (17.4%) of MDR, which supporting the hypothesis of rising trend of MDR-TB when compared with reports from hospital based studies till 2014.⁽²⁴⁾ High level of MDR-TB (17.4%) in the present study could be attributed to the fact that it was carried out in the tertiary care hospital where patients being referred from different part of the district. However, pure community based studies are needed to confirm these findings.

Epidemiological studies are important to track the disease origin and its spread in the community which in turn is helpful for in the disease control and surveillance.^(24,26) Several methods such as biotyping, bacteriophage typing, or antibiogram have its own

limitations.⁽¹¹⁾ Molecular characterization by using DNA based techniques, such as RFLP using IS986 and IS6110 has been tried extensively. But lack of applicability of some of the probes such as IS6110 for Indian isolates indicates need for alternative molecular technique.^(19,27)

RAPD-PCR technique used in the present study has successfully been used earlier for *M. tuberculosis* strain discrimination.^(11,12,18,19) RAPD uses short oligonucleotide primers of an arbitrary sequences and low stringency PCR to amplify discrete DNA fragments that can be used as genetic markers.⁽¹²⁾ RAPD can be used for study of genetic heterogeneity based on DNA sequence.⁽¹²⁾ As per some studies, the degree of polymorphism obtained by RAPD-PCR was almost the same as that obtained by RFLP.⁽²⁸⁾ Tazi et al reported that, the values obtained with RAPD data were almost always higher than those obtained with the MIRU data.⁽¹⁸⁾ This suggests that a RAPD technique allows detection of more mutational events than the MIRU techniques.⁽¹⁸⁾

In our study, RAPD fingerprinting profiles of *M. tuberculosis* isolates showed excellent strain discrimination. Both the primers amplified scoreable fragments in the strains analyzed. Patterns obtained by RAPD-PCR with both the primers were stable and reproducible. Notable polymorphism was revealed with both the primers. Our results showed that *M. tuberculosis* strains from this group of patients were highly diverse (90%). Diverse DNA fingerprinting indicates that source and type of infections within the population were varied. It also indicates availability of multiple phenotypes as well, that calls for continuous monitoring of DST and treatment regimen. High degree of polymorphism among *M. tuberculosis* isolates have been reported from India and other part of world as well.^(5,11,18,19,29) Whereas, 76% similarity was reported by Rehim et al from Egypt, where all strains belonged to very close genotypes.⁽²⁰⁾

Association of specific RAPD pattern was not observed in our study suggested that no any particular clones of *M. tuberculosis* have played any dominant role in HIV related TB patients. We found that majority of the tuberculosis in this group of patients might be due to reactivation of latent tuberculosis. However, TB is endemic in India therefore it is difficult to comment, as exogenous infection may have been acquired from areas other than the presently studied area. Similar RAPD profiles generated by two strain 6 and 7 (**Fig. 1**) indicates transmission. Recent findings have proved out that transmission of tuberculosis may not require close and prolonged previous contact and minor contact in the community may be sufficient.⁽³⁰⁾

Conclusion

Our findings showed that RAPD-PCR technique is having potential to yields valid as well as reproducible results in standardized assay conditions. Study of

profiles generated by RAPD-PCR indicates that multiple sources of open cases of tuberculosis are available in the community and some of them are MDR-TB as well. This may create difficulties in front of the strategies implemented to control tuberculosis in the community. However, more studies and vigorous testing of the isolates would be needed. This is the first genetic analysis of *M. tuberculosis* strains from HIV-infected patients from this area. Therefore, results generated by RAPD-PCR should be compared with other molecular methods. However, generating this type of information will definitely facilitate the tracking and monitoring these microorganisms, leading to more appropriate measures for tuberculosis control.

Acknowledgement

We are sincerely acknowledge Roy Subarna, Scientist E, National Institute of Traditional Medicine, For providing facility to perform RAPD-DNA fingerprinting of *M. tuberculosis* isolates at Regional Medical Research Centre of Indian Council of Medical Research, Belgavi, Karnataka.

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