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Line probe assay for detection of *Mycobacterium tuberculosis* complex: An experience from Central India

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***Background & objectives*: *Mycobacterium tuberculosis* complex may sometimes not be detected in sputum samples of suspected multidrug-resistant tuberculosis (MDR-TB) patients by line probe assay (LPA) even though they are smear positive for acid-fast bacilli (AFB). This retrospective analysis was attempted to understand and document our experience with LPA for detection of *M. tuberculosis* complex and diagnosis of MDR-TB under programmatic conditions.**

***Methods*: One thousand two hundred and ninety four sputum samples of MDR-TB suspects that were smear positive for AFB, and received from February to November 2013, were tested by LPA for the presence of *M. tuberculosis* complex and resistance to isoniazid (INH) and rifampicin as per the diagnostic mandate of an accredited reference laboratory. As per the mandate, those samples that were negative for *M. tuberculosis* complex were cultured, and the growth again tested by LPA. A retrospective analysis of the results was carried out.**

***Results*: *M. tuberculosis* complex could be detected in 1217 (94.04%) but not in 77 (5.9%) of smear-positive sputum samples. Of the 1217 positive samples, 232 (19.1%) were MDR, 130 (10.6%) were rifampicin monoresistant and 101 (8.3%) were INH monoresistant. Seven hundred and fifty four (61.9%) strains were found to be pansensitive. Overall, 5.1 per cent of the sputum samples were negative for *M. tuberculosis* complex by LPA and culture. In at least 10 (0.77%) sputum samples smear positive for AFB, *M. tuberculosis* complex could not be identified by LPA though *M. tuberculosis* was present, as evidenced by culture positivity.**

***Interpretation & conclusions*: LPA is a robust technique for diagnosis of drug-resistant TB that has provided the basis for rapid and effective control of drug-resistant TB in India. While the reasons for concomitantly negative LPA and culture results of smear-positive sputum samples from MDR-TB suspects may be many, the possible presence of non-tubercular mycobacteria in these samples and the likelihood of inappropriate therapy in these patients cannot be ruled out. Addition of culture to the diagnostic algorithm may enhance the diagnostic yield.**

Key words Acid-fast bacilli - *M. tuberculosis* control band - MDR-TB - non-tubercular mycobacteria - smear positive

India has a high burden of multidrug-resistant tuberculosis (MDR-TB). The annual status report of TB India, 2014, mentioned that in 2012, the MDR-TB amongst notified new pulmonary TB patients was

2.2 per cent, whereas amongst notified re-treatment pulmonary TB patients, it was 15 per cent[1]. Given the global situation of MDR-TB and an urgent need for detection of drug resistance amongst TB patients, line probe assay (LPA) was introduced. LPA is a rapid technique based on polymerase chain reaction (PCR) that is used to detect *Mycobacterium tuberculosis* (MTB) complex as well as drug sensitivity to rifampicin (RPM) and isoniazid (INH) through the Revised National Tuberculosis Control Programme (RNTCP) of India. It is used for diagnosis of drug-resistant TB under programmatic conditions[2]. Only sputum samples that are smear positive for acid-fast bacilli (AFB) are tested by LPA. However, there are instances where MTB complex is not detected by LPA even though the samples are smear positive for AFB. Here we describe our experience with detection of MTB complex and diagnosis of MDR-TB under programmatic conditions by LPA in a tertiary care centre and hospital in central India.

Material & Methods

The laboratory at the department of Microbiology, Bhopal Memorial Hospital and Research Centre, Bhopal, Madhya Pradesh, India is an accredited reference laboratory for TB, with a mandate to diagnose drug-resistant TB. Sputum samples from MDR-TB suspects are received from various districts across the State of Madhya Pradesh. LPA is carried out routinely on these samples and results are sent online to the respective district TB centres.

A total of 1528 diagnostic sputum samples from MDR-TB suspect patients were received from February to November 2013 in the Microbiology laboratory. Of these 1528 patients from whom samples were received, 945 were classified as 'smear positive at diagnosis, retreatment case'; 306 patients as 'any follow up smear positive'; 140 patients as 'failure'; 131 patients as 'retreatment, smear positive at the fourth month'; four patients as 'smear negative at diagnosis, retreatment case' and two patients as 'contact of known MDR-TB case'. Smear microscopy was carried out on these samples using Ziehl-Neelsen stain. Smear-positive samples were processed by NALC-NaOH (N- acetyl-L-cysteine - sodium hydroxide) decontamination method[3]. DNA was extracted from the decontaminated samples using GenoLyse® kit (Hain Lifescience GmbH, Nehren, Germany) as per manufacturer's instructions. The extracted DNA was processed for LPA using GenoType®MTBDRplus version 2.0 (Hain Lifescience GmbH, Nehren, Germany) for detection of MTB complex and rifampicin and/or INH resistance according to the manufacturer's instructions. A positive *M. tuberculosis* control (TUB) band indicated the presence of members of *M. tuberculosis* complex in the sputum sample. Samples with no TUB band were cultured on Lowenstein-Jensen (L-J) medium[3] since accredited liquid culture services were not available in our laboratory at that time. DNA was extracted from colonies of those samples that showed growth of AFB. DNA extraction was carried out using GenoLyse® kit as per manufacturer's instructions[4]. LPA was carried out on the extracted DNA using GenoType®MTBDRplus (Hain Lifescience GmbH, Nehren, Germany) for detection of MTB complex and rifampicin and/or INH resistance according to the manufacturer's instructions[5]. H37Rv was used as positive control and DNA extraction and master mix reagents were used as a negative control as per the manufacturer's protocol[5]. LPA was carried out only on the smear-positive samples[6]. The study was approved by the institutional ethics committee.

Results

Of the 1528 sputum samples, 1294 were smear positives and 234 were smear negative. Of the total 1294 LPA tests done, there were 77 such samples, for which TUB band was not present (*i.e.,* 5.9% of all the samples tested by LPA). TUB band was present in 1217 samples, of which 232 (19.1%) were MDR, 130 (10.6%) were rifampicin monoresistant and 101 (8.3%) were INH monoresistant. Seven hundred and fifty four (61.9%) strains were found to be pansensitive. Of the 945 patients who were smear positive at diagnosis, on retreatment, 117 (12.4%) were MDR, 65 (6.9%) were rifampicin monoresistant, 61 (6.4%) were INH monoresistant and 480 (50.8%) were pansensitive. Of the 306 patients who were smear positive at any follow up, 55 (18%) were MDR, 25 (8.1%) were rifampicin monoresistant, 22 (7.1%) were INH monoresistant and 159 (52%) were pansensitive. Of the 140 patients with failure, 27 (19.3%) were MDR, 23 (16.4%) rifampicin monoresistant, 13 (9.3%) INH monoresistant and 53 (37.8%) pansensitive. Of the 131 patients on retreatment, smear positive at fourth month, 31 (23.7%) were MDR, 16 (12.2%) rifampicin monoresistant, five (3.8%) INH monoresistant and 62 (47.3%) pansensitive. Of the four patients classified as smear negative at diagnosis, on retreatment, only one was smear positive and was rifampicin monoresistant. Of the two patients who were contacts of known MDR TB cases were MDR.

All the 77 samples with no TUB band were cultured on L-J medium. There was growth of AFB in 18 samples. When LPA was carried out on culture isolates of these 18 samples, TUB bands were seen in 10 of them. Hence, MTB complex was detected in 10 of these culture isolates by LPA. The remaining eight were non-tubercular mycobacteria (NTMs). Speciation was not carried out. Thus, 10 out of 1294 (0.77%) sputum samples were wrongly identified initially as having no member of MTB complex. In addition, 67 (5.1%) of the total 1294 sputum samples were negative for MTB complex by LPA as well as by culture.

Discussion

Molecular diagnostic tools for the diagnosis of MDR-TB effectively address the issue of the long turnaround time associated with culture and sensitivity testing though high cost has hampered wide applicability of these tests. With the introduction of LPA for the rapid diagnosis of drug-resistant TB, there has been a significant reduction in time to initiation of treatment in MDR suspect cases[7]. A multisite validation study from India found LPA to be a sensitive and specific tool for the detection of rifampicin resistance in AFB smear-positive sputum specimens[8], though, in a study from Punjab, India, 2.7 per cent specimens were detected as invalid[9].

In our study, of the total 1294 LPA tests done, TUB band was not observed in 77 (5.95%) such samples. Our findings indicated that there were at least 0.77 per cent AFB smear-positive sputum samples that were wrongly identified as having no member of the MTB complex by LPA. It is also important to review decontamination procedures, especially centrifugation and time of exposure to reagents to ensure optimum results. However, since 0.77 per cent of samples negative for MTB by LPA were culture positive, it may not be entirely correct to depend solely on molecular assays for diagnosis of MTB infection. We could not relate this with the grading of sputum samples since smear microscopy was done on concentrated sputum samples after decontamination. It has been documented that 97 per cent of smear-positive specimens give interpretable results within 1-2 days using LPA for TB[10].

Although both molecular tests, LPA and Cartridge Based Nucleic Acid Amplification Test (NAAT), have been endorsed by the World Health Organization (WHO)[11,12], there is no clarity regarding the superiority of one over the other[13]. The provision of these tests free of cost through the Revised National Tuberculosis Control Programme (RNTCP) has facilitated wider acceptance of these powerful tests in clinical practice, at primary health centres in India. Both these molecular tests have significantly reduced the need for a primary culture of sputum samples and subsequent drug sensitivity testing (DST) of the mycobacterial isolates. However, it has been pointed out in the WHO policy statement on LPA that the LPAs are not a complete replacement for conventional culture and DST, and mycobacteriological culture is still required for smear-negative specimens[6].

Reasons for false-negative LPA may include the following: reagents not equilibrated to room temperature; addition of insufficient reagents, improper mixing of reagents, addition of reagents in incorrect amounts; improper immersion of strips in the reagents during incubation; improper washing of strips and improper sampling, storage, transport or preparation of specimen[5]. In view of the above, a repeat DNA extraction of all the 77 samples, which did not show TUB band, was carried out and then LPA was done again. However, the results remained the same. Further, 5.1 per cent of sputum samples were smear positive but negative for MTB complex by LPA. In addition, no mycobacteria were isolated from these samples by culture. This might be due to the presence of PCR inhibitors or any possible technical issue with the process of DNA extraction. These results could also be due to the presence of non-viable NTM. It has been documented earlier that a considerable number of NTMs are not identified and also not notified to the public health authorities[14]. With effective case detection and management of TB by the RNTCP of India, the epidemiology of a possible endemic infection with NTM may be unmasked. The major limitation of the study was that this was a description of our experience with LPA for diagnosis of MTB and drug resistance, not a planned study. Since the service had been newly introduced, the procedures for sample collection and transportation were still in the process of being streamlined.

In conclusion, our analysis showed that it was difficult for a single test (in this case, LPA) to be 100 per cent sensitive in its target detection. Therefore, an additional test may be the solution in the existing set-up. In addition, the presence of NTMs in smear-positive clinical samples must be documented to avoid erroneously identifying a patient as an MDR suspect. Further prospective studies on larger sample sizes would be needed to address the issues identified in this study.

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***Conflicts of Interest*:** None.

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