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Editorial

MOLECULAR METHODS IN THE DIAGNOSIS OF TUBERCULOSIS

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Early diagnosis of tuberculosis (TB) and initiating optimal treatment would not only enable cure of an individual patient but will also curb the transmission of infection and disease to others in the community. Of the several distinct components of TB control programme, case finding remains the corner stone for effective control of the disease. Currently, sputum smear microscopy by Ziehl-Neelsen (ZN) staining method is the simplest and most rapid test available to detect acid fast bacilli (AFB) in clinical specimens. Although it is a robust technique and quite inexpensive with a specificity of almost 100%, it suffers from a low sensitivity ranging from 22 to 78% and requires at least 5×10^3 bacilli per ml of sputum. In most of the disease endemic developing high burden countries (DEDC) such as India, smear microscopy at present is the only cost effective tool for diagnosing patients with infectious pulmonary tuberculosis and to monitor the progress of treatment.

The presence of active tuberculosis is also being diagnosed in DEDCs by other conventional laboratory procedures including specimen digestion and decontamination, isolation by culture on solid, and/or liquid media and identification and drug susceptibility testing of recovered isolates by standard phenotypic methods. Because of the slow growth of mycobacteria, the above procedures may require a turn-around time of 8-12 weeks or even longer. Additional limitations in relation to culture is the very limited availability of quality assured culture facilities in DEDC. Further more, the diagnosis of tuberculosis in children is especially difficult as they can not easily produce sputum and clinical features are non-specific and chest radiographs are often difficult to interpret. Though the demonstration of mycobacteria in various clinical specimens by culture remains as a gold standard, this is not always possible in children due to pauci bacillary nature of illness. The same is true in extra pulmonary manifestations and quite often also in patients suffering from HIV and tuberculosis. To meet these special situations, new techniques are very much needed and among them molecular amplification assays such as PCR have been shown to be promising alternative even for developing countries¹.

Molecular amplification techniques are mainly indicated for detection of organisms that require prolonged incubation and also that cannot be grown *in vitro* or the current culture techniques are too insensitive. Several molecular methods have been developed during the last decade for the direct detection and identification of *M. tuberculosis* in clinical specimens. These methods are able to potentially reduce the diagnostic turn around time from weeks to days, which acquire a greater relevance under special circumstances. If PCR is used in the diagnostic process as well as to identify disease caused by Non-tuberculous Mycobacteria (NTM) and to directly detect drug resistance of *M. tuberculosis* complex in clinical specimens, it will become less cumbersome for the patient, and reduce the delay both in diagnosis and also in the start of treatment.

The basic principle of any molecular diagnostic test is the detection of specific nucleic acid

sequence by hybridization to a probe, a complementary sequence and followed by the detection of the hybrid. DNA and RNA fragments have been proposed for the amplification target in *M.tuberculosis* and the most frequently used targets are IS6110, a repetitive element, 16S ribosomal DNA (rDNA), and 16S rRNA.

Although a large number of studies have been published on the ability of nucleic acid amplification assays (NAA) to detect directly *M.tuberculosis* from clinical specimens, it is nearly impossible to draw any definitive conclusions based on these studies due to their varied sensitivity, specificity and predictive values. Additional issues which include nucleic acid isolation procedures, gene targets for amplifications, sample size, cycling parameters, the type of patient population and type of samples cannot be ignored. Considering all the above mentioned issues, the US Food and Drug Administration (FDA) prescribed culture as a mandatory requirement in conjunction with the performance of any amplification-based test.

Many commercial direct amplification tests (CDATs) are available. These include: COBAS AMPLICOR Mycobacterium system (Roche Molecular system, Branchburg, NJ) which uses the 16SrRNA gene; Amplified *M.tuberculosis* Direct (AMTD) assay (Gene Probe, Inc, San Diego, Calif) in which 16S rRNA is the target amplified and which is identified with an acridinium ester-labelled *M.tb* complex specific DNA probe; and LCx MTB assay (Abbott LCx probe system by Abbott Laboratories, Abbott Park, Ill) which uses a Ligase chain reaction method wherein detection is performed by a micro particle enzyme immune assay with the LCx fluorimetric analyzer. Another test is the BD Probe Tec energy transfer system DTB (Becton Dickinson Biosciences, Microbiology Products, Sparks, Md.), in which the large sequences of IS6110 and 16S rRNA gene are both co-amplified. Detection is subsequently made by the increase in fluorescence polarization. Some CDATs are also available for the detection of both *M.tuberculosis* and its resistance to rifampicin, e.g. the INNO-LiPA Rif.TB assay.

However it should be noted that many of these tests have not yet been approved by the appropriate regulatory agencies. In 2000, the Centers for Disease Control and Prevention updated its recommendations for the use of CDATs in the diagnosis of active TB. Accordingly, it was recommended that sputum specimens be collected on three different days for AFB microscopy and culture. If AFB smears are negative, CDAT is performed on the first specimen collected, otherwise it is performed on smear positive specimens. If smear and CDAT are positive, that confirms TB. If the smear is positive and CDAT is negative, a test for inhibitors needs to be performed. If inhibiting substances are not detected on at least two consecutive smear-positive CADT negative samples, the patient is presumed to have NTM. If a specimen is smear negative and CDAT positive, and the same result is obtained from an additional specimen, the patient can be presumed to have TB. Lastly, if both the smear and CDAT are negative in two consecutive specimens, the patient can be presumed to be free from active TB. It also recommends that the decision regarding therapy depends upon clinical status of the patients^{2,3}.

In diagnostic Mycobacteriology, the introduction of PCR or CDAT was considered as an exciting new milestone. The probabilities of confirming a diagnosis of tuberculosis in paucibacillary situations are higher with PCR than with the routine diagnostic procedures such as sputum smear microscopy followed by Chest X-rays⁴. The advantage of the test is that it can be performed from a single specimen and yields results within a day. This makes the diagnostic process shorter and more patient-friendly, and in turn it may reduce transmission of infection. However, the disadvantage of PCR is that it may also detect nonviable tubercle bacilli and PCR negative data must be considered more carefully because of false negatives due to the presence of inhibitors of PCR in some samples. These techniques, however, should be performed always in conjunction with microscopy and culture, and the results should be always interpreted with the patients' clinical data.

Although molecular testing methods have enhanced diagnostic potential in detecting *M.tuberculosis* from clinical specimens, to differentiate between mycobacterial species and also detect drug resistance, the application and cost effectiveness of such technically demanding testing methods in large DEDC such as India with limited resources both in terms of man power with adequate technical expertise and laboratories with technical infrastructure, requires to be studied extremely carefully. However, in the future after the establishment of quality ensured culture laboratories at the State level, the National Reference Laboratories in India, along with laboratories in the major referral teaching hospitals and laboratories in the corporate sector, may be able to take up molecular based methods in special situations as described earlier. Adequate quality assurance of each of these molecular tests would have to be ensured. Until then, molecular based diagnostic methods wherever it is practised at present in India should be used only as an added adjunct in the diagnosis of tuberculosis under special circumstances and always in conjunction with the existing established diagnostic techniques i.e. sputum microscopy and culture, and X-ray.

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