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Research Article

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Evolution of Rapid TB Diagnostics technique and its potential impact on Tuberculosis epidemics in India

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Abstract India has the highest burden of both TB and MDR TB cases. The first step in controlling the prevalence of TB is early diagnosis and early treatment initiation of detected cases. Early treatment initiation leads to killing of most of the actively dividing bacteria in the initial phase of treatment, minimizing the probability of transmission of infection. ZN microscopy and solid culture & DST were used as the diagnostics technique earlier. ZN microscopy has a lower sensitivity to detect MTB and solid culture takes a two month protocol time, which eventually delays diagnostics. Due to the low sensitivity of ZN based microscopy and long protocol period for culture tests, it was very tough to diagnose TB early and accurately. Newer techniques improves the limitations of conventional one and also provides a more accurate and rapid detection of drug resistant TB. We review here the newer and rapid diagnostic techniques which have been implemented in India under Revised National TB Control Programme. We discuss the LED Fluorescence Microscopy and Real time PCR based tests for early diagnosis of TB, Reverse hybridization based rapid genotypic DST and Automated Liquid culture and DST by MGIT 960. Newer techniques improve the rapid case detection and early treatment initiation, which definitely controls the spreading of tuberculosis. However, we still need some more rapid and accurate techniques especially for drug susceptibility testing for other drugs than rifampicin, like isoniazid, streptomycin, etc for more effective patient management.

Keywords MDR TB, DST, Rapid Diagnostics, Rifampicin

Introduction

Tuberculosis (TB) is an infectious disease caused predominantly by *Mycobacterium Tuberculosis,* most commonly transmitted by inhalation of infected droplet nuclei. TB disease usually affects the lungs, but can involve any part of the body. India accounts for one fourth of the global TB burden i.e. 2.2 million out of 9.6 million new cases annually [1]. Tuberculosis (TB) is one of the top 10 causes of death worldwide [2]. Multidrug-resistant tuberculosis (MDR-TB) poses a formidable challenge to TB control due to its complex diagnostic and treatment challenges. According to WHO, The annual global MDR-TB burden was estimated at around 490 000 cases, or 5% of the global TB burden; however, less than 5% of existing MDR-TB patients are currently being diagnosed as a result of serious laboratory capacity constraints [3]. Alarming increases in MDR-TB, the emergence of extensively drug resistant TB (XDR-TB), potential institutional transmission, and rapid mortality of MDR-TB and XDR-TB patient with HIV co-infection, have highlighted the urgency for rapid screening methods. For providing better treatment facility to the patients in order to save the patient's life and limiting the emergence of drug resistant TB, it is necessary to diagnose the patient in early stage of disease. The Laboratory plays a decisive role in TB diagnostics and drug susceptibility testing. The rapid case detection and drug susceptibility testing is important for early treatment initiation and effective patient management, that will control the transmission of TB cases in our society. For a long time, the laboratories used only two methods, i.e. Microscopy using ZN staining and Culture (mainly by Lowenstein Jensen medium).

ZN based staining and microscopy -

Ziehl Neelsen staining and microscopy was the only method available for initial microbiological confirmation of TB. In developing countries, smear microscopy is still a TB confirming microbiological technique. The sensitivity of ziehl-neelsen staining and microscopy is only 60-70% and it requires 5000-10000 AFB per ml to diagnose. This technique detects AFB (Acid Fast Bacilli) and cannot differentiate between MTBC (Mycobacterium Tuberculosis Complex) and NTM (Non Tuberculous Mycobacteria), hence cannot confirm TB. India has this facility on DMC (Designated Microscopy Centre) over 1 lac population. The testing is supervised by district and state level supervisors to assure quality work under RNTCP.

This technique misses a good number of extra pulmonary, patients living with HIV and pediatrics cases. ZN microscopy is a rapid to perform but inefficient to detect low bacterial loads and also can't differentiate between live and dead bacilli, culture technique becomes more reliable.

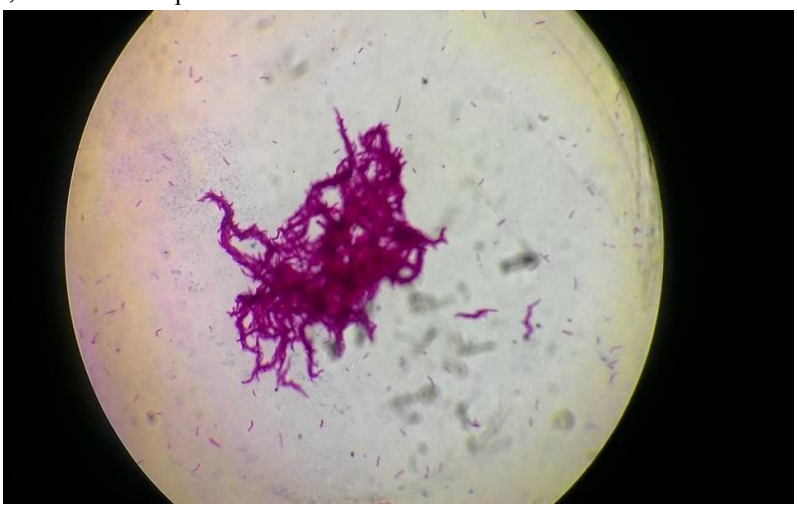

Figure 1: AFB showing cords after ZN based staining and microscopy

LJ medium based Solid Culture and DST (Gold standard) -

Culture improves the sensitivity by around 30% for detection of TB cases. It can detect few numbers of bacilli and considered as the gold standard for TB diagnosis. The most used culture technique was LJ media which contains malachite green for its antimicrobial property, which suppress growth of other microorganism up to a level. As, the MTB species is a slow growing bacilli with generation time of 15-20 hours, it takes 3-4 weeks to develop visible colonies on LJ media. But, due to the long incubation period the chances of contamination also increases. So, LJ based culture test is a more sensitive method but delays the diagnosis of patients and hence not meeting the requirement of an effective rapid technique for better patient management.

Figure 2: MTBC colonies on LJ media

New techniques

The Revised National TB Control Programme has been implemented all over the India till 2005. One of the main focuses in RNTCP was to ensure rapid diagnosis of TB cases. The Programmatic Management of Drug Resistance Tuberculosis (PMDT) was implemented till 2013. For the early detection of TB/DRTB cases it is necessary to implement rapid and more accurate techniques. We review here the new and rapid diagnostic techniques which have been implemented in India under Revised National TB Control Programme.

LED Fluorescence Microscopy

Conventional fluorescence microscopy is more sensitive than Ziel-Neelsen technique and takes less time in reading slides, but its use has been limited by the high cost of mercury vapor light sources and need of a dark room.

Light emitting diodes (LED) has been developed to allow benefit of fluorescent microscopy without the associated cost. WHO recommended the use of LED microscope as an alternative to conventional fluorescent microscope in resource limited settings. LED provides a cheap and reliable light source with a long lifespan and no need of a darkroom. Fluorescence microscopy is 10% more sensitive than the conventional Ziel-Neelsen microscopy [4]. The principle of fluorescence microscopy is based on staining the slides with solution containing fluorochrome dye (Auramine O). After primary staining the slides is decolorized with acid alcohol solution and counterstained with $KMnO_4$ (for background quenching of fluorescence). LED microscopy showed 84% sensitivity and 98% specificity against culture as the reference standard [5].

The LED microscopy takes less time to read slides as the fluorescent bacilli can be seen at lower magnification. As, LED microscopy is rapid and more sensitive than the zn based microscopy , WHO recommends its use on microscopy centre replacing the conventional ZN microscopy.

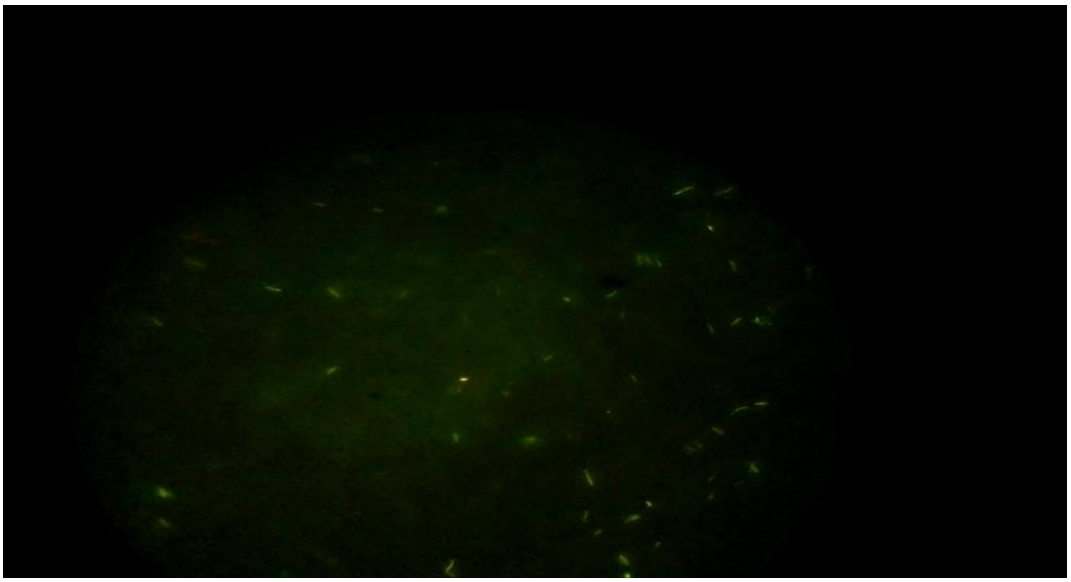

Figure 3: AFB showing under Fluorescence Microscope

Automated system based Liquid Culture and DST method

Culture is the gold standard for TB diagnostics. It is used as a reference material for validation of other techniques. Apart from the high sensitivity than microscopy technique, culture also gives the facility of species identification and DST. As compared to the solid media, Liquid media gives positive result in less time. Liquid culture increases the sensitivity up to 20% for detection of MTB [6]. Due to the three dimensional growth of bacteria it gives more rapid results than solid culture. The technique used in India under RNTCP is automated MGIT 960 (Mycobacterial Growth Indicator Tube). The MGIT 960 detects the consumption of oxygen. The oxygen acts as a fluorescence quencher and as the bacteria uses oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within MGIT tube when visualized under UV light. The machine is not specific for MTB, as it just detects fluorescence. The media and other content of the MGIT tube supports the MTB growth and resist the growth of other contaminants. The media used for liquid culture is Modified Middlebrook 7H9

broth. Additionally growth supplement, OADC (oleic acid, albumin, dextrose and catalase) is use to encourage the growth of MTB. In addition to this, combination of antibiotics is used to minimize the contamination. The antibiotics used are PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocilin). As per the guideline, the protocol time for giving a culture negative result is 42 days for liquid culture and is 56 days for solid culture. Also, liquid culture gives positive results earlier. So, if a patient is on Multi Drug Resistant (MDR) TB or Extensively Drug Resistant (XDR) TB treatment, we will have the results available earlier to decide the further extension of particular drugs. Handling liquid culture is more risky than handling a solid culture, as it creates much aerosol. So, it is recommended to use liquid culture under Bio Safety Level 3 (BSL 3) facility to ensure safety of the lab personnel. Under RNTCP, a lab can give liquid culture services only if they have BSL3 facility. WHO recommended both the liquid and solid based culture and DST for lab giving diagnostics facilities for TB.

Figure 4: MTB positive liquid culture tube

Molecular Diagnostics

The revolution in TB diagnostics comes with the introduction of DNA based detection of MTB and its drug susceptibility test. There are so many techniques available for TB detection, such as Line Probe Assay (LPA), Nucleic Acid Amplification Test (NAAT), Loop Mediated Isothermal Amplification Test (LAMP), etc. All these techniques are based on amplification of specific DNA sequence and its detection. Currently under RNTCP, two of these are used, *i.e.*, Line Probe Assay (for first and second line anti TB drugs) and CBNAAT (for DST of rifampicin only

Line Probe Assay (LPA)

Line probe assay for first line anti TB drugs is a multiplex PCR and reverse hybridization based assay has been shown to be a rapid and accurate method to detect the MTB complex and its susceptibility with rifampicin and isoniazid (for first line LPA test). As, we all know Rif and Inh are the two main first line drugs and if the bacilli becomes resistant to these two drug, it is said to be MDR (Multi drug resistant) TB. The increased understanding of the molecular basis of resistance to anti tuberculosis drugs and the consequent optimization of molecular methods leads to development of these techniques. The LPA for Rif and Inh is developed by HAIN Lifesciences and named Genotype MTBDR line probe assay. It detects the wild type and most common mutation governing resistance for that drug. For example, specific sequence of β subunit (*rpoB*) gene of RNA polymerase was used for Rif detection. For Inh detection two genes are analysed - catalase peroxidase (kat g) gene and inhA gene. Line probe assay technology involves the following steps: First, DNA is extracted from *M.tuberculosis* isolates or directly from clinical specimens. Next, polymerase chain reaction (PCR) amplification of the resistance-determining region of the gene under question is performed using biotinylated primers. Following amplification, labeled PCR products are hybridized with specific oligonucleotide probes

immobilized on a strip. Captured labeled hybrids are detected by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. Line probe assay performance characteristics have been adequately validated in direct testing of sputum smear-positive specimens and on isolates of *M. tuberculosis* complex grown from smear-negative and smear-positive specimens. Direct use of line probe assays on smear-negative clinical specimens is not recommended [7]. The most impressive thing about this technique is its turnaround time. LPA result can be available within 48 hours. As per the updated PMDT guideline, isoniazid mono resistant patient will receive a different combination of drugs based on its level of resistance. So, LPA is the only molecular technique available in India for detection of resistance for both rifampicin and isoniazid, but recommended only for smear positive specimen.

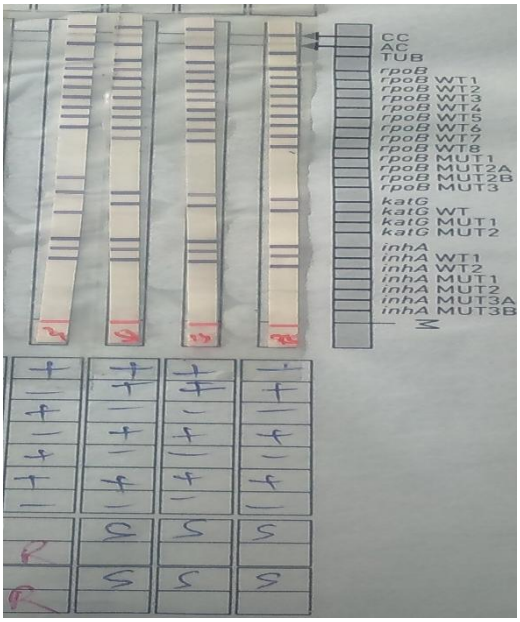

Figure 5: First line LPA blot

Cartridge Based Nucleic Acid Amplification Test (CBNAAT)

Cartridge based nucleic acid amplification test is a more rapid technique as it gives result only in 3 hours. The technique has been developed by Cepheid as "Xpert MTB/Rif" and is a real time PCR based detection of MTB and its susceptibility to Rif. If we compare this with LPA, it takes less time and less lab facilities than LPA. This technique gives DST result of only one drug but still playing great role in patient management as this technique is made available on district level under RNTCP. Xpert MTB/Rif achieved an overall pooled sensitivity of 88% and a pooled specificity of 99% [8]. Besides these advantages, this technique has the only drawback is it gives susceptibility result of only rifampicin. As per the updated PMDT guideline, if a patient is detected as rifampicin resistant by CBNAAT, the patient will take the medication of MDR TB along with isoniazid until the susceptibility of isoniazid is known by culture and LPA. After getting isoniazid DST result, the medication will follow accordingly. Like, if the infectious bacilli are sensitive to isoniazid, it will be added to the regimen and if the result is resistant to isoniazid, it will be stopped. So, if we see retrospectively, an MDR TB patient resistant to both rifampicin and isoniazid will take isoniazid drug unnecessarily for 1-2 months if initially detected by CBNAAT. Also the isoniazid mono resistant cases will be missed if diagnosed by CBNAAT.

However, apart from the great advantage to used these DNA based techniques, there are some drawbacks also. Like, the test can detect a silent mutation and predicts as resistance. Also epigenetic resistance can't be detected by these methods. So there are some limitations and for some cases, where phenotypic DST will be preferred.

Immunochromatographic tests for identification of MTBC -

For culture based DST to be done, it is necessary to first confirm that the positive culture belongs to MTB complex. Previously, this was done by some biochemical methods which can differentiate MTBC from NTM. Three tests are available for this. The BD and the SD Bioline assays detect MPT64 antigen, while Capilia

detects MPB64 antigen; both Mycobacterium tuberculosis complex-specific secretory proteins. Both liquid mediums and solid mediums can be used as samples. The test have 2 bands, a target (T) and a contol (C) band. The T band shows the presence of MTBC and C band is an internal positive control. Research shows that these techniques acquire sensitivity, specificity, PPV and NPV of 99.19%, 100 %, 100% and 97.3% respectively [9].

Discussion

The Revised National TB Control Programme was launched in 1997 in India with a phase wise scale up manner and covers the entire country by the end of 2005. The success of the RNTCP majorly depends on the rapid and quality assured diagnostics of TB. The newer techniques developed was rapid and accurate. The LED fluorescence microscopy increases the sensitivity by 10% from that of conventional ZN based microscopy. Also, it takes less time to read the slide. In India, the LED fluorescence microscopy is replacing the conventional microscopy. DNA based detection of drug resistant TB cases is becoming the milestone in the way of effective control over spreading of TB. As these techniques are rapid, it provides the scope of early treatment initiation.

Culture is considered as the gold standard for TB diagnostics. The emergence of MGIT 960 based liquid culture and DST provides a more rapid culture system. The use of liquid culture is not just limited to diagnostics of TB, but also helps in more effective patient management put on drug resistant TB treatment. As it gives the culture result of drug resistant treatment follow up samples earlier than conventional solid culture, it becomes smooth to evaluate the treatment accordingly. Although we are detecting the MDR/Rif resistant cases efficiently by CBNAAT, the other mono resistant or poly resistant cases missed. So, we need some more rapid diagnostics techniques in future for rapid detection of susceptibility for other drugs also to modify the treatment accordingly.

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