TUBERCULOSIS THEN AND NOW: A REVIEW ON CONTINUING DIAGNOSTIC PROGRESS

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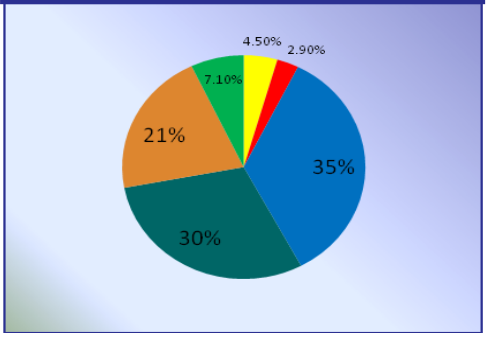
HOW TO CITE THIS ARTICLE:
Mihir K.R. Goswami. "Tuberculosis then and now: a review on continuing diagnostic progress". Journal of Evolution of Medical and Dental Sciences 2013; Vol2, Issue 31, August 5; Page: 5923-5935.

Despite substantial success n implementing standardized care and improving rates of cure in recent years, the global burden of tuberculosis (TB) remains enormous. Lack of rapid and accurate diagnosis and case detection are major obstacles to TB control. TB diagnosis, even today, continues to rely heavily on tools such as direct smear microscopy, solid culture, chest radiography and tuberculin skin testing: tools that often perform poorly, and require infrastructure frequently unavailable in the periphery of the health system where patients first seek care. The limitations of the existing diagnostic toolbox have been exposed by the human immunodeficiency virus (HIV) epidemic[1, 2] and by the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). Diagnostic delays and health system failures often result in missed or late diagnosis, with serious consequences for TB patients.[3]

In the past few years, there has been an unprecedented level of interest and activity focused on the development of new tools for TB diagnosis, largely because of agencies such as the Foundation for Innovative New Diagnostics Working Group (NDWG), the Global Laboratory Initiative (GLI) (another Stop TB Partnership Working Group), the World Health Organization (WHO), and the Special Program for Research and Training in Tropical Diseases (TDR). [2,4-6] Funding agencies such as the Bill & Melinda Gates Foundation, the Global Fund to Fight AIDS, TB and Malaria (GFATM), and UNITAID have provided the much needed resources and impetus to push the new tools agenda, in keeping with the Global Plan to Stop TB[7].

Tuberculosis (TB) is the leading cause of death worldwide attributable to a single infectious disease agent, and is an impediment to human development in developing countries. The impact of the condition is evident by the fact that about 1/3rd of the world population is infected with tuberculous bacilli with up to 10% lifetime risk of developing the disease; in particular, pulmonary TB, especially the reactivated latent infection, is the major source of the infection in communities.[8] The situation is worsening in many countries, particularly attributable to the synergy with the HIV epidemic and the appearance of multi-drug resistant (MDR) or extensively-drug resistant (XDR) tuberculosis.[9]

Figure 1. Global estimates of TB incidence



Therefore, improved knowledge of health professional on the current issues concerning the disease, and advancements related to management, is important for effective disease control since there is a global effort to eliminate the disease by the year 2050.[10]

Epidemiologic estimates: If left untreated, each person with active TB disease will infect on average between 10 and 15 people every year. In the past, WHO estimated that the largest number of new TB cases occurred in the South-East Asia Region, which accounted for 35% of incident cases globally (Figure 1).[11] The estimates appear particularly critical for India since it has more new TB cases annually than any other country.

Need for augmented methods in TB management: Despite the development of potentially curative chemotherapy, TB still remains a leading cause of human mortality in the developing world; limitation in early diagnosis is possible contributor. Essentially, the urgently needed and ongoing development of new tools and methods that can improve the diagnosis depends largely on the progress made by basic and applied research.[12]

Advances in diagnosis

An update on conventional methods: Even in modern world, TB case detection still remains dependent upon sputum smear and culture, radiography and clinical symptomatology, and not all TB patients receive a bacteriological diagnosis. In fact, this has led to achievements in this direction also, with progress in the existing methods.[13]

Progress in imaging: Recently, serial pulmonary [(18)F]-2-tluorodeoxy- D-glucose positron emission tomography (PET) has been investigated as a promising non-invasive method to monitor disease activity and responses to anti-TB chemotherapy.[14,15] The technique, though highly expensive, could be useful for the management of patients with MDR and XDR TB in selected cases.

Progress in sputum smears examination by microscopy: Recognition of the advantage of fluorescent microscopy for enhancing sensitivity over that of ordinary light microscopy, without loss in specificity, mark an achievement in conventional TB microscopy.[16] The specificity could be further improved by attaching a stronger light source.

Further, a systematic review on the sputum processing in sputum smear testing demonstrated that centrifugation in conjunction with any of several chemical methods is more sensitive, and overnight sedimentation preceded by chemical processing is more sensitive while having similar specificity.[17]

Progress in culture examinations: Over the past, a series of culture examination systems has been developed using liquid media for rapidly detecting Mycobacterium tuberculosis (M. tuberculosis). These liquid cultures are more rapid and sensitive than solid medium cultures. Therefore, WHO recently endorsed the use of liquid TB culture and drug susceptibility testing (DST) for M. tuberculosis in low-resource settings.[18]

New diagnostic test using mycobacteriophages to identify M. tuberculosis from biological specimen require only two days of turnaround time in the laboratory. However, though they have a high specificity, they lack sufficient sensitivity to substitute the conventional culture techniques.[19]

Other systems based on phenotypes have been devised mainly for DST. The microscopic observation drug susceptibility is one such system where the characteristic growth of M. tuberculosis in the liquid medium is checked under an inverted light microscope. Similarly, in another system (nitrate reductase assay), bacterial growth is confirmed from the bacterial activity to reduce nitrate to nitrite in liquid media that is indicated by change in colour of the media.[20, 21] Other colorimetric redox indicator methods have also being evaluated for the rapid detection of MDR in M. tuberculosis, and can be applied to clinical sputum samples with performance comparable to genetic methods in their sensitivity and specificity.[22, 23].

New TB diagnostic tests : Rapid detection of individuals with TB can be difficult in clinical practice, seeing that only 44% of all new cases are identified by presence of acid-fast bacilli (AFB) on sputum smears.[24] The gold standard for the diagnosis of TB is the detection of M. tuberculosis, the causative microorganism of TB. However, culture growth of the organism may take $\geq$ 2 weeks on average. Accordingly, the extemporized decision to initiate anti-TS treatment can be difficult in cases where AFB is not found on sputum smear microscopy despite the clinical suspicion of TB. In such a scenario, the clinical diagnosis of active tuberculosis relies on different methods, such as the tuberculin skin test (TST), chest radiography, amplification of M. tuberculosis nucleic acids and/or pathological examinations from biological specimens.

In global terms, building capacity and enhancing universal access to rapid and accurate laboratory diagnostics are necessary to control TB. As remarkable efforts have been made globally to accelerate the development and expansion of new diagnostic technologies, mentioned below are some of the recent advances in the diagnostic domain of TB across several new and established methods. Recently, the WHO has endorsed some of these novel methods (Box 1[17]); while some techniques are simple, others have complex requirements, and, therefore, it is important to carefully determine how to link these new tests and incorporate them within the diagnostic algorithm. The potential and successful implementation of these methods is expected to provide prompt diagnosis in the real world also.[25]

Box 1. New TB diagnostic tests Recommended by WHO

- Liquid media for culture and drug susceptibility testing (DST)
- Molecular line probe assays for rapid screening of MDR-TB
- LED-microscopy
- Non-commercial culture and DST methods MTB/ RIF assay

Molecular methods

Nucleic acid amplification tests (NAAT): Performed on the bronchopulmonary specimen, NAAT represents one of the most frequently used molecular tests for laboratory diagnosis of pulmonary TB. It is a rapid diagnostic method, and results can be available within a day after obtaining sputum or bronchoalveolar lavage (BAL) fluid. Nevertheless, diagnostic accuracy of the tests is highly heterogenous since NAAT amplification targets are not standardized.

The clinical value of NAAT performed on respiratory specimens for diagnosis of pulmonary TB has recently been formed. In individuals with positive AFB sputum smears, the sensitivity of NAAT to

detect M. tuberculosis nucleic acid on these specimens was more than 95%.[26.27] Thus, the presumptive diagnosis of TB can be rapidly confirmed when AFB are found on sputum or BAL smears. On the contrary the estimated sensitivity of NAAT in individuals with negative AFB sputum smears for diagnosis of active TB is highly heterogeneous, and is not consistently accurate enough to be routinely recommended for TB diagnosis.[28],[29] Nevertheless, an earlier meta-analysis and an independent recent study showed that in individuals with a negative sputum smear the specificity of NAAT for the diagnosis of active tuberculosis was 97% and 98%.[30.31] Therefore, a positive result in a M. tuberculosis-specific NAAT performed on a respiratory specimen may be suggested to be highly indicative of pulmonary TB. Yet, false positive results can be there in individuals with a past medical history of TB; and a small percentage of patients with smear negative TB have a positive sputum or BAL NAAT result.

Line probe assays : These are NAAT that are aimed at detecting common genomic mutations responsible for antibiotic resistance from a biological probe or culture by DNA hybridization.[32,33] The tests involve DNA extraction, multiplex NAAT, solid phase reverse hybridization on the test strip, and detection of mutations such as in the rpoB gene, in the katG gene and the inhA gene promoter regions.[34]

A meta-analysis showed that while the pooled sensitivity in resistance detection on clinical specimen for rifampicin was similar to conventional DST following culture, the pooled sensitivity for isoniazid-resistance testing was less optimal at 85%.[35] Genotype MTBDRsl assay represents the newest version of the line probe assays, and, in addition, can detect genetic mutations related to drug resistance of strains of M. tuberculosis, including those for fluoroquinolones and injectable drugs (amikacin), thus, enabling rapid diagnosis of XDR TB, together with direct testing on clinical specimen.[36]

Advances in serology testing : The wide profile of the disease comprises formidable factors against sensitivity and specificity of the expected diagnostics, and these disease characteristics have led to a long list of systems so far proposed and developed as serological diagnostics, each having diverse set of characteristics (Table 1).[37]

Table 1. Types and nature of serodiagnostic methods[5]

Antigens	38kDa, 16kDa, 88kDa, MPT51, malate anythase, CFP-10, TbF6 polyprotein, antigen 85B, antigen A60, antigen 5, tuberculophosphatide, lipoarabinomannan, Rv3425, alphacrystallin, 2,3-diacyltrehalose, 2,3,6,- triacyltrehalose, 2,3,6,6 – tetraacyltrehalose2- sulfate (SL-1), cord factor
Compound	Protein, lipid, polysaccharide (and their complex)
Composition	Single antigen, multiple antigen
lg class	lgG, lgA, lgG (single or combined)
Laboratory technique	Enzyme-linked immunosorbent assay, immunochromatography, immunodot rapid test, kaolin agglutination test

WHO/TDR evaluated commercially available TB tests relating to their performance, reproducibility and operational character, using 355 well-characterized archived serum samples to evaluate 19 rapid TB tests. It was noted that the sensitivity of these rapid tests ranged from 1 % to 60%, and specificity, from 53% to 99%. Patients with sputum smear-negative TB and HIV-positive patients exhibited poorer test performance. Further, while none of the assays performed well enough

to replace microscopy; combinations of existing potential candidate antigens may enhance sensitivitys,[38] Currently available serological tests cannot be recommended for diagnosing TB exclusively.[39]

Progress in cellular immunodiagnosis -IGRA : They represent indirect marker for infection, and are used to evaluate the presence of persistent mycobacteria-specific T cell responses in-vivo (TsT) and ex-vivo (interferon-g release assays; IGRA).[40-41] However, the tests cannot distinguish between individuals with latent TB, active TB or past TB.[42-43]

Tuberculin skin test (TST) : TST, developed as an 'allergy-test for the diagnosis of TB in children; has been the standard test for immunodiagnosis of TB, and despite recent invention of IGRA, it is still much more widely used as a screening method to identify individuals with a positive immune response against M. tuberculosis.[44] However, the results of the test may present with wide-interindividual variations in terms of characteristics of presentation. Further, while the sensitivity of the test decreases from a cut-off of 5 to 10 and 15 mm, the specificity increases with increase of the cut-off used to define positive indurations,

Recently, a phase I trial of a skin test that used recombinant early secretory antigenic target (ESAT)-6 instead of tuberculin demonstrated safety and tolerability of such a test.[45] Such a skin test, in conjunction with culture filtrate protein (CFP)-10 antigens, might increase the diagnostic sensitivity, and may perhaps overcome some of the limitations currently related to TST use and application. If such a trend continues, and clinical trials show superiority of this test to the TST, it may possibly be made widely available for the diagnosis of latent TB infection in resource-limited settings where the use of IGRA is prohibited by their operational costs.

IGRA –A FEW MORE WORDS: IGRA introduction might be regarded as one of the most important development in the diagnosis of M. tuberculosis infection over the last decade. The procedure represents the coupling of the discovery of antigens relatively specific to M. tuberculosis - EsAT-6 and CFP-l 0 and the development of simplified technologies of measuring interferon-γ Two commercialized systems are available for the latter technology QuantiFERON-Gold (QFT-G) measures interferon-y in IU/mL using an enzyme-linked immunosorbent assay (ELISA) and T-SPOT.TB counts the cells releasing interferon-y visualized as spots with the ELISA-spot (ELISPOT) technique. Findings of the diagnostic performance of these tests have accumulated, and characterized over the last few years. The former test, i.e. QFT-G, is now available as an' in tube' version (QFT-G-IT) that also includes the antigen TB7.7 in addition to EsAT-6 and CFP-10.

Though IGRA were originally intended to diagnose latent TB infection, but in absence of a gold standard of TB infection the active disease is usually used as a surrogate for infection when quantifying sensitivity. In contrast, specificity is measured in subjects with low risk of M. tuberculosis infection, for instance, healthy young subjects without known contact with TB cases. The sensitivity and specificity of IGRA is high and obviously superior to TST; yet the variability in sensitivity may be greatly ascribed to the difference in patients' characteristics. In general, it is commonly interpreted that these assays are superior to the TST to detect latent TB infection.[46, 47] Of the two IGRA tests, T-SPOT. TB seems to be more sensitive than QFT-G, and vice versa for specificity.[38] The negative predictive value (NPV) for TB is higher than 95% if combined IGRA and TST test results are negative, and, accordingly, the best use of

IGRA in non-immunocompromised hosts is to rule out active TB.[48, 49, 50]

Other biomarkers : It has always been a research Priority in the field of TB to identify and evaluate other

Table 2: Selected methods under investigation	
Method	**Investigational target**
FACS analysis of short-term stimulated blood or cell cultures	Frequency of CD27+ lymphocytes; identification of intracellular IFN-γ identification of IFN - γ -and IL-2-secreting cells
Chemokine production following stimulation with ESAT-6/CFP10/TB7.7	IFN- γ - inducible protein IP-10
IFN- γ - production following stimulation with ESAT-6/CFP10	Identification of IFN- γ - secreting cells
IFN- γ - production following stimulation with antigens different from ESAT-6/CFP10	IFN- γ production following stimulation with heparin-binding hemagglutinin IFN- γ production following stimulation with Rv3879c peptides in addition to ESAT-6 and CFP10 IFN- γ production following stimulation with RD-1 selective peptides
Proteomics	Mass spectrometry analysis of serum proteins to identify a TB specific fingerprint
Detection of MTB- specific cell wall components	Lipoarabinomannan
Gold nanoparticle probe assay	Hybridization of MTB-specific DNA with gold nanoparticle probes
Loop mediated isothermal amplification	Hybridization of MTB-specific DNA

Biomarkers for active TB or latent TB infections. To improve the accuracy of assays based on the IGRA Technology for TB and latent TB infection, various other combinations of M. tuberculosis specific antigens and cytokine presentations are being explored.[5]

Tabel 2 shows a selection of methods under development for the diagnosis of TB[5.] In most of these test result are available within 24 hour. Amongst this growing list of biomarkers under investigation, interferon-induced protein (IP)-10, a CXC chemokine, and monocyte chemoattractant protein (MCP)-2, a CC chemokine, have been clinically evaluated.

Immunophenotyping by fluorescence-activated cell sorting : Flow cytometry is an especially promising tool to further improve the diagnostic accuracy of local immunodiagnostic assays for diagnosis of AFB smear-negative TB.42 In sequence, with aim to obtain a rapid diagnosis of TB in suspects with negative AFB sputum smears, immunophenotyping of antigen-stimulated cells has also been performed through fluorescence activated cell sorting of BAL cells or sputum cells.[51,52] Nonetheless, despite the fact that multi-colour flow cytometry analysis allows for a better identification of cells reacting following antigen encounter, the method is technically more demanding, compared with IGRA, for immunodiagnosis of TB.

Proteomics : Proteomics, the science of the structure and function of proteins, has identified proteins significant as diagnostic biomarkers or as therapeutic targets in a range of diseases, including TB.[53,54] The technique when applied to analysis of serum proteomic fingerprinting, or individual recognition of proteomic profile, to distinguish subjects with/without TB, exhibited a fairly good diagnostic efficacy with sensitivity of 93.5% and specificity of94.9%.47 Further, with aim to translate proteomic signatures to conventional test formats, the investigators identified and

Improved laboratory diagnosis of tuberculosis- Indian experience

There exists an overwhelming need for improving TB diagnostics in India through the use of cost effective, patient-friendly methods appropriate to different tiers of the native health system. Over the past few years' substantial progress has been made in India in the field of TB diagnosis, and serious efforts have been made to facilitate the development of diagnostic tests for pulmonary TB, extra pulmonary TB and MDR-TB.' Advances in the Indian context encompass the following:

- Varied approaches have been attempted towards improving smear microscopy and rapid culture, and for differentiation between the M. tuberculosis complex and non-tuberculous mycobacteria.
- Several laboratories have developed in-house PCR assays for diagnosing TB with high accuracy.
- Approaches for distinguishing M. tuberculosis and/or M. bovis infection and disseminated M. avium complex infection in HIV-AIDS patients have also been described.
- Serological tests to detect antigens or antibodies to M. tuberculosis specific components by using cocktails of Excretory/Secretory protein antigens, Ag8S complex antigens, Hsp 6S antigen, RD 1 antigens and Rapid Reverse Line Blot Hybridization assays to detect MDR-TB (mutations to rifampicin, isoniazid and streptomycin) have also been developed.
- Other methods like measurement of adenosine deaminase activity and use of luciferase reporter phages have also been explored for TB diagnosis.

It is expected that the validation and application of these methods in laboratory and public health settings would result in improved TB diagnosis, and contribute to effective disease management in India.

Measured transthyretin and amyloid A from highly informative peaks, together with other known parameters, i.e. C-reactive protein (CRP) and neopterin, providing good diagnostic accuracies. Aside from serological diagnosis, Iipoarabinomannan is also considered as an attractive urine marker in an antigen capture ELISA-based system for detecting TB.

Table 3:

Summary of findings from several systematic reviews on TB diagnostic tests		
Diagnostic Test	**Disease/Site**	**Major Findings/Results of Systematic Reviews**
Diagnosis of active TB		
Sputum smear microscopy	*Pulmonary TB*	▪ FM is on average 10% more sensitive than conventional microscopy. Specificity of FM and conventional microscopy is similar. FM is associated with improved time efficiency ▪ Centrifugation and overnight sedimentation preceded by any of several chemical methods (including bleach) are more sensitive than direct microscopy; specificity is unaffected by sputum-processing methods ▪ When serial sputum specimens are examined, the mean incremental yield or increase in sensitivity from examination of third sputum specimen ranges between 2% and 5%
NAATs	Pulmonary and extra pulmonary TB	NAATs have high specificity and positive predictive value. However, they have lower (and highly variable) sensitivity and negative predictive value for all forms of TB, especially in smear-negative and extrapulmonary disease. In-house ("home brew") NAATs produce highly inconsistent results compared with commercial, standardized NAATs
Commercial serologic antibody detection test	Pulmonary and extra pulmonary TB	Serologic tests for pulmonary and extrapulmonary TB produce inconsistent estimates of sensitivity and specificity; none of the assays performs well enough to replace microscopy
ADA	TB pleuritis, pericarditis, peritonitis	Measurement of ADA levels in pleural, pericardial, and ascetic fluid has high sensitivity and specificity for extrapulmonary TB
IFN-γ	TB pleuritis	Pleural fluid IFN-γ determination is a sensitive and specific test for the diagnosis of TB pleuritis
		(Continued on next page)

Phage amplification assays	Pulmonary TB	Phage-based assays have high specificity but lower and variable sensitivity. Their performance characteristics are similar to sputum microscopy
Automated liquid cultures	Pulmonary TB	Automated liquid cultures are more sensitive than solid cultures; time to detection is more sensitive than solid cultures; time to detection is more rapid than solid cultures

Diagnostic Test	Disease / Site	Major Findings/Results of Systematic Reviews
TST	Latent TB infection	▪ Individuals who have received BCG vaccination are more likely to have a positive TST; the effect of BCG on TST results is les after 15 years; positive TST with indurations of greater than 15 mm are more likely to be the result of TB infection than of BCG vaccination. ▪ The effect on TST of BCG received in infancy is minimal, especially 10 years after vaccination. BCG received after infancy produces more frequent, more persistent, and larger TST reactions. NTM infection is not a clinically important cause of false-positive TST, except in populations with a high prevalence of NTM sensitization and a low prevalence of TB infection.
T-cell-based IGRAs	Latent TB infection	IGRAs have excellent specificity (higher than the TST) and are unaffected by prior BCG vaccination
Diagnosis of drug resistance		
Phage amplification assays	Rapid detection of rifampicin resistance	When used on culture isolates, phage assays have high sensitivity, but variable and lower specificity. In contrast, evidence is lacking about the accuracy of these assays when they are directly applied to sputum specimens
LPAs: INNO-LiPA Rif.TB (LiPA) And Geno Type	Rapid detection of rifampicin resistance	LiPA is a highly sensitive and specific test for the detection of rifampicin resistance in culture isolates. The test has lower

MTBDR assays		sensitivity when used directly on clinical specimens. The Geno Type MTBDR assays have excellent sensitivity and specificity for rifampicin resistance even when directly used on clinical specimens
Colorimetric redox-indicator methods and NRAs	Rapid detection of rifampicin and isoniazid resistance	Colorimetric methods and NRAs are highly sensitive and specific for the rapid detection of rifampicin and isoniazid resistance in culture isolates

CONCLUSION: TB still remains a concern in most parts of the world, and seeing the impact it has in individual and national and global terms, identification and development of effective methods for early diagnosis is a major activity in research domain of TB, together with applying their validity in real world clinical practice; indeed, a low case detection rate is still a major limitation in TB control worldwide. Today, technology has made it possible to rapidly identify individuals with TB, and a good combination of these diagnostic methods is a critical element of TB control. Many of these methods are quite promising and will find way in widespread use in clinical practice, thus, further approximating the goal of TB control.

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Date of Submission: 04/07/2013.
Date of Peer Review: 05/07/2013.
Date of Acceptance: 10/07/2013.
Date of Publishing: 30/07/2013