TRUE FACETS OF TB DIAGNOSIS IN 2012: HYPES AND REALITIES

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Tuberculosis takes a heavy toll of ~5000 lives every day from the disease; responsible for the 86% of DALY burden. Despite having drugs to treat TB efficiently, we have failed to control the disease. *Mycobacterium tuberculosis* has exploited it to their advantage evolving with multiple mutations making it resistant to first-line and second-line drugs. Most of the high-burden countries are low-medium income countries, their national TB program (NTP) still use sputum smear microscopy as the tool of diagnosis. Many new molecular tools are emerging, but confuse the larger TB clinical scientific community at the NTPs. Coherent information need to be disseminated, encouraging TB scientific community to generate evidences within NTPs assessing new tools through critical analyses in terms of value addition and cost benefit before considering rolling out in the program. It is also imperative that the scientific community need to have an open mind to use different tools in the right permutation and combination than being exclusive of one another. This article portrays an overview of the diagnostics landscape in 2012 with pros and cons of different tools to be able to generate a step-wise algorithm for optimal exploitation of the tools within available resources in each of the settings.

Keywords: *Mycobacterium tuberculosis*, diagnosis, drug-resistant TB, culture, genotypic tools, phenotypic tools, MGIT, GenXpert, LPA

Abbreviations: DALY (Disability-adjusted life years), a health economics calculus is a means of measuring the ‘cost-effectiveness’ of a given health intervention that took into account morbidity, mortality and age for comparison and impact assessment.

Introduction

Tuberculosis (TB) kills one individual every 18 s and infects an individual every second. TB is the major killer from a single infectious agent and this magnitude of the mortality is responsible for the loss of 86% of the DALY burden of TB while rest is due to illness, making significant dent in the economy and on the society [1]. Despite having the chemotherapeutic regimens to treat the patients successfully, we have failed to contain the scourge created by this disease which has been further enhanced with the emergence of multi-drug resistance/extensively drug-resistant MDR/XDR-TB. According to Global TB Control WHO Report 2011 covering data from 2010, there were 8.8 million new TB cases in 2010 [2]. Also, it claimed progress towards Millennium Development Goal (MDG) target of reducing incidence rates and mortality rate by 2015 while far off the target with regards to reduction in prevalence rates. It is also estimated that about 65% of the cases are treated with the level of 87% treatment success rate among new cases of smear positive pulmonary TB cases in 2009.

There are two paradoxical hurdles – first the delay in detecting TB amongst the healthcare seeking population

due to unavailability of optimal tools at ease of resources and time in the peripheral clinics for active case finding and treatment to avert further transmission; and the second is crucial in reducing the number of never seeking care TB patients, which contributes to the major share of the mortality; in specific high-risk TB subgroups such as prison inmates, HIV patients, urban slum dwellers and those with limited access to health services. WHO Report also highlighted issues on children – in 2009, almost 10 million children became orphans as a result of parental deaths caused by TB.

H1N1 swine flu which struck the globe in 2009 creating havoc, killed only 18,500 individuals (as per lab confirmations) raised sensational alarm in every sector raising prompt alertness. This resulted in fuelling several prioritised initiatives with ~15 billion USD that came up with a vaccine within 6 months’ time frame. Unfortunately, despite the magnitude of the global health crisis, TB, being most prevalent in the developing and resource-limited regions has never raised attention to such an extent. The entire investment on R & D for TB was 575 million USD for year 2010 (http://g-finder.policycures.org/gfinder_report/search.jsp). There is an absolute need to create an insur-

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mountable awareness and activism across sectors to gain global political clout for further investments.

Current tools

Sputum smear microscopy by Ziehl–Neelsen staining discovered by Robert Koch in 1880s still remain the basis of diagnosis along with chest radiograph and corroborative delayed type of hypersensitive skin testing with tuberculin (which was also a discovery from Robert Koch from the 19th century and originally made to be a vaccine and failed its purpose), wherever plausible and feasible along with clinical features. Sensitivity of the sputum smear microscopy ranges between ~60% for overall detection of TB and ~70% for detection of pulmonary smear positive TB.

Culture has been the gold standard of the TB diagnosis and was facilitated with advent of automated liquid culture technology MGIT (Mycobacteria Growth Indicator Tube) endorsed by WHO in 2007. Traditional microbiological labs used to do solid culture with egg-based media Lowenstein Jensen/Ogawa slant since many decades which has been hallmark of confirmed TB diagnosis for the last century.

Deland and Wagner developed in 1969 a technique for semi-automated detection of the metabolism of bacteria by measuring the $^{14}\text{CO}_2$ liberated during the growth and decarboxylation of ^{14}C -labeled substrate incorporated in the growth medium [3]. This radiometric technique used initially for blood culture using BACTEC 460 instrument was introduced commercially for mycobacterial recovery and drug susceptibility testing (DST) in 1980. Multiple evaluations of BACTEC 460 TB system demonstrated added advantages over solid media in significant time savings, especially for smear negative specimens [4–6].

The automated liquid culture, MGIT introduced by Becton Dickinson International (BD) used BACTEC exploiting a principle of quenching – radioactivity was replaced with oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate, embedded at the bottom of the tube containing Middlebrook 7H9 medium. Free O_2 gets consumed with bacterial growth and gets replaced with CO_2 . Depletion of free O_2 takes away the inhibition of the fluorochrome resulting in fluorescence within MGIT tube when submitted to UV light [7]. In Manual MGIT, tubes are read manually and in MGIT 320 or 960, fluorescence is read automatically.

This also allows standardized procedures with quality control reagents for rapid identification and DST with first and second-line drugs. Scores of studies including at national TB control program level across the globe have compared the added advantages of automated liquid culture over solid culture, to cite a few a department health study conducted at Bangkok, Thailand ($n = 2566$) showed 53% positive on liquid culture vs 39% in solid culture with time to detection shortened to 11 days vs 27 days, respectively, with significant value addition [8]; another Indian study showed 41% culture positive in MGIT culture vs 24% in solid culture with time to detection of 9 days vs 38 days, re-

spectively ($n = 14,957$). In the Indian study, MGIT showed its significant value addition in case of sputum smear negative samples with 18% positivity by MGIT vs 1% over solid culture with time to detection of 16 days vs 48 days, respectively [9]. There are also reported evidences from comparative evaluation for *Mtb* complex and NTM in liquid culture (MGIT 960) vs solid culture (LJ/Ogawa) clearly indicating substantial added value of liquid culture in terms of sensitivity as well as reduced time to detection [10]. Significant proportion of cases ranging ~25% would be missed if only solid culture would be used [11]. Similar trends have also been demonstrated with liquid automated culture in different body fluids from patients with extra-pulmonary TB [12]. All these proportion can vary in different settings with different population, prevalent clinical strains and the environment, hence, there is absolute need to generate adequate evidences in each setting to be able to make right judgement in the usefulness of each of these tools in patient diagnosis within the National TB Control Program with critical analyses of cost-benefit as well as value addition.

FIND (Foundation of Innovative and new Diagnostics) have been instrumental in the WHO's endorsement of MGIT liquid culture in 2007, LPA (Line probe assay) in 2009 and GenXpert in 2010 with hope of LAMP (Loop mediated isothermal amplification) and Point of Care (POC) assays in coming years.

Genotype MDRTB Plus line probe assay (LPA) is a great tool for rapid identification of DR-TB only in positive sputum smear samples. This assay has multiple steps of DNA extraction, PCR amplification, and reverse hybridisation of amplified nucleic acids on the specific DNA probes bound on the strips. This needs specific laboratory set-up. Even though automation has been achieved in reading the results, it still remains as a useful tool at the Reference lab levels. Global Laboratory Initiative (GLI) of WHO/Stop TB Partnership has categorically indicated that this is not a replacement test for phenotypic DST. This can be used as a triage test to guide further treatment decision and cannot be used to define XDR TB for surveillance purposes (<http://www.stoptb.org/wg/gli/assets/html/4th%20GLI%20meeting%2017-19%20April%202012%20AGENDA.htm>).

GenXpert is a rapid, integrated compact cartridge-based nucleic acid amplification test which had turn over time of around 3 h (sputum processing and 2 h of assay time) has been an asset for TB diagnosis to detect *Mtb* complex as well as Rifampicin resistance mutation [13]. WHO recommendations were specific primarily for MDR-TB suspects and HIV+ve individuals or individuals with unknown HIV status in high burden settings with suspicion of TB as well as secondarily for HIV-ve individuals not at risk of MDR TB with either abnormal chest X-ray or negative sputum smear with suspicion of TB. It also recommended doing phenotypic DST for first line and second-line drugs once found positive for *Mtb* complex with positive Rifampicin resistance. In no way, it was meant to replace any existing tools, but as an important tool to be used in the right context for rapid MDR-TB detection as

well as aiding diagnosis in difficult to diagnose cases with negative sputum smear microscopy [14, 15]. However, the hopes of putting it at sub-district level and the push for rapid roll out without other logistics in place raise some serious concerns [16]. It is essential to create logistics for immediate culture and DST of the GenXpert positive (both for *Mtb* and Rif resistance) samples before start of the second-line treatment to enable precision therapy for the patient based on drug susceptibility pattern through phenotypic testing. Unless this system is not built in parallel, the ambitious presumptive treatment with standard 7 drug second-line therapy would enable pathogen to evolve its strategies faster and successfully beating our efforts to control it. Currently, only about 5% of the DR-TB patients are being treated with second-line drugs due to limited availability of the second-line drugs and rapid detection of DR-TB patients without available second-line therapies could lead to serious ethical, social and medical dilemmas. It is also quite a daunting task for the treating physicians to manage the serious adverse effects of the second-line drugs in patients and to juggle the therapies optimally. Based on these concerns, rapid roll out of GenXpert in the sub-district level clinics could be catastrophic unless we addressed the concerns simultaneously. This also points us **to be inclusive than to be exclusive** of one another on policy to use different diagnostics tools wisely in systematic manner with defined algorithm from the evidences generated in the settings from pilot studies.

Stop TB partnership through GLI have raised concerns over the Positive Predictive Value of the GenXpert result which is dependent on the prevalence, and that if the prevalence of Rif-resistance is <15%, this could lead to a significant number of false-positive results, leading to unnecessary treatment of patients with 2nd line TB drugs (<http://www.stoptb.org/wg/gli/assets/html/4th%20GLI%20meeting%2017-19%20April%202012%20AGENDA.htm>). Hence, it is essential to assess prevalence in terms of resistance by periodic surveillance studies for which liquid culture is the perceived gold standard and optimal tool.

If we draw an analogy with respect to the progress made in TB diagnostic tools and aerospace industry over the last century, the growth has been phenomenal in the latter due to collective consortia efforts with multistakeholders across different disciplines as well as geographic boundaries over decades. This needs to be imbibed into the TB scientific community to be able to come up with rapid progress in the field. It is also worth considering to use the existing and upcoming GenXperts as major workforce by running the instruments almost round the clock than the way it is used now in a normal work day schedule. This may give us better return and create momentum in combating the menace of DR-TB.

With regards to Indirect assay using human immune responses as diagnosis, IFN γ Release Assay (IGRA), a FDA approved test for latent TB is not a diagnostic test. This is a smart test, but sub-optimal as it detects infection and does not distinguish disease. With advent of our understanding human immunology better, it may be a misnomer to restrict

the assay to IFN γ alone and it may be worth considering with open mind to include more cytokines/chemokines in the assay and may even consider to change the term from 'IGRA' to 'Immune Response Assay' to be broader in capturing the pattern recognition with set of markers.

Phenotypic methods

Phenotypic analysis of DST is required to characterise the genotypic detection of DR-TB for deciding on therapeutic regimens of the patient optimally. This can be well performed with MGIT system for first-line drugs. Second-line DST with MGIT is being optimised at several European Supranational labs. Apart from this, there are other poor man's options such as microscopic observation broth-drug susceptibility assay (MODS), Slide DST, Phage-based assays, Colorimetric methods with MTT and resazurin as redox indicators and Nitrate reductase assay [17–22].

It is also essential to corroborate genotypic diagnosis with phenotypic characterisation to follow-up the patients on therapy. There are reports in deterioration of patient even after specific therapy following DST results, in these cases the drug-resistance patterns were shown to drift with modifications of phenotypic characters in terms of susceptibility and resistance [23]. Hence, proportional concentration method with multiple concentrations is required to address this issue than using a single critical concentration. This is particularly necessary for patients deteriorating following therapy.

A review elegantly emphasised that the term 'resistance' with regard to *Mtb* is by no means a simple homogeneous category, but is quite heterogeneous and frequently composed of low-level, moderate-level and high-level drug resistance which presumably has important biological implications [24]. It also highlighted the variances such as low-level drug resistance, in part, not corresponding to clinical resistance; conversely, in the presence of a high-level resistance phenotype, the drug is of little, if any, clinical benefit. The clinical implications of moderate levels of resistance are less clear and need to be addressed in prospective studies with standardized protocols for quantitative DST of both first-line and second-line drugs for assessing the impact of resistance heterogeneity on treatment results, i.e. by correlating data from quantitative resistance testing with clinical outcome. Given the limited number of drugs available for the management of XDR TB till the upcoming drugs are licensed, it is wise to spare those than using those for drug sensitive patients.

Gaps in our knowledge and the need-based approach

Despite many recent progresses in our understanding the immunopathogenesis of the disease, we have much paucity in the knowledge on the basics of the disease biology. In terms of biomarkers of the disease, we need set of biomarkers which can serve as Diagnostic Markers; Prog-

nostic markers for reactivation/disease amongst latently infected individuals; Markers of relapse predicting at the beginning the high-risk group at the time of diagnosis of primary disease; Markers of treatment failure (drug resistance) to identify the vulnerable group which can eliminate the cycle of failures with standard therapy helping the patient for precision therapy as well as to reduce the public health risk. If we can determine 'Surrogate markers of immune protection,' this would accentuate the screening of potential vaccine candidates harnessing effective TB vaccine development; similarly 'Surrogate marker of bacterial clearance (clinical end-point)' would help the assessment and development of potential drug candidates [25, 26]. New 'Markers for infection' would also benefit in our efforts to eliminate the disease in low prevalent settings.

All these demand multiple harmonised, standardised prospective cohort studies in high and medium prevalent settings to decipher the natural history of infection to assess diversities – commonalities and uniqueness and to be able to design effective intervention strategies. This is much more essential if we envisage developing a universal TB diagnostics or universal TB vaccine. More and more studies demonstrate the clinical strain diversities and the heterogeneity of human immune responses.

To make appropriate permutation and combination for precision, it is required to have open access and standardised harmonised validation protocol in head to head comparisons which can only be achieved through creation of systems within NTPs to evaluate new diagnostic tools within the program settings.

MDR/XDR-TB

In a very recent NEJM article, two leading figures from the field have very aptly commented that the fault lines of MDR TB pandemic are largely manmade [27]. Inequities in health and health care delivery structure have facilitated the pathogen to flourish with its evolved armoury. New cases resistant to almost all available drugs are being reported and these patients are literally on an accelerated 'death warrant'. Despite new tools to diagnose DR-TB, second-line drugs and heightened public awareness, only 5% of the affected DR-TB patients have access to the right therapy. Bold strategies, political will and generous funding are critical to arrest this pandemic.

Augmented immunotherapy may save many of these lives and further R & D need to be invested on priority to exploit the approach.

Point of Care Assay

Several leading advocacy groups along with the scientific community have worked together to draw out a blueprint based on consultative consensus process qualifying minimum acceptable specifications of point of care diagnostics for TB [28]. It has several key points including the basic

one of at least higher sensitivity over Sputum Smear Microscopy to diagnose active pulmonary TB as well as ability to diagnose active TB in HIV/TB co-infected patients and children, followed in importance by paucibacillary, DR-TB and extra-pulmonary TB (EPTB); allowing direct treatment initiation and ideally also provide DST info; to be used at the place where patients are treated – in community, to enable to communicate the result to the patient on the day of collection (could be longer if it also provides DST); designed to be performed by nurses/community health workers; adapted to non-invasive samples – capillary blood, urine or breath; need to be qualitative and YES/No answer is sufficient; aim to be adapted to broader population (HIV+ve, children) rather than super high sensitivity in a restricted population.

New tools in the horizon

Researchers around the world are engaged in novel innovations in the diagnosis of TB as well as drug-resistant TB. A product named 'Genedrive' from EPISTEM, UK has very recently obtained CE-IVD certification as a test for TB and Drug-resistant TB based on nucleic-acid amplification platform [29]. Another cartridge-based test from Wave 80 technologies, USA is in late stage of development. Academics from the Netherlands have developed tests combining multiplex ligation-dependent probe amplification assay with luminex technologies to be able to genotypically test all drug resistant mutations in a single test [30]. EU supported TMREST consortium is involved in a newly developed integrated Lab-on-Chip versatile platform for fast detection of MDR [31]. Handful of leading chemists are busy deciphering the pattern recognition of volatile organic compounds in breath or other body fluids to be able to develop electronic nose for TB [32, 33].

A review published at the end of last year portrayed emerging alternative approaches such as Reported Phage assays, breath tests as well as phenotypic assays such as MODS, NO antibiotics resistance data which can bring enormous value addition in resource-limited settings with high burden of TB and drug-resistant TB [34]. We need to have an open mind to use any tools within our available means to be able to win our battle against this resilient *Mycobacterium tuberculosis* using cost-effective analysis as well as multiple menus to choose from than having gala buffet dinner menu.

Two approaches to dissect B-cell mediated immune response through immune proteome and peptide microarray chips have expanded our knowledge and may help towards better diagnostics tools for TB [35, 36].

Way forward

Current funding level for TB diagnostics in year 2010 as per G-finder is ~183 million USD (http://g-finder.policy-cures.org/gfinder_report/search.jsp). We need this to be

significantly increased with heightened advocacy and political commitments with fine balance of science in the area of basic science and real lives in clinics to translate research knowledge into products of public health importance. To achieve that, we also need positioning strategies to create national Biorepositories, standardised harmonized protocol and study designs with consortia approach, open access and shared consensus and to work in partnership, synergy in symbiotic mode.

There is absolute need to do head to head comparative studies in the field in operational mode to establish optimal test (may be different depending on the environment and scenario) before deciding on which one to use based on evidences in each setting. This also entails mapping the landscape of natural history of infection and the immune responses including transmissibility and reactivation.

We need to be determined to adapt, improvise, innovate and act to move forward, letting the action speaks of our collative efforts. We should consider thinking out of the box for diagnostics and asking the right question than running after Hi-Tec to do our basics right, for example, sharpening our current tool, smear microscopy through simple innovation. Possibly biosignatures (multiple patterns than single biomarker) would be the answer which requires coherent data collection from surveillance in standardised and harmonized manner.

Culture of *Mtb* is highly sensitive and the current gold standard for diagnosis but unfortunately suffers from many drawbacks, notably delays [37]. It also stated that the drawbacks of conventional microbiological methods for TB diagnosis and resistance detection have largely been overcome by modern molecular technologies; however the much needed point-of-care TB test will remain elusive if expectations stay unrealistic and research and funding strategies are not changed. Development of new technologies, better use of existing tools, and adequate capacity to care for the increasing numbers of cases that will be diagnosed with scale-up of TB diagnostics; all need to be addressed simultaneously.

Several recent publications have demonstrated Rifampicin monoresistance in adults and children with susceptibility to INH confirming the absolute need of phenotypic confirmation of INH resistance on genotypically confirmed positive Rifampicin resistant cases for patient management appropriately [38–40].

A multi-centre studies using direct DST of *Mtb* for rapid detection of MDR-TB using MGIT culture system has demonstrated added advantage of quicker method to reliably diagnose MDR-TB in an average of 11 days with time saving of more than 8 days [41]. This involved putting for DST directly on sputum sample than waiting for initial culture followed by DST.

In a study with HIV-TB co-infected patients in South Africa, blood culture was the only investigation yielding positive mycobacterial results in 59% of patients and thus found useful test in that setting. In this cohort, 64% patients with culture confirmed *Mtb* complex disseminated disease had a CD4 count of < 50 cells/mm³ and 2/3rd of

these had a CD count of < 25 cells/mm³. In a subsequent study in rural South Africa in patients with HIV, diagnosis of MDR/XDR-TB was made by blood culture alone in 12 patients with additive yield for diagnosis of DR-TB suggesting use of mycobacterial blood cultures in all patients suspected of DR-TB in similar settings [42].

Famous German physicist Max Planck had stated in a quote that the virtues never get established outright, but gets established as weaker things perish over time. Hence, time will tell which tool becomes the winner in the long run. We need to be realistic and open as it may not be one fit for all.

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Notes

The paper portrays views of the author in his personal capacity as a clinician scientist and these does not reflect views of the organisation he was/is affiliated to. Author is currently CEO, Vaccine Grand Challenge Program, Department of Biotechnology, Ministry of Science & Technology, Govt of India, New Delhi. He led the coordination of Grand Challenges in Global Health Consortium of 15 institutions on 'Biomarkers of Protective immunity against TB in the context of HIV/AIDS in Africa' based at Max Planck Institute for Infection Biology, Berlin and at the interim was a freelance consultant in global health, vaccines, tuberculosis and translational medicine.

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