Biomarkers for Infectious Disease Diagnostics in the Developing World:

Diagnosis of Tuberculosis in HIV Positive and HIV Negative Individuals

Katherine Tynan, Andy Blasband, Paul Neuwald, Laura Penny and Mickey Urdea

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Halteres Associates, LLC
5858 Horton Street, Suite 550
Emeryville, CA 94608
510-420-6733
www.halteresassociates.com

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Introduction

Tuberculosis (TB) is a chronic disease and the clinical manifestations of TB are highly variable at different stages of the infection. One set of important factors that affect the clinical presentation include host factors such as age, immunodeficiency states, co-existing diseases and prior BCG immunization. In addition, factors such as the interactions between the host and the microbe at the anatomic sites of involvement play a role in the clinical manifestations presented. Before the HIV epidemic, 85% of reported tuberculosis cases were limited to the lungs, with the remaining 15% involving non-pulmonary sites. This proportion is substantially different in HIV infected individuals, with more extra-pulmonary involvement associated with worsening immune compromise.[1]

Any diagnostic method that is used in resource limited settings should have certain minimal performance characteristics, including acceptable sensitivity, specificity, predictive value, turn around time (TAT), simplicity, and robustness. In particular it would be preferable to keep false negative (i.e. potentially infectious) cases to a minimum. However, given that there is a cost to over-diagnosis and concomitant over-treatment of false positives, there is a tipping point where the test characteristics will best match the available resources. With the right inputs it is possible to calculate these parameters, but it is beyond the scope of this document. Diagnosis of TB presents a number of other unique challenges in that biomarkers indicative of disease will vary in patients with early exposure, active disease, co-infections and latent infection (LTBI). Clearly the choice of biomarker(s) that are specific for the indication will be a critical factor for success.

One group of patients that is briefly discussed in this report but is not dealt with extensively is children. There are critical differences in the immune systems of children and adults specifically as it relates to mounting a response to *M. tuberculosis*. The disease in children is more likely to be severe, due to deficiencies in macrophage function, dendritic cell function and deficiencies in the development of Th 1 type T cells, all of which are essential to effective TB immunity. Young children under 5 years of age are at particular risk (up to 20%) of developing disease following infection, generally from a diseased parent. Many will present with disease within one year following infection, most within 2 years. For infants particularly, the time-span between infection and disease may be quite short and the presentation of primary TB (PTB) is as an acute rather than chronic pneumonia. Further confounding their diagnosis children rarely have sputum smear positive TB, few children under six will produce a sputum sample and so the diagnosis is often presumptive.

A second group of patients which is covered extensively is HIV infected patients with active TB disease. The increasing global burden of tuberculosis (TB) is linked to human immunodeficiency virus (HIV) infection. In 2003[2] worldwide ~9% of all new TB cases in adults (aged 15-49 years) were attributable to individuals co-infected with HIV, but the proportion was much greater in the WHO African Region (31%) and some industrialized countries, notably the United States (26%). In the general population, only 5-10% of the people infected with TB bacteria will go on to develop active TB disease at some point in their lives. However, the risk of developing TB is estimated to be doubled within the first year of acquiring HIV-infection and the annual risk for TB is ~10%. Consequently the HIV pandemic presents a massive challenge to global TB control.

A key element for appreciating the complex biology of TB is understanding why individuals infected with *M. tuberculosis* experience such different clinical outcomes. Approximately 70% of the people exposed to *M. tuberculosis* clear the pathogen from the bodies with no adverse effects: ~30% of exposure cases become "infected" and of these, approximately 10% go on to develop PTB or "active disease". The other 90% control the infection and are considered "latently" infected. Of the latently infected individuals 5-10% will re-activate and present with "active disease" at some future date.

The web of clinical outcomes results in a host of diagnostic and therapeutic questions. The following three TB-related clinical indications are addressed in this report and the decisions they inform are outlined below:

- **TB (1):** Case detection of active TB in HIV negative patients
    - Detection of TB biomarker(s) in HIV negative patients with previously untreated clinical symptoms of active TB
    - Decision: To initiate treatment with a front line multidrug therapy
- **TB (2):** Case detection of active TB in HIV positive patients
    - Detection of TB biomarker(s) in HIV positive patients with previously untreated clinical symptoms of active TB
    - Decision: To initiate treatment with a front line multidrug therapy
- **TB (3):** Case detection of multiple drug resistant TB
    - Detection of all or most common genotypes of MDR in patients with resistant TB
    - Decision: To initiate treatment with appropriate targeted therapy
    - Detection of multidrug resistant TB also has significant implication for epidemiological surveillance of populations, the details of which are not discussed in this document.

Active case detection in HIV positive and negative patients and an understanding of acquired drug resistance are the areas of greatest need for TB diagnostics today. To gain an understanding of the gap between the current tests on the market, and the need for better tests for these indications, we examined the biomarker and specimen combinations currently used in clinical practice, the resultant accuracy and limitations of these tests, and ultimately the applicability of these biomarkers to resource limited sites.

1. Active Cases of TB: Status of Currently Available Biomarkers

In many health outposts in the developing world, clinical evaluation is the only tool available for the diagnosis of chronic TB. A cough persisting for more than 3 weeks is the most common symptom of pulmonary TB, and patients may present with cough and other non specific symptoms such as fever or fast breathing, which do not distinguish TB from a variety of other infectious diseases that are common in the developing world. The clinical picture is used to select patients for AFB (acid fact bacilli) microscopy in many locations, and if available, evaluation via radiography.

The use of chest radiography to diagnose pulmonary TB is popular where there is access to an X-ray machine (CXR), due to its speed and simplicity. The use of clinical findings in combination with skilled interpretation of radiographic images provides a sensitivity of ~90% for advanced pulmonary TB. However, its major drawbacks for resource limited sites include the infrastructure and expertise required to properly conduct and interpret the radiography, and the high false positive rate, which in some settings can be as high as 20%.3 It should also be noted that patients with relatively early TB often lack definitive pulmonary changes. The presentation of pulmonary TB differs in early and late HIV infection and is intimately linked with the level of immunosuppression. In the early stages of HIV infection the clinical picture often resembles post-primary pulmonary TB with sputum smears being positive and CXR revealing cavities. In later stages of HIV infection the clinical presentation is more typical of primary PTB with sputum smears being negative and CXR showing infiltrates with no cavities (atypical of PTB).

In pediatric cases, there are no specific features on clinical examination that can confirm that the presenting illness is due to TB. Respiratory symptoms and disease are extremely common in childhood, particularly before 5 years of age. As mentioned above, sputum is not available in pediatric patients because young children usually swallow their sputum. Gastric aspirates and laryngeal swabs are generally not useful unless facilities are available for *M. tuberculosis* culture. This means that bacteriological confirmation is usually not possible. In most cases of suspected Pediatric TB (PTB), the child is presumed to have TB if he/she has been treated with broad-spectrum antibiotics with no clinical response. In addition, children, less than 5 years of age are particularly susceptible to severe forms of disseminated disease (military TB in which widespread dissemination through the body via the blood occurs) and extrapulmonary forms of TB (especially TB meningitis). As in adults, the natural history of TB in a child infected with HIV depends on the stage of HIV disease. Early in HIV infection, when immunity is good, the signs of TB are similar to those in a child without HIV infection. As HIV infection progresses and immunity declines, dissemination of TB becomes more common. Tuberculosis meningitis, military TB, and widespread tuberculosis lymphadenopathy occur.

There are two basic strategies for laboratory-based diagnoses of tuberculosis: 1) The direct detection of *Mycobacteria tuberculosis* or it products, and 2) The indirect strategy, which includes measurements of humoral and cellular responses of the human host against tuberculosis.

1A. Culture Tests

Culture remains the gold standard in mycobacteriology, in resource-unlimited laboratories due to it high sensitivity and specificity. The limit of detection (LOD) is approximately 100 bacilli per mL of sputum. In high prevalence countries, 80 - 90% of the patients presenting with symptoms will be culture positive. Unfortunately the sensitivity of culture is lower in advanced AIDS patients due to fewer bacilli in the sputum. The main limitation of culture is the turn around time (TAT), which takes 3-6 weeks using classical methods, and 50% less than that in some semi-automated commercial liquid systems. Example systems include the BACTEC MGIT960 system described below, the BACTEC 460TB (radiometric system from Becton Dickinson) or the automated ESP Culture System II (Trek Diagnostic Systems).

The status of the biomarkers that are currently used in commercially available culture tests for the diagnosis of *M. tuberculosis* is presented in Table 1.

Table 1. Status of Culture Tests Used in Commercially Available Assays for Detection of Active *M. tuberculosis* in HIV-/+ individuals.

Test name and description	Specimen type and Biomarker	Sensitivity & Specificity	Types of patients for which this test works well	Issues with respect to application in a resource limited setting
Bacilli growth on Lowenstein-Jensen (LJ) and Middlebrook 7H10/7H11 agar, 7H9/7H12/Dubos media	Sputum, others (especially in the case of extra-pulmonary TB): gastric aspirate, blood, CSF, bone marrow biopsy. The biomarker is the observation of acid fast bacilli	Limit of detection ~100 bacilli/mL Sensitivity 85% Specificity 99%[3,1]	In high prevalence areas, 80-90% of patients with symptoms will be culture positive. Lower sensitivity (~20%) in HIV + patients. Detects ~ >85% of cases. Fluids other than sputum have few bacilli; therefore only culture or nucleic acid amplification approaches have adequate sensitivity.	Medium complexity test. Requires significant infrastructure and technical skills. Advantageous in that positive samples can be removed, distinguished from other mycobacteria and drug susceptibility ascertained. Main limitation: TAT 3 - 6 weeks. Generally run at a reference or district lab level. The positivity of culture largely depends on the decontamination technique (i.e., the chemical used for decontamination) and the centrifugation method adopted.
Example: BACTEC MGIT 960: Mycobacteria growth indicator tube (MGIT) contains modified, non-radioactive 7H9 broth + a fluorescence quenching-based oxygen sensor. The system cultures 960 clinical specimens simultaneously (other than blood). Also used extensively for anti-microbial sensitivity testing.	Sputum, others (extra-pulmonary TB): gastric aspirate, blood, CSF, bone marrow biopsy /bacilli	Limit of detection 100 bacilli per mL [4,5]	Same as above	Same limits as above but faster TAT (7days (87%) - 9 days- (96%)). More costly and places higher demands on infrastructure

1B. Microscopy Tests

The use of microscopy to detect acid-fast bacilli (AFB) in stained smears is still the only widely available diagnostic tool available for identifying TB in most developing countries. However, this method has an effective LOD of 5,000 bacilli/mL and under less optimal conditions is 10,000 bacilli/mL. Poor sensitivity is further exacerbated by HIV co-infection, resulting in only 16% of all TB cases reported with a laboratory confirmed diagnosis of AFB.[6]

The status of microscopy-based approaches for the diagnosis of *M. tuberculosis* is presented in Table 2.

Table 2. Status of Microscopy Tests for Detection of Active *M. tuberculosis* in HIV-/+ individuals.

Test name description	**Specimen type/Biomarker**	**Sensitivity & Specificity**	**Types of patients for which this test works well**	**Issues with respect to application in a resource limited setting**
Acid Fast Bacilli Smear (i.e., carbol-fuchsin stains examined by light microscopy using oil immersion and the fluorochrome stains examined by fluorescence microscopy). Due to higher sensitivity of fluorescent staining, this method allows screening of a large number of smears under low power in less time (subject to false positives). Processing and bacilli concentration steps (centrifugation) increase sensitivity. In resource-limited sites, no preprocessing of the sputum occurs, so sensitivity is on the lower end of reported ranges	Sputum/bacilli	Limit of detection ~5000 AFB/mL (minimally needed for a consistently positive result) Sensitivity 40-60% Specificity 99%.[3]	Test is very dependent on the number of bacilli. In high prevalence areas present with advanced cavities in their lungs and so harbor many bacilli. Patients are required to provide 3 specimens (leading to high patient drop out) and results take 4-5 days The number of bacilli seen in a smear may reflect disease severity and patient infectivity. Therefore it is important to report the number Does not detect extra-pulmonary TB or discriminate between TB and other mycobacterium	Medium complexity test. Expert microscopy interpretation will result in 60% (~5,000 AFB/mL) of culture positive individuals being identified. Poorer skills will result in 40% (~10,000 AFB/mL) identification. In HIV+ co-infection, ~ 20% of culture positive individual will be positive. Other factors influencing sensitivity includes staining technique, centrifugation speed, reader experience, and prevalence of TB. Generally run at a regional or peripheral lab level

1C. Nucleic Acid Amplification Tests

Nucleic acid amplification (NAA) approaches, such as PCR, TMA, SDA, have been developed for platforms that can be used in resource-rich diagnostic labs. Most tests identify the Mycobacteria Complex which includes: *M. tuberculosis, M. bovis, M. bovis BCG, M. africanum, M. canetti, and M. microti*. None of the currently available tests are validated for the detection of *M. tuberculosis* complex in non-respiratory specimens, and no standardized methods exist for processing specimens. In specimens that are smear positive, the sensitivity is approximately 100%. However, in specimens that contain fewer organisms, are AFB smear negative or are extrapulmonary, the NAA tests generally detect 48-53% of patients with culture positive tuberculosis.[7]

Commercially available NAA tests for the detection of *M. tuberculosis* are presented in Table 3.

Table 3. Status of Nucleic Acid Amplification Tests for Detection of Active *M. tuberculosis* in HIV-/+ individuals.

Test name Description	**Specimen type/Biomarker**	**Sensitivity & Specificity**	**Types of patients for which this test works well**	**Issues with respect to application in a resource limited setting**
Roche Amplicor MTB – DNA amplification of 16S DNA via PCR	Sputum, bronchial washings and alveolar lavages; 100 µL volume is tested; potentially very few bacilli /mycobacterial DNA	Limit of sensitivity is 100 bacilli/mL. Sensitivity is measured relative to culture. Sensitivity 66.3-94.2% Specificity ~99%.[7, 8]	FDA approved for detection of *M. tuberculosis* in untreated patients who have AFB positive smear. Cannot assess the viability of the organism and is inappropriate for monitoring the patient during and after treatment.[9]	TAT 6-8 hours. High cost, high complexity, high infrastructure required. In practice, lower sensitivity than culture due to small specimen size and preprocessing (removal of inhibitory substances, etc.) on sputum which inadvertently reduces the bacilli count. Reduced sensitivity in HIV+ individuals due to reduced bacterial load.
Gen-Probe MTB Direct Test – Transcription mediated amplification (TMA) of TB 16S rRNA	Sputum, bronchial specimens, or tracheal aspirates; 450 µL volume is tested /mycobacterial RNA	Limit of sensitivity is 100 bacilli/mL. Sensitivity is measured relative to culture. Sensitivity 85.7-97.8% Specificity ~99%.[7, 10]	FDA approved for detection of Mycobacteria complex in untreated patients with AFB positive smear and negative concentrated sediments. RNA is considerably less stable than DNA; test is likely to be detecting viable organisms	TAT 6-8 hours. High cost, high complexity, high infrastructure required. In practice lower sensitivity than culture due to small specimen size and preprocessing (removal of inhibitory substances etc.) which inadvertently reduces bacilli count. Reduced sensitivity in HIV+ individuals due to reduced bacterial load.
BD-Probe TEC Assay: is based on strand displacement amplification (SDA) an isothermal technique. The target analyte in this assay is IS6110 DNA - some *M. tuberculosis* strains lack this sequence	Sputum, bronchial washings and alveolar lavages /mycobacterial DNA	100 bacilli/mL Sensitivity is measured relative to culture. Sensitivity 82.7-97.1%.[7] Specificity 94-99%	Not currently used in clinical practice, so unable to locate claims Cannot assess the viability of the organism and is inappropriate for monitoring patient during and after treatment.	TAT 6-8 hours. High cost, high complexity, high infrastructure required. In practice lower sensitivity than culture due to small specimen size and preprocessing (removal of inhibitory substances etc.) which inadvertently reduces bacilli count. Reduced sensitivity in HIV+ individuals due to reduced bacterial load.

Test name Description	Specimen type/Biomarker	Sensitivity & Specificity	Types of patients for which this test works well	Issues with respect to application in a resource limited setting
Example of new potentially cheaper assay format: Eiken LAMP assay for TB – loop mediated isothermal amplification of the gyr B gene of Mycobacterium. Presented at a recent StopTB/FIND conference.[11]	Sputum /mycobacterial DNA	Sensitivity is measured relative to culture. Claim limit of sensitivity is 10 bacilli per mL	Not currently used in clinical practice so unable to locate claims; however it seems to have the following advantages. TAT ~ 2 hours. Ins-trumentation appears to be relatively low cost. Results show good correlation with Amplicor assay Work is under way to use LAMP technology directly on unprocessed sputum specimens – initial results look promising	Medium cost, medium complexity testing with infrastructure required. Reduced sensitivity in HIV+ individuals due to reduced bacterial load.

1D. Phenotypic Tests

Another culture-based method that is used to diagnose Mycobacterium infection utilizes mycobacteriophage: The technology uses a commercially produced bacteriophage (Actiphage™) that is specific to mycobacteria. The Actiphage are generally added to culture isolates although some studies have added them directly to the clinical specimen, where they infect TB bacilli and replicate. Then, a specific potent virucide (Virusol™) is added to destroy the extra-cellular phage, while the TB bacilli and replicating phage within them remain unaffected. The Virusol is then neutralized, so that any phage that are subsequently released from the TB bacilli are not destroyed. Sensor cells,™ a non-pathogenic, rapidly growing mycobacterial strain also susceptible to the Actiphage, are added and the mixture is incorporated into an agar layer in a Petri dish and incubated overnight at 37°C. A positive result is indicated by a zone of clearing (plaque) in an opaque lawn of sensor cells. Plaques are representative of viable TB bacilli that were in the original specimen.[12]

The status of commercially available phenotypic tests for the diagnosis of *M. tuberculosis* is presented in Table 4.

Table 4. Status of Biomarkers for Phenotypic Detection of Active *M. tuberculosis* in HIV-/+ individuals.

Test name Description	**Specimen type/Biomarker**	**Sensitivity & Specificity**	**Types of patients for which this test works well**	**Issues with respect to application in a resource limited setting**
Fast Plaque TB (Biotec Laboratories Ltd.) This test directly detects the presence of mycobacteria. Luci-ferase reporter phages are under develop-ment. This system is better suited for rapid drug susceptibility testing on cultures.[13,12]	Sputum and cultured bacilli (limited information on direct detection in sputum)	Sensitivity is measured relative to culture. Most reports cover detection post culture however in newly diagnosed smear positive TB patients sensitivity was 70-75% with specificity of 99% in sputum	Detect presence of mycobacteria	Similar complexity to culture. Reduced sensitivity in HIV+ individuals due to lower bacterial load in sputum

1E. Immunodiagnostic Tests

A wide variety of immunodiagnostic approaches are used to diagnose TB. There are over 40 commercially available serology kits for detecting anti-mycobacterial antibodies in serum kits. These kits use a variety of native and recombinant TB antigens, including the 38kDa, 16KDa, 6kDa, LAM, ESAT-6, CFP-10 proteins. Sensitivity and specificity are variable and as yet none of the commercial tests can outperform the smear test. This is due to a number of factors: humoral response to *M. tuberculosis* is heterogeneous, HIV positivity can influence titers, and prior exposure of the patient to mycobacterial antigens, through inactive (past) TB, or environmental exposure to other mycobacteria can affect specificity.[14] Systematic studies to determine the causes have not been conducted. The development of new serology reagents for TB diagnostics stalled many years ago. Many researchers believe that it would be possible to identify appropriate TB antigens and their corresponding antibody responses if new studies were funded (personal communication Dr. S. Laal, NYU Medical Center).

The status of commercially available immunodiagnostic-based tests for the diagnosis of *M. tuberculosis* is presented in Table 5.

Table 5. Status of Biomarkers for Phenotypic Detection of Active *M. tuberculosis* in HIV-/+ individuals.

Test name Description	**Specimen type/Biomarker**	**Sensitivity & Specificity**	**Types of patients for which this test works well**	**Issues with respect to application in a resource limited setting**
Example 1: MycoDot Lipoarabinomannan (LAM) (Mossman Associates). LAM is a cell wall glycolipid of mycobacterium and modulates host immune response. **Example 2:** Rapid Test TB (Quorum Diagnostic/Omega) recombinant 38-kDA antigen from MTB and purified from *E.coli*. Test strip is incubated with 100 µL of serum. 38kDA antigen appears to be secreted and preferentially produced by multiplying bacilli, best correlated with advance or muti-bacilliary pulmonary TB. **Example 3**: Anti A60 IgG (Anda Biologicals) ELISA based. The A60 antigen is an antigenic complex recognized by 85% of anti-tuberculosis antibodies produced during infection and disease. This type of complex is present in all members of the *Mycobacterium*, *Corynebacterium* and *Nocardia* species.	Serum or whole blood and other body fluids/ (host response antibodies to mycobacterial proteins/antigens)	MycoDot: Sensitivity 93%, (<70% in HIV + cases) specificity 95% Rapid Test TB: Sensiti. 25% Specifi. 87%.[15,4] Anda A60 IgG Specificity is limited to the three genera Sensitivity 81% Specificity 88%	The credibility of the claims for these test are limited by the lack of regulation and limited field trials	HIV co-infection roughly halves the sensitivity. A significant problem with all of these assays is the considerable variability of the host response. Non-tuberculosis mycobacterial infections may cause cross-reactivity and loss of specificity. Choice of analyte has been poor and some of these markers are associated with advanced disease (LAM, 38kDa). Low specificity leads to high false negative rates in high prevalence areas. ELISA and EIA respectively format kits requires infrastructure.

There are special challenges in the detection of active cases of TB in HIV- individuals. In sites with sufficient resources, it is possible to identify TB in approximately 90% of HIV negative cases, using a combination of clinical presentation, AFB smear, nucleic acid amplification, and sophisticated culture approaches. Because of the time required for some of these tests, and iterative approach that is often used in employing these tests, even patients who are initially smear negative will convert to smear positivity and will be ultimately diagnosed with TB. There are no good studies that estimate the degree to which current testing algorithms used in resource-rich sites fail to identify TB in HIV positive individuals, but it is clear that the tendency of HIV positive individuals to have extra-pulmonary sites, as well as their limited immune response to TB, reduces the sensitivity of current testing algorithms.

2. Latent TB: Status of Currently Available Biomarkers

As previously noted, approximately10% of infected individuals go on to re-activate to an active TB disease state. Latency can only be understood as a dynamic process and infected individuals are defined

as those people infected with TB but who are able to limit the growth of the mycobacteria. Latent TB can persist throughout a person's life in an asymptomatic and non-transmissible state through efficient cell-mediated immunity. The granulomas subsequently heal, leaving small fibrous and calcified lesions. However, if an infected person cannot control the initial exposure in the lung, or if a latently infected person's immune system becomes weakened by immunosuppressive drugs, HIV infection, malnutrition, diabetes mellitus, aging, or other factors, the center of the granuloma can become liquefied in an unknown manner and then serves as a rich medium in which the now-revived bacteria can replicate very rapidly. At this point, viable *M. tuberculosis* can escape from the granuloma and spread within the lungs (active pulmonary TB). It is at this stage that *M. tuberculosis* can enter alveoli and bronchi of the lung, and the infected individual becomes infectious because the bacilli can be disseminated to others by coughing or expectoration.

The tuberculin skin test (purified protein derivative or PPD) was until recently the only tool available for detecting LTBI. The tuberculin test does not measure immunity and by itself, it does not indicate the presence or extent of TB disease; it only indicates infection occurred at some time. This illustrates a key point in the validation of any new test: in the absence of a "gold standard" it is hard to measure success. Both of the new tests to be discussed were validated using a nebulous standard, where specificity was estimated using data from people with no identified risk for *M. tuberculosis* exposure, and sensitivity was estimated using data from untreated (< seven days) patients with culture confirmed *M. tuberculosis.*

The two IFN-γ assays based on RD1 antigens (deleted in all sub-strains of BCG) are rapidly becoming the gold standard for the identification of latent TB in resource rich, low prevalence countries. There is limited information on the role and utility of these assays in immuno-compromised individuals, patients with extra-pulmonary TB, in children, and populations in high-incidence countries. In addition, due to the lack of specificity of the PPD test, there are likely to be many PPD false positive individuals who are also negative using the IFN-γ assay. How these results will be resolved remains a question. It should be noted however that unlike PPD, RD1 based tests can discriminate between MTb infected and BCG vaccinated individuals since all sub-strains of BCG are deleted for RD1. Finally, none of the current tests have the ability to predict progression from infection or latency to active TB disease.

The status of commercially available tests for the diagnosis of latent *M. tuberculosis* infections (LTBI) is presented in Table 6.

Table 6. Status of Tests for the Detection of Latently Infected Individuals (LTBI)

Test name Description	**Specimen type/Biomarker**	**Sensitivity & Specificity**	**Types of patients for which this test works well**	**Issues with respect to application in a resource limited setting**
Tuberculin Skin Test: Tubeculins (PPD (purified protein derivative, Parke Davis) or RT23) are employed as the test antigen in the **Mantoux test**. Results read within 48-72 hours. The only standard method for detecting latent TB. The test is based on the fact that infection with MTB produces a delayed-type hypersensitive reaction to certain antigenic components of the organism contained in the "tuberculins" (PPD, RT23).[1]	Skin puncture (delayed type hypersensitivity to MTb)	Difficult to determine – see below	Measure of exposure, will be positive in the absence of active disease. Potentially useful in children.	PPD shares a large number of antigens with BCG and other environmental mycobacteria, leading to high false positive rate in endemic countries. Many factors cause false negatives including errors in proper storage of tuberculin, administration, interpretation, and timeliness of return visits (Commonly, up to 30% of individuals tested do not return to have their results read). Sensitivity is low in HIV+ immunosuppressed patients
Gold QuantiFERON –TB (Cellestis Ltd): enzyme-linked immunosorbent assay (ELISA) detects the release of interferon-gamma (IFN-γ) in whole blood from sensitized persons when it is incubated (16-24 hours) with mixtures of synthetic peptides simulating two proteins present in *M. tuberculosis*: early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10).[16,17]	Heparinized whole blood Gamma interferon production from T cells	Sensitivity 80% (relative to culture positive). Specificity >98%. Note: there is no accurate standard to compare a new test for LTBI. Specificity of the QuantiFERON-TB Gold test were estimated using data from people with no identified risk for *M. tuberculosis* exposure and sensitivity was estimated using data from untreated (< seven days) patients with presence of culture confirmed *M. tuberculosis*	FDA approved for detection of LTBI. Cannot separate active or latent TB except on the basis of positivity in healthy individual's with-out recent expo-sure. The test's ability to fore-cast progression from the latent to active disease state had not been determined	Current TAT is 2 days, requires laboratory, infrastructure and is costly. Suitable for implementation at a regional laboratory level. The test has not been evaluated for use with children (<12 years), infants, or HIV+ subjects and in TB endemic countries.

Test name Description	Specimen type/Biomarker	Sensitivity & Specificity	Types of patients for which this test works well	Issues with respect to application in a resource limited setting
T SPOT –TB (Oxford Immunotech Ltd) ELISPOT (Enzyme Linked Immunosorbent Spot) assay. PBMCs are incubated (16-24 hours) with ESAT-6 and CFP-10) [16, 17]	Heparinized whole blood (PBMCs) (Gamma interferon production from T cells)	Similar to Gold QuantiFERON	CE Marked in Europe and awaiting FDA approval. Measure of exposure. Testing is indicated for diagnosing LTBI. Cannot separate active or latent TB except on the basis of positivity in healthy individuals without recent exposure.	Current TAT is 2 days. Requires laboratory, infrastructure and is costly. Suitable for implementation at a regional laboratory. Validity for children, HIV+ subjects and in TB endemic countries not known.

3. Drug Susceptibility of TB: Status of Currently Available Biomarkers

The development and dissemination of *M. tuberculosis* strains that are resistant to antibiotics poses an enormous challenge to TB control programs, both in the detection of an antibiotic resistant strain, and in providing access to second line therapies that might treat it. The resistance mechanisms and responsible genes are known for many of the front-line drugs and some of the frequently used second line drugs. There are approximately 10 known genes (rpoB, KatG, inhA, ahpC, kasA, ethA, EmbB, pncA, gyrA, rrs) that account for a significant proportion of resistance to 10 of the most commonly used anti-mycobacterial agents(for review see reference [7]). The mutations are largely point mutation and there is some correlation between the degree of resistance (MIC) and the type of nucleotide substitution. There has been a significant increase in multi- and extreme drug resistant TB (MDR/XDR–TB) most notably in Eastern Europe and hotspots in Russia and China. XDR is defined as MDR-TB that also has resistance to 3 or more of the 6 major second line drugs. The emergence of these strains highlights the importance of having tests which are accessible to as large a fraction of the at risk populations as possible. In general approximately 60% of drug resistant TB in local communities is due to transmission from person to person and thus indicates the ineffectiveness of control programs.

The purpose of tests to detect drug susceptibility aids in the selection of the most appropriate treatment regime that is available. Drug resistance is defined as a decrease in the *in vitro* susceptibility of *M. tuberculosis* of sufficient degree to be reasonably certain that the suspected resistant strain is different from a wild type strain that has never come in contact with the drug. Conventional culture-based methods for evaluating drug susceptibility take four to six weeks to complete. Newer methods that have been developed are either phenotypic, or rely on determining the mycobacteria genotype using nucleic acid amplification (NAA)-based methods. The most common genotypes that provide drug resistance in the strains in existence today are generally known, and are discussed in a later section.

The status of currently available tests used in to determine the drug susceptibility of *M. tuberculosis* is presented in Table 7.

Table 7. Status of Tests for the Detection of Drug Susceptibility of *M. tuberculosis*

Test name Description	**Specimen type**	**Sensitivity & Specificity**	**Types of patients for which this test works well for**	**Issues for application in resource limited setting**
Phenotypic Methods: Generally indirect methods (direct would be from the clinical specimen) which require prior culture of the mycobacteria to obtain sufficient numbers of bacteria to inoculate media and agar plates with varying concentrations of drugs to be tested. In many systems, a single critical concentration of the drug is tested (e.g. the BACTEC 460, MGIT 960, MB/BactT system, and the ESP II systems). The E-test system (AB BIODISK) has a series of strips with a gradation of drug concentrations.[1,7]	Mycobacteria growth by culture (occasionally set up directly on patient samples) For a valid test 50-150 colonies must be obtained on the drug free medium	Each method must be able to identify 1% of the mycobacterial population – when more than 1% of a population is resistant, then the population as a whole may soon be resistant.	Identification of resistance to front line therapies is based upon the "proportion method" and is currently considered the standard reference method worldwide. For the proportion method, the ratio between the number of colonies growing on drug containing medium and the number of colonies growing on drug free medium indicates the proportion of drug resistant bacilli present in the bacterial population.	TAT is on the order of weeks. Requires laboratory infrastructure and trained personnel. More expensive with increasing sophistication
Phenotypic Method (Direct): Example: Fast Plaque TB-Response* (Biotec Laboratories Ltd.). The protocol is almost exactly as describe previously in this document for direct detection of active TB, except prior to incubating overnight (18-24 hrs) the different drug and drug concentrations to be tested are added to individual tubes and plated individually. Results of the test are read as plaques (zones of clearing) on a lawn of Sensor cell growth. Very high correlation to other methods.[12,18]	Sputum (Decontaminated with NALC-NaOH) or cultured bacilli. (limited information on on direct testing of sputum)	Results in 48 hours from a sputum specimen. ~95%/99% relative to culture The results are interpreted as follows (example): RIF- plate must have 100 plaques or more to interpret results RIF+ <50 plaques = Rifampacin susceptible RIF+ 50 plaques = Rifampicin resistant	Only tested on smear positive individuals (i.e., very dependent on the number of bacilli present) Approximately 10-15% of smear-positive culture-positive specimens may not be detected by the test, due to the presence of inhibitory factors in the sputum.	Requires laboratory infrastructure and trained personnel. Cost. Suitable for implementation at a regional laboratory level

Test name Description	Specimen type	Sensitivity & Specificity	Types of patients for which this test works well for	Issues for application in resource limited setting
Genotyping Methods: the major question in the application of this technology is the correlation of susceptibility determined by phenotypic methods, as this is still the gold standard. Methods being applied include DNA sequencing, PCR, and INNO-LiPA Rif TB (Line probe assay, Innogenetics)[19,7]	DNA extracted from cultured bacilli or patient sputum	High sensitivity and specificity (98-99%) relative to culture methods		Generally highly technically demanding, and need expensive costly equipment and reagents. Suitable more implementation at a regional laboratory level. Not all drug resistant MTB have mutations that have been detected thus far.

* FIND/TDR has worked with Biotech Laboratories to get this test on the market.[20]

In sites with sufficient resources it is possible to identify drug sensitivity/resistance for all TB cases using a combination of sophisticated culture approaches (phenotyping) and nucleic acid amplification (genotyping). It should be noted that when a group of 17 Supranational TB Reference Laboratories submitted data on ~17,459 isolates tested phenotypically between 2000-2004, the results illustrated little or no standardization of quality assurance and limited reproducibility for some of the drugs (The data were presented at a StopTB meeting by S. Shah of the CDC 2005).[21] The lack of "gold standards" is a key issue for these laboratories. The bottom line is that time-consuming drug susceptibility testing, particularly using phenotyping methods, postpones effective treatment of patients suffering from MDR-TB, since treatment is normally initiated with a first-line drug. These patients go on to newly infect members of their households and communities with MDR-TB. In addition it has also been shown that rapid initiation of the appropriate therapy in HIV+ individual significantly affects their survival.[22]

The relative merits of the major diagnostic approaches discussed above are presented diagrammatically in Figures 1 and 2 (next page). In these figures, the ideal approach would be in the upper right quadrant, because of its high predictive power (clinical utility in resource limited settings), and the low level of resources required for successful implementation. The figures identify the approaches that are easy to perform, but are limited by the biology of TB or its hosts. Such approaches appear in the lower right quadrants. In addition, approaches with high predictive power, but that currently require significant resources, are seen in the upper left quadrants.

Figure 1. Map of Currently Available Diagnostic Approaches for Detecting Active Cases of TB.

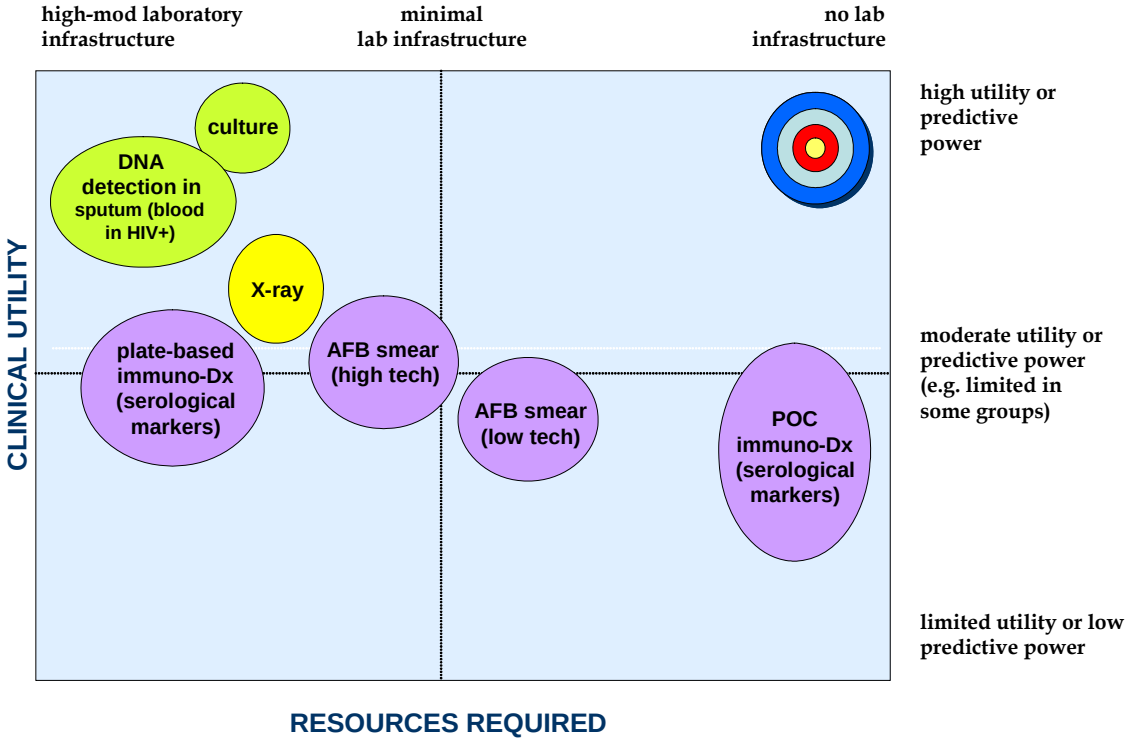
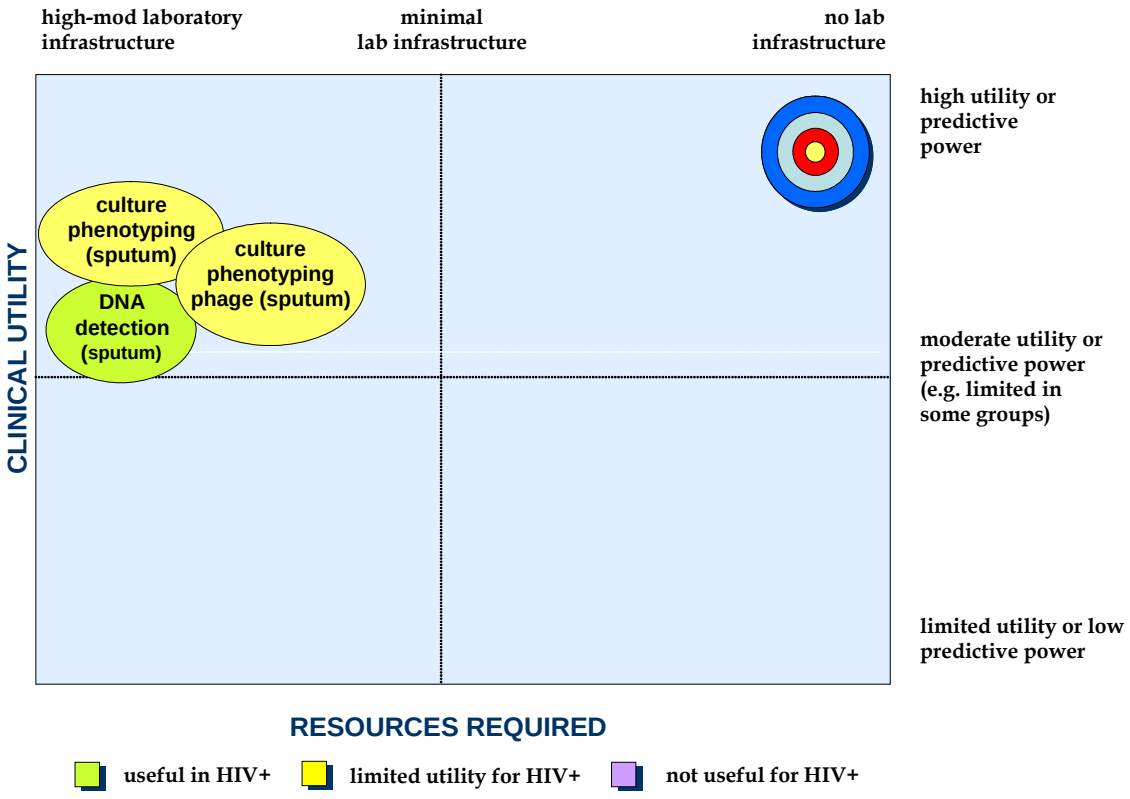


Figure 2. Map of Currently Available Diagnostic Approaches for Diagnosing TB Drug Susceptibility



TDR/FIND is in the process of conducting a sponsored comparative evaluation of drug susceptibility diagnostic methods and their cost effectiveness in Peru (Inno-LiPA rif TB, Fast Plaque TB-Response and more traditional phenotypic methods).

4. Current Deficiencies in the Diagnosis of TB in Resource-Limited Settings

In resource limited settings, there are many challenges to implementing tests that might provide the most clinical value. Many of these issues were identified in the course of a comprehensive review of 19 commercially available serology kits conducted by Jane Cunningham, Medical Officer of WHO/CDS/TDR/PDE.[14] The study concluded that the performance of the tests varied widely. Many of the tests showed high lot-to-lot and reader-to-reader variability, specificity was poor (<80%), tests with specificity over 90% detected <40% of the TB patients from endemic regions (host response variability), and HIV co-infection diminished performance of the assays. In conclusion, none of the assays perform well enough to replace microscopy.

None of the current tests is ready for deployment to resource limited sites for the following reasons.

- Culture of MTb is not available in resource-limited settings and methods that rely on growth in culture are impractical for deployment in resource limited settings, even if they were easy to perform.

- X-ray is too expensive today, requires considerable training, has a slow TAT, and is too nonspecific, especially in HIV+ patients

- AFB smear tests require a minimum of 5,000 AFB and multiple specimens for accurate detection, and the success of implementation is highly variable. In addition, limited sensitivity in HIV+ individuals combined with the difficulty in obtain sputum samples from children and HIV infected individuals, make it challenging to perform well in resource limited settings.

- Nucleic acid detection methods for *M. tuberculosis* DNA or rRNA are highly specific, and have high sensitivities in sputum from smear positive individuals. However, the complexity and TAT (6-8 hours) limits deployment of these tests. Sensitivity can be compromised by poor technique in sputum processing.

- Point of care serology-based tests, do not yet have adequate performance (sensitivity, specificity, or reproducibility) to recommend their implementation. However it may be possible to identify appropriate MTb antigens that provide sensitivity and specificity for antibody detection particularly in HIV+ TB patients.

- Tests of TB exposure that are used to determine active versus latent infection will be of little use in regions where the prevalence of TB is very high.

The deficiencies of the current approaches are summarized in Table 8. Cells that are filled in light blue are the characteristics that limit the utility of the test in resource-limited settings.

Table 8. Summary of Current Deficiencies in Diagnostic Tests for TB

Clinical Decision	Test	TAT	Specimen types	Sensitivity/Specificity (Limit Of Detection)	Resources & Infrastructure
Active TB infection in HIV- and HIV+ individuals	Culture	Days - weeks	Sputum and many others	85%/99% (100 bacilli) in HIV - 20% lower for HIV+	High
	X-ray/clinical presentation	1 - 2 hours	NA	variable, good in HIV- patients with advance TB, poor in HIV+ and children	High
	Acid Fast Bacilli Smear	minimum 3 days	Sputum only	40-60%/99% (5000 AFB per mL), HIV+ low end, 20% of culture +	Low-Moderate
	NAA for MTC i.e. Roche Amplicor, Genprobe	6-8 hrs	Sputum and many others	66-94%/99% (100 bacilli), depends on specimen.	High
	POC serology approaches	Min - hours	Blood (Serum)	25-90%/88-95% generally 50% reduction of sensitivity in HIV+ cases	Limited/No resource (test dependent)
	Phage detection	1 day – 2 weeks	Sputum and culture	70-75%/99% data unavailable for HIV+	High
Latent TB infection	Skin test	2 – 3 days	N/A	Difficult to say in high prevalence countries.	Low
	Interferon gamma	1.5 – 2 days	Whole blood/PBMCs	80% (relative to culture +)/98%, no data for HIV+. Not an indicator of active infection	High
Drug Susceptibility	Phenotyping	2-8 weeks	Sputum and culture	Variable but if well executed high sensitivity and specificity	High
	Phage	2 days – 8 weeks	Sputum and culture	Variable but if well executed 95%/99% relative to culture	High
	Genotyping	4 hours – 8 weeks	Sputum and culture	98-99% relative to culture methods	High

In summary, therefore, there are three major categories of problems with TB tests that are available today when it comes to implementing them in resource limited sites.

First, there are many tests for useful biomarkers that have acceptable specificities and sensitivities, but that are impractical for settings such as health outposts for one or more reasons. These problems include a turn around time that may be as long as several weeks, laboratory infrastructure and supply chain complexity that can not be managed, and training requirements that are too high for the personnel that are likely to perform the test.

Secondly, there are tests for biomarkers that simply don't have the clinical utility (predictive power) necessary for TB diagnosis in developing countries. AFB smear and current POC serology tests utilize biomarkers that have a ceiling on their predictive power, due to the biology of *M. tuberculosis* and its host, no matter how well the current tests are implemented.

Finally, there is the "hidden" problem of specimen type. Although sputum is the most commonly used specimen in the diagnosis of TB in resource-rich settings, it is often impractical to collect a good quality specimen, especially for HIV+ individuals and pediatric cases. Sputum also generally requires processing to remove inhibitors. Blood specimens, such as unprocessed finger prick specimens, can be practical in resource limited settings, but if fractionation is required, it becomes impractical in these same locations. Nasopharyngeal swab collections are also practical for respiratory diseases but may result in a potential decline in sensitivity of Mycobacterium isolation in culture. The yield of material is limited and, due to the hydrophobicity of Mycobacteria, the organisms may be entrapped within the fiber matrix and not readily transition into solution or onto media. False negative cultures are possible, especially if few pathogens are present. As mentioned previously, extrapulmonary TB (EPTB) can occur at any age and both young children and HIV positive adults are particularly susceptible. Up to 25% of TB cases may present with EPTB and up to 50% of HIV+ patients may have EPTB. EPTB represents a particular challenge with regard to specimen collection and processing. A variety of clinical materials will have to be considered, including urine,[23, 24] gastric aspirate, bronchial washings, cerebrospinal fluid, material from abscesses, bone marrow, and other biopsy specimens. Other than urine, for which there are promising results, none of these is a practical specimen type for resource limited sites.[24]

In conclusion therefore, there is no single test today which is suitable for identification of active TB regardless of HIV status that could be deployed at resource limited sites.

5. Opportunities to Improve the Clinical Performance of Biomarkers

Many improvements could be made to existing test technologies that would allow the currently available biomarkers to deliver adequate performance in resource limited settings. Below we discuss a small number of these possible technological improvements. For a more comprehensive discussion of these opportunities, refer to the Technology Paper and StopTB/Find strategic plan. Also, for the purposes of clarity we did not thoroughly review all the potential methodological improvement to existing biomarkers that would enable them to move from laboratories at the regional level to peripheral health centers. For instance there are many opportunities to improve culture methods that would significantly impact therapy decisions but that will never be suitable for deployment at resource limited sites.

5A. Modifications to POC Immunodiagnostics

The secreted *M. tuberculosis* antigen lipoarabinomannan (LAM) is excreted in urine regardless of the anatomical location of the mycobacteria. A plate-based ELISA has been developed that can be performed on unprocessed urine.[24] Initial studies indicate that this test has a useful sensitivity (80.3% compared to positive sputum culture) when performed in a regional hospital setting. Conversion of the ELISA based format into a format suitable for POC testing, such as a lateral flow device, would eliminate some of the hurdles to deploying this test to resource limited sites. The company developing this test, Chemogen, has recently converted the test to a strip format and is working with FIND to conduct a large clinical study in Tanzania.[25] It is likely that this single marker test will not have sufficient sensitivity; however, perhaps additional complementary urine-borne markers will be found presenting a reasonable alternative to sputum.

5B. Modifications to Nucleic Acid Amplification Methods

The high sensitivities and specificities that can be achieved with the nucleic acid based biomarkers (see Table 3) make it attractive to attempt to implement these kinds of tests on a technology platform that is feasible for resource limited settings. Such a platform would allow tests to be run for both case detection and drug susceptibility. There are quite a few nucleic acid analysis platforms that are under development for field use, primarily for biosecurity applications, but almost none of these have addressed the issue of sample preparation. A notable exception is the Cepheid GeneXpert platform. Though it is generally designed to be run in a laboratory, it can be run on battery power, can function at a fairly wide range of ambient temperatures, provides a fairly rapid result, and the self-contained, single use cartridges have supply chain requirements that are probably close to those required for widespread deployment in health outpost settings. One significant remaining challenge is the cost of the cartridge, and therefore the cost per test. Therefore efforts to explore ways in which to reduce the manufacturing costs of the cartridges would increase the accessibility of tests run on this platform. Cepheid was recently funded by the FIND organization to develop a GeneXpert cartridge for TB drug susceptibility testing. This cartridge will use nested PCR to detect TB-specific rpoB gene sequences that confer drug resistance directly from sputum, and eventually from blood. Such a test would serve not only to detect the presence of *M. tuberculosis*, but also to determine the most appropriate therapy. This product is at least 18 months away from commercialization. No information concerning the product in development is available at this time. (www.cepheid.com, personal communication, Dr David Persing).

5C. Modifications to Sample Collection and Processing Methods

The fact that sputum is the specimen type that often contains the known biomarkers for TB, (e.g., whole bacilli, *M. tuberculosis* protein and carbohydrate antigens, and the nucleic acids of *M. tuberculosis*) poses a significant challenge for the diagnosis of TB. Unfortunately, sputum is a very impractical specimen type to work with in resource limited settings, because it requires strict collection protocols, significant processing using reagents, equipment and training that are unlikely to be available, and because it cannot reliably be obtained from large groups of patients with TB, including children, patients who are HIV+, and patients with extra-pulmonary TB. In addition, the processing steps for sputum, including decontamination of the specimen (removal of other confounding bacteria before culture), removal of inhibitory components (for nucleic acid amplification methods), centrifugation (to concentrate the bacilli for smears) or reduction in viscosity, may all inadvertently result in loss of sensitivity through reduction in the number of bacilli in the processed specimen. Though simplified and improved methods to process sputum might enable the wider use of some methods, such as nucleic acid amplification approaches, it would not eliminate the issue that sputum cannot be reliably obtained from young children and many HIV infected individuals. Therefore it would also be beneficial to explore ways to improve the reliability of obtaining a respiratory specimen from children and HIV positive individuals.

It may be possible to improve sputum processing by incorporating a method of "panning" for bacilli. Panning is a general term used to describe the capture and concentration of a desired organism in a biological specimen via binding of the organism to a capture surface. The capture ligand typically is an antibody or peptide that binds specifically to the organism. The ligand is routinely attached to a solid support such as a membrane, micro-array surface or micro beads (e.g., magnetic beads) This technique,

in the form of a peptide-mediated magnetic separation, has been used to facilitate the selective isolation of *M. paratuberculosis* in bulk milk.[26] In this example, paramagnetic beads were coated with a peptide specific for *M. paratuberculosis*. The authors combined the magnetic bead separation with PCR to detect as few as 10 bacilli per milliliter. In another example, *Bacillus anthracis* was captured via an antibody specific for the *B. anthracis* surface array protein SAP.[27] The inventors in this example were able to detect concentrations at least as low as 1800 cfu/mL without culturing. The capture antibody in this example was attached to a solid support, namely a microtiter dish.

Nasopharyngeal swab collections have been shown to be practical for other respiratory diseases. Quidel Corporation has a commercial test based on a nasopharyngeal collection device which is used with a rapid differential diagnosis of acute influenza type A and type B virus infection. Whether or not this device could be used for TB testing is not known, but it could be worth exploration.

A promising technical approach that could circumvent the sample processing steps to a large extent is under development by Bioscale in Boston, Mass. Based upon a new highly sensitive acoustic sensor technology, bacteria and viruses from crude samples such as whole blood and feces been detected with the method. As few as 1,000 organisms have been detected. As yet, the method has not been applied to TB, but the company has intentions of investigating the opportunity.

Because a substantial percentage of patients with HIV develop TB disease in extra-pulmonary sites, there are many other specimen types that would be useful to be able to test, but that remain impractical for resource limited sites.

6. Evaluation of Known Biomarkers That Have Not Yet Been Clinically Validated

The Stop TB New Diagnostics Working Group has indicated that "the prominent roadblock for the development of suitable antigen or antibody assays is the lack of suitable immunological targets."[6] In parallel with efforts to discover novel biomarkers for TB, it is worthwhile to consider whether there are molecular entities which are already known, but that simply have not yet been evaluated extensively on a clinical basis or by using systematic product development criteria. To effectively mine these biomarkers it will be necessary to look at analytes of a particular class in combination to determine if there is the potential to develop accurate multi-analyte tests.

6A. Evaluation of Known Molecules as Biomarkers for Detection of Active Cases of TB

Immunological Approaches: A variety of sources exist for information on *M. tuberculosis* antigens that have yet to be evaluated as either analytes or as the binder in a serology-based test. It is not clear that these antigens, or combinations of these antigens, have been evaluated in any serious, systematic, and well-funded commercial development program. Antigens of *M. tuberculosis* that have appeared in the public literature are presented in Table 9.

Table 9. Known *M. tuberculosis* Proteins Whose Utility as Biomarkers of TB Disease Have Not Been Evaluated Systematically

Antigen	**Reference**
TbH9 (Mtb39)	Molecular Characterization and Human T-Cell Responses to a Member of a Novel *Mycobacterium tuberculosis mtb39* Gene Family (1999) Davin C. Dillon et al.[28]
TBH6	Use of Multiepitope Polyproteins in Serodiagnosis of Active Tuberculosis (2002) Raymond L. Houghton, et al.[29]
81-kDa malate synthase (GlcB) protein (Rv1837c; same as the 81 –kDa protein	Antigens of *Mycobacterium tuberculosis* Recognized by Antibodies during Incipient, Subclinical Tuberculosis. (2005) Krishna K. Singh, et al.[30]
Ppe-c	Immunogenicity of the *Mycobacterium tuberculosis* PPE55 (Rv3347c) Protein during Incipient and Clinical Tuberculosis (2005) Krishna K. Singh, et al.[31]
MPT51 (Rv3803c)	Same as above.[31]
Mtb81	Mass Spectrometric Identification of Mtb81, a Novel Serological Marker for Tuberculosis (2000) Ronald C. Henderickson et al.,
TB9.7, TB15.3, TB16.3 and TB51	Assessing the Serodiagnostic Potential of 35 Mycobacterium tuberculosis Proteins and Identification of Four Novel Serological Antigens (2005) Karin Weldingh et al.,
* Please note list is list based on telephone conversations with experts and is not meant to be comprehensive.	

Antigens of *M. tuberculosis* could be clinically validated as biomarkers within a one to three year horizon. The time to implementation as a product will be longer, and will depend largely on speedy access to clinical samples to validate the tests.

A number of companies such as Chembio are developing multi-antigen serology tests whose performance might be improved by the addition of unique combinations of some of these yet-untested antigens. In their TB/HIV co-infection test, Chembio reports 100% sensitivity and specificity for HIV antibodies (typical of many HIV serology tests), in the TB only cases they reported 66.5% sensitivity (97.5 % specificity) and in the TB/HIV+ cases 46.9% sensitivity (99.6% specificity) for TB antibodies using five *M. tuberculosis* antigens in the test (Mtb-8, CFP10, 38kDA, TBF10, TBF6) (all TB cases were culture positive).[35] It may very well be possible to increase the accuracy of these types of tests by including broader or better biomarker combinations. Tong and colleagues at the University of Leiden have recently tested 54 TB antigens with the aim of identifying the best combinations of antigens that maximally discriminate between TB-infected and uninfected individuals.[36] Such systematic evaluations of *M. tuberculosis* antigens could provide a rich source of new material for further study, in addition to the list provided above. The Bright Ideas Fund of the World Health Organization (WHO) has funded Karin Weldingh at the Staten Serum Institut (Denmark) to look at antigen combinations for immunodiagnostic test formats.[37] Results of this work were not reviewed for this report.

A novel antigen/antibody identification effort is underway at Chembio Corporation in collaboration with Peter Andersen's lab at SSI, Steven Reed's lab at IDRI and others (personal communication Javan

Esfandiari , Chembio). The serology project was partially funded by NIH (SBIR Phases I and II), whereas the antigen detection project was funded by WHO/TDI. The large-scale screening for relevant antigens and antibodies was facilitated using the Multiantigen Print Immunoassay (MAPIA), a method designed by Chembio scientists. The long-term goal of their studies has been to develop an accurate and inexpensive POC test for rapid TB detection. Although preliminary results were evidently encouraging, the lack of well-characterized antibody reagents and published data on the presence of diagnostically important antigens in biological fluids in human TB has made this work technically challenging and time-consuming.

A TB reactive skin patch to detect active TB infection has been investigated by Sequella Corporation. According to their web site an international prospective, randomized blinded Phase III clinical trial on the TB Patch is ongoing in South America. Submission of the worldwide registration application(s) was expected to begin in 2006. The basis of the test is the TB antigen, MPT64 a 23-kD secreted protein restricted to members of the Mycobacterium tuberculosis complex which elicits T cell responses and cutaneous delayed-type hypersensitivity (dth) reactions in *M. tuberculosis*-infected animals. Patients with tuberculosis and their tuberculin-positive contacts respond to the protein, but recipients of BCG vaccine strains lacking the mpt64 gene do not. Despite its obvious non-invasive advantages the main disadvantage of this approach is that the patient must return to the clinic after 72 hours for interpretation of the skin response results (historically, 30% of individuals tested do not return to have their results read). Preliminary results suggest high sensitivity (94%) and specificity (100%) but it is not clear how this was ascertained. It remains to be seen whether a single biomarker of this type will be sufficiently sensitive to detect exposed and non-exposed individuals at various disease stages, particularly in high prevalence populations. The technological approach, however, does not preclude the use of additional TB antigens; however, it will not be possible to overcome the turn around time or need for an additional visit. The patch could have a role in the diagnosis of children but is likely to have low sensitivity in immunosuppressed HIV+ patients.

Volatile Organic Approaches

The second class of known molecular entities that are under evaluation as biomarkers are volatile organic compounds that are produced in the breath of individuals with active *TB disease*, and can be detected via either gas chromatography (GC) or mass spectrometry (MS) techniques. When grown in the laboratory, *M tuberculosis* generates a very distinctive pattern of volatile organic compounds which can be detected in the air. There is some evidence that these same volatile organic compounds are also present in the breath of infected patients, consequently it should be possible to detect pulmonary TB rapidly and accurately. While there is limited literature on metabolite research for *M. tuberculosis,* there is extensive data on a non-invasive clinical diagnostic test for the diagnosis of *Helicobacter pylori* infections based on the detection of urea in the breath of infected patients. In an analogous scenario, patients actively infected with pulmonary TB may exhale detectable levels of these compound(s) in the breath, which might allow active cases to be distinguished from latent infections. Metabolites found in the breath are volatile and hence do not require any specimen processing prior to analyses. Analyses of volatile compounds are routinely performed using GC, though for increased sensitivity and specificity, GC is coupled with MS.

GC-MS has been employed for over 20 years to diagnose disease and in recent years utilized for volatile metabolite discovery and diagnosis.

There are several companies that are pursuing this line of research. Michael Phillips of Menssana Research indicated that they have completed a Phase I pilot study with NIH support,[39,40] and that the results are to be published in the journal Tuberculosis. For culture positive individuals, the sensitivity was estimated at 94.1%, and the specificity at 95.1%. The NIH has funded a Phase II multicenter international validation study in the USA, Mexico, Philippines, and UK, which is now in progress. They are still in the product development phase, which requires the breath specimen to be collected on to a sorbent trap at the point of care; and then shipped to a laboratory for analysis. The next phase of product development will employ their new remote diagnosis system, in which the breath specimen is collected and analyzed at the point of care. Menssana does not yet have an estimate of the projected cost per assay because they are still in the development phase.

KIT (Royal Tropical Institute, Netherlands) and Cranfield University in the UK are working together in the development and optimization of a portable prototype gas/volatile sensor array and associated computational analysis, for the rapid detection, identification, and analysis of *M. tuberculosis* in culture, sputum, and ultimately breath specimens.[41,42] Their aim is to develop a simple breath analysis device capable of distinguishing tuberculosis from other lung diseases. Their work has been supported by FIND and the Bright Ideas Fund of the World Health Organization (WHO). Rapid Biosensor System Ltd., has also been funded by WHO for a project "Aerosol Immunosensor for TB Screening.[37] Other academic laboratories working in this area include David Walt's laboratory at Tuffs University in Boston.[43]

While it is obvious that a GC-MS base diagnostic would not be practical in resource limited environments, developing technologies utilizing carbon nanotubes may provide a field-deployable and sensitive volatile detection diagnostic tool. Work at Nanomix (Emeryville, California) has shown that nanotubes can be derivatized with a polymer to allow the attachment of capture probes on the surface the nanotubes. The binding of a target to the capture probe changes the electrical properties of the nanotube device allowing for sensitive label free detection. Currently, Nanomix has produced an ammonia (NH_3) detecting nanotube that can detect environmental ammonia in poultry houses. While challenges still exist for reliable and reproducible manufacture of nanotubes, if the manufacturing issues can be resolved, nanotubes may provide a means for the simple instrumentation for the detection of TB-specific volatile organic compounds (Leah Fine personal communication).

Another company that is developing technology for measuring volatile organics is Aperon (www.aperon.com). Aperon uses a proprietary technology called "Sol-Gel", which is a glass-based matrix with a three dimensional structure that allows analytes such as volatile organics or proteins to be incorporated, while retaining the specificity and reactivity of the analyte molecules. They have evaluated multiple specimen types including breath, blood, and urine. They are currently developing a sensor to detect nitric oxide in the breath of asthmatics as a measure of inflammation. The current instrument relies on detecting an optical signature (i.e., molecules that generate, emit, or transmit are detectable in such a system). In addition to exquisite sensitivity in the parts per billion (ppb) range, Sol-Gels potentially enable the development of low-cost, highly sensitive, miniaturized biosensors. In theory, multiple

analytes could be detected on a sensor array of Sol-Gels of different dimensions. Depending on the concentration of the analyte(s), detection is complete in less than one minute of exposure to the matrix. They are currently in clinical trials for their first generation product for asthma, and do not currently have plans to develop biomarkers or a diagnostic device for TB (Bhairavi Parikh, personal communication)

If the method is successful, the analysis of volatile organics could deliver clinically validated biomarkers in the three to five year timeframe. Because the pattern of volatile organics is not likely to be affected by HIV status, this approach to the diagnosis of active TB cases looks promising. There are a number of key hurdles which need to be overcome for implementation of this approach including, confirmation that adequate sensitivity can be achieved with a field deployable device, determining that specificity can be achieved given a potential background of multiple other disease states and,, the development of a field-usable instrument that is practical for resource limited sites.

6B. Evaluation of Known Molecules as Biomarkers for TB Drug Susceptibility

Biomerieux and Avesthagen (www.avesthagen.com, Bangalore) are working with Affymetrix microarrays to define a set of mycobacterial genes that can be used to identify species, detect virulence genes, and detect drug resistance polymorphisms. Biomerieux apparently plans to launch a first generation of their microarrays which will be sold in North America and Europe within the next 12 months. The second generation microarray under development by Avestagen will assay over a thousand genes amplified by NASBA, using sputum specimens directly (currently have a four step extraction procedure). They estimate that the final product will assay approximately 200 genes and cost around $100. (Personal communication from Dr. Rajyashri and Daraius Morawala at Avesthagen)

It should be noted that a percentage of all drug resistant isolates do not have an identified mutation. For example from 152 INH-resistant isolates examined at UCSF, ~10% had no mutation in katG or in the promoter of inhA and ahpC. (Midori Kato-Maeda personal communication, paper submitted)

These approaches could to deliver clinically validated biomarkers for drug susceptibility in the one to three year timeframe. Despite the inability to identify a mutation in a portion of samples, deployment of a test to identify MDR TB at resource limited sites will probably require the development of a simplified NAT format. Cepheid Corporation is actively pursuing such a device.

7. Approaches for the Discovery of Novel Biomarkers for TB

In addition to improvements that could be made to existing diagnostic test methods through technology improvements or the implementation of known biomarkers into these tests, there are also opportunities for the discovery of new biomarkers that might be better suited for deployment in resource limited settings.

7A. New Biomarker Discovery for Active Case Detection

Bioinformatics Approaches. It is clear from a review of the literature that a systematic query of the sequence databases using bioinformatics tools has not taken place. Without too much effort it should be possible to take all *M. tuberculosis*-specific sequences and screen for potentially immunogenic motifs that are not deleted in known clinical stains. This could form the basis of a systematic biomarker discovery

program for host antibodies that are diagnostic for active TB (personal communication Dr. Michel Klein). This would be a low risk project that could be completed in less than three years. This same approach could be taken to increase the pool of available TB antigens for direct detection of MTB. Using bioinformatics approaches, the sequence databases could be mined for all the secreted proteins, monoclonal antibodies could be developed for the novel proteins, and these antibodies could be systematically screened for their sensitivity and specificity. . This is another low risk project that could be completed in less than three years. It is unknown at this time how predictive the existing software tools are for *M. tuberculosis*.

The Broad Institute in Massachusetts is currently sequencing approximately thirty strains of MTb. The results will be made publicly available in 2007. This will greatly increase the effort and success in the design of probes and the understanding the biology of the organism.

Proteomics Approaches. FIND is funding Proteome Systems (Australia) to develop "novel biomarkers" for a rapid antigen-based diagnostic test to be deployed in a point of care device.[49] In a recent press release Proteome Systems indicated that they have detected TB proteins in human sputum and blood, including those from HIV co-infected individuals. They plan to develop a number antibodies to determine the optimal panel of antibodies to implement.[50]

Milagen, Inc. is a developer of immunodiagnostic products that claims to have generated a complete set of antibodies to TB proteins using a "DNA vaccine" approach. (http://beta.milagen.com/home, Moncef Jendoubi personal communication) If true, the reagents would be very useful in the development of new diagnostic reagents.

The following three approaches are considered higher risk than the preceding approaches.

Mycobacterial Transcript Profiling. There is a real need for the development of quantitative approaches to understand the basic biology of the mechanisms that enable mycobacterial persistence and replication within the human host. Gene expression profiling approaches may provide useful insights into which genes are important for persistence and replication. This approach is being taken by a consortium of researchers, lead by the Stephan Kaufman of the Max Planck Institute in Berlin. The group is analyzing a comprehensive set of gene expression profiles to examine those molecular and immune responses that correlate with protection from TB disease. Rachman et al., recently published a genome-wide expression analysis of *Mycobacterium tuberculosis* from clinical lung specimens.[44] For pulmonary *M. tuberculosis,* they identified transcribed genes involved in active fortification and evasion from host defense systems. This work should provide insight into mycobacterial pathogenesis and host responses, which may in turn lead to identification of host response biomarkers. Examining gene expression at the mycobacterial level *in vitro* may give insight into the dynamics of transcription regulation during the different stages of disease.[45] Given the complexity of this disease, the limit to access of clinically relevant lung specimens and the lack of applicability of animal models, there are significant challenges to identifying appropriate diagnostic markers. The relevance of *in vitro* studies to *in vivo* is a major question.

Host Response Transcript Profiling. A number of groups have conducted comprehensive transcript profiling of the host response to *M .tuberculosis* exposure. Analysis of the expression data shows induction of cytokines and chemokines, ribosomal proteins, and the interferon-response gene STAT1. Some of these changes, observed using microarray systems, have been validated by quantitative reverse transcription polymerase chain reaction experiments. Whether any of these markers are unique enough to TB disease and could be converted to immunological reagents remains to be determined.[46] Jenner and Young recently completed an extensive review of host responses against pathogens using transcriptional profiling in a series of 32 studies that involved 77 different host–pathogen interactions.[47] They include (by way of reference) some of the work by Gary Schoolnik's group at Stanford on TB infected macrophages. The review reaches many interesting conclusions at the broadest possible level, and concludes that host cells respond with broadly common transcriptional programs, components of which are preferentially up-regulated according to the host cell type and the pathogen involved. Pathogens can interfere with these responses, which can enhance their virulence in the host. Other academic laboratories looking at *in vivo* gene expression of *M .tuberculosis* include John McKinney's group at Rockefeller. His group is examining mouse and human tissues via quantitative real-time RT-PCR with fluorescent probes ("molecular beacons"). Not too surprisingly, the mouse is not a very good model system for human disease, however the McKinney group is now focused on the comparative analysis of *M. tuberculosis* gene expression in the lungs of humans with latent infection versus active disease looking for gene expression profiles consistent with protection and pathogenesis.[48] Systematic transcript profiling of lymphocytes and blood from actively infected individuals is likely to provide insights into host responses and latency biomarkers within a two to five year timeframe.

Major Histocompatibility Arrays. Peptide microarrays containing major histocompatibility (MHC) proteins are used to measure the host response to antigen presentation. In brief, MHC molecules (both Class I and II) are paired with peptides of the antigen(s) of interest and immobilized to a solid support with cytokine capture antibodies. The peptide microarray is then used to detect and characterize CD4+ and CD8+ T cell host response to disease biomarker(s) paired to MHC molecules. Cytokines released by T cells are identified in traditional sandwich assay. Further details are reported elsewhere.

MHC peptide arrays have been successfully used to detect and characterize HIV, Vaccinia and Influenza specific T cell clones. The technology has also been used to correlate long-term survival to cytokine response patterns in a melanoma cancer vaccine trial and to monitor changes in recognition of epitope binding sites during a malaria vaccine trial. Most recently, MHC peptide arrays have been used to detect low frequency autoimmune CD4+ T cells in a type 1 diabetic patient. MHC peptide arrays have the potential to detect host-derived biomarkers upon exposure to *Mycobacterium tuberculosis* and to distinguish the concomitant host immune responses to active versus latent TB infections, HIV+/-, and PPD+ but TB negative. (Dr Lawrence Stern, Professor of Pathology, University of Massachusetts Medical School, Dr. William Kwok, Benaroya Research Institute, Dr. Mark Davis, Stanford University and a new company called Immunocyte (Hopkinton, MA). The advantages include increased sensitivity over current flow cytometry assays, the ability to recover T-cell clones directly from the array, the benefit of small sample sizes and the capability to do multi-parameter analysis. This approach is could deliver biomarkers in 5 or more years.

7B. New Biomarker Discovery for Progression to Disease:

No good markers of TB progression in humans presently exist. Factors related to the progression from infection to disease or latent infection to active disease include 1) the intensity of the initial exposure, 2) recent rather than remote infection, and 3) additional medical conditions such as HIV infection that weaken immunity. A better understanding of the immunologic and genetic factors associated with the human response to TB exposure is needed to predict which persons who are exposed to TB and which with latent infection are at highest risk of progressing to active disease. Numerous investigational approaches are currently being used that may identify biomarkers related to progression.

Mathematical Modeling Combined with Hypothesis Testing. In mouse models of TB, if the number of colony forming units (CFUs) in the whole lung exceed 10^8, rapid progression to death is nearly certain. Whether an equivalent threshold exists for humans is unclear. Through the development of a series of cellular mathematical models, Marino and colleagues[51, 52] have consistently found that levels of extracellular bacteria (bacterial load) are the most informative marker of disease progression. In all of the models, except one, they conclude that, if bacterial levels can be contained intracellularly (within low levels of infected macrophages), then infection can be controlled. In the case of latent infection their models predict that that all of the bacteria are harbored within a few infected macrophages within the granuloma. In should be possible to either construct a prospective study or look at data on bacterial load and outcomes. These approaches could deliver biomarkers in the 3-5 year time horizon.

Clinical Algorithms: In the case of HIV positive, TB infected individual there may be the potential to put together a clinical algorithm combining multiple types of biomarkers which may be informative for disease progression. In a Zambian study researchers indicated that a chest radiographic pattern can both predict CD4 count and survival in HIV-infected patients[53]. They go on to suggest that typical upper-lobe cavitatory disease is associated with a preserved CD4 count and excellent outcome, whereas patients with atypical patterns had low CD4 counts and 50% mortality at 1 year

Mutation Analysis of Virulence Genes. Approximately 200 genes and the proteins they encode are necessary for *M. tuberculosis* virulence.[54] Considerable work is being carried out to characterize these genes in clinical isolates of *M. tuberculosis* to understand at the protein level how these differences result in changes in the ability to cause disease.[55] A new National Institutes of Health (NIH) initiative to fund a comprehensive mutational analysis of the *M. tuberculosis* genome is now underway (NO1 30036, awarded to W. Bishai, Jr.).

Candidate Genes. It would be very useful to identify new biomarkers with higher discriminating power for predicting progression to active TB. Studies conducted by Singh et al., (Table 9) have shown that antibodies to MTb malate synthase, MPT51 and PPE-C are detectable ~6 months prior to development of clinical TB in HIV+ patients who eventually became TB+. The presence of antibodies is unaffected by the CD4 status of the patients. These antibodies are absent from sera of HIV+ patients who are at low risk of TB. Thus, emergence of antibodies to these antigens could serve as biomarkers of incipient infection with MTb and identify HIV+ subjects who are progressing to clinical TB. Other candidates include limited evidence of an association between IFN-γ response to ESAT-6 and subsequent progression to active TB.[56] There is some indication that cellular immune responses (e.g., cytokine assays

– TNF-*a*) may act as indicators of incipient disease in immunocompromised patients. Combinations of biomarkers may be needed to predict progression. The recent paper by Ordway et al suggests that elevated interleukin-4 (IL-4δ2) is associated with clinical illness, or progression toward disease, and may be one avenue to explore.[57]

Host Genetics Analysis. In a less hypothesis driven analysis, specific human population cohorts are being analyzed to elucidate the reasons for their high susceptibility to TB.[58, 59] As part of the 2005 Grand Challenge to look at molecular and immune responses that correlate with protection from tuberculosis, a consortium of researchers is conducting a large-scale SNP association study, with the hopes of identifying genetic factors that alter susceptibility to pulmonary and meningeal *M. tuberculosis* disease (Mark Seielstad, Singapore). In the long term, this approach will provide insight into the individual variation in host responses to exposure and may lead to the identification of biomarkers that might predict susceptibility to disease by *M. tuberculosis*. Lalita Ramakrishnan at the University of Washington is using zebra fish to explore the basis of the differential susceptibility to MTb among different individuals. Using the optically transparent larvae it is possible to monitor real time host-pathogen interactions. In addition, specific host genes (candidate genes) involved in immune expression can be modified to identify their role in infection as well as perform genetic screens to identify new host susceptibility genes. Thus, systematic approaches are now being applied to population genetic analyses of both the pathogen and the host. These studies are expected to pay dividends in the longer term.

Recently, researchers have identified three variations of a human gene, SP110, which are associated with tuberculosis susceptibility in West Africa[60]. The variations were identified in samples obtained in The Gambia, and two of these variations also were found in samples obtained from the Republic of Guinea and Guinea-Bissau. The function of SP110 in disease progression is unknown, but it is thought to control the death of infected cells.

7C. New Biomarker Discovery for TB Drug Susceptibility

Genetic Mapping, Sequencing, and Phenotype Correlations. As mentioned above, the resistance mechanisms and responsible genes are known for many of the front-line drugs and for some of the frequently used second line drugs as well. There are approximately 10 known genes (rpoB, KatG, inhA, ahpC, kasA, ethA, EmbB, pncA, gyrA, rrs) that account for a significant proportion of the resistance to 10 of the most commonly used anti-mycobacterial agents (for review see reference [7]). The only rational approach to identifying new biomarkers for drug susceptibility is cloning and phenotypic screening, to identify the genes conferring the resistance, and subsequent sequencing of many clinical isolates to determine the frequency of the mutation. The final key will be to identify a simple, self-contained, and rapid technology platform, perhaps like Cepheid's GeneXpert system, that can be implemented at resource-limited sites. As noted above, a significant percentage (10-15%) of drug resistant clinical isolates do not have an identified mutation. As a consequence of these observations, it will be necessary to continue the evolution of simplified phenotyping methods such as phage infection for deployment at regional centers.

8. Clinical Sample and Study Design Issues for Biomarker Discovery and Validation

One of the main impediments to discovering and evaluating biomarkers that can be used in developing countries is access to well characterized clinical samples with clinical history, smear status, culture status, geographically matched controls, and outcomes data. To this end, WHO/TDR/FIND has funded the development of a TB specimen bank. They have obtained serum, urine, saliva and sputum from patients (HIV+/-) and controls. The projected number of available aliquots is ~26,500. The samples represent a valuable resource of geographically distributed, well characterized samples. A number of other academic institutions have rich sample collections including John Hopkins, Case Western Reserve and New York University Medical Center. There are several well organized groups in Africa and Asia which collaborate extensively to enable multi-center trials to be conducted.

For the ideal specimen bank, samples that have smear and culture status, clinical history, and matched controls are highly desirable. All samples must be quality controlled to the extent that a total reactivity measure should be available, as storage conditions can significantly affect the quality of the samples. A portion of the samples should be from the same individuals, taken over time to monitor disease progression and treatment efficacy markers. Few specimen collections have focused on children, especially TB meningitis in pediatric cases.

In the event that new sample collection, shipment, and processing are to be investigated, it is necessary to freeze samples away for future work. This will be very difficult in some locations. For nucleic acids, sample storage materials such as Whatman cards can be considered. It is also necessary to consider that samples might be used for both the discovery of new markers and for validation of the clinical utility; and these two applications have different requirements. As a result, larger volume samples should be collected and frozen for discovery research, while smaller sample volumes would be used for clinical validation and can be stored in different ways, depending upon the intent. Although sputum samples are often processed using the NALC procedure, it should be remembered that this process will eliminate most everything but *M. tuberculosis* itself. This might be acceptable if *M. tuberculosis* detection is the only planned use for a sample, but the use of the sample to detect any other marker (such as HIV) would be compromised. It could be necessary to set up different collection and storage protocols for different testing methods.

For the purpose of looking at host factors, serum or saliva could be useful for protein markers; however, intracellular host mRNA is a useful material in the study of immune disorders. There are protocols for collection and processing whole blood to preserve RNA. At this time however, it does not appear that these methods are being employed in sample collections, possibly due to the logistics, the costs, or both.

In order to investigate the use of volatile organics as biomarkers, samples from individuals in developing countries need to be collected. There has been recent progress in this area. In the case of breath samples it is possible to use a glass collection device and have the subject breath (exhale) ten times or so into the device. The glass device is placed in a chilled (approximately -70°C) steel sleeve during the exhaling. The subject's breath condenses onto the surface of the glass. The device is removed from the steel sleeve and allowed to warm. The frozen breath condensate thaws and then runs to the bottom of the glass device

where it is collected. The devices to date are primitive (mostly home made), but appear to perform adequately.[61]

Ideal Sample Bank:

- **Access to clinically validated samples with appropriate QC**
  - **Adequate geographical representation (host and pathogen variability)**
  - **Disease cases with different clinical isolates (mycobacterial variability)**
  - **Adequate representation from high prevalence area and multiply infected individuals (HIV+, other respiratory pathogens, etc.)**
  - **Individuals infected with different but related mycobacteria (endemic in the environment)**
  - **Pediatric cases; both HIV+/-**
  - **Extra-pulmonary TB cases**
  - **Drug resistant cases**
  - **Pedigreed endemic controls (people who live in the same area, preferably age and sex matched, but are not infected)**
- **Adequate sample quantities for analysis**
  - **For discovery, a relatively small number (100s) of high volume (as large as practical) samples will be needed**
  - **For validation, a large number (1000s) of medium volume samples will be needed**
- **Multiple sample types**
  - **Sputum**
  - **Serum or plasma**
  - **Urine**
  - **Saliva**
  - **Breath condensates**
  - **Blood cells (PBLs)**
  - **Nasopharyngeal swabs**
- **Time course samples**
  - **Natural disease course**
  - **Before and during intervention; successful, unsuccessful and switched therapy**
  - **Evolving drug resistance cases**
  - **Normal population during same period**
  - **Other respiratory diseases with and without TB during the same period**

The requirements for samples and study design are slightly different for MTb infection versus drug resistance. The CDC and TDR repository stores much of the reference material that has been collected, as do the groups at UCSF and the National Public Health Institutes in the Netherlands. Such studies require well-characterized isolates whose mechanism of drug resistance has been verified by sequencing and quality controlled relative to phenotypes (MICs). For the assessment of known mutations leading to resistance, isolated DNA, blotted Whatman cards, or NALC precipitates will suffice. It would also be useful to collect MIC-demonstrated resistant strains and conduct a surveillance of mutations over time.

The information will be useful in determining if SNP or sequencing approaches will ultimately be necessary in field tests.

An interesting model to look at is the recently proposed "National Tuberculosis Archive" in the United States.[62] While collections of genotypic, epidemiological and clinical data are available in databases, they are not integrated. There is evidence from some locations (e.g., San Francisco and New York) that understanding the molecular characteristic of an outbreak and its phylogenetic signature can enhance public health control measures. A significant effort to permit universal access to all pertinent data is a significant need and a recommended activity.

9. Recommendations for the Improvement of TB diagnostics

The following recommendations are presented for consideration, given the deficiencies of current diagnostic test methods for deployment in resource limited settings, the opportunities for improving the deployment of existing biomarkers, and the approaches that might be used for discovering novel biomarkers that are more appropriate for use in resource-limited settings.

The most promising paths forward for the development of diagnostic tests for resource limited settings are summarized in Figures 3 and 4 (next page). In these Figures, the current approaches are shown as solid spheres (consistent with Figures 1 and 2) and the potential future approaches are shown as open spheres. The position of each sphere on the graph illustrates the resource requirements (x-axis) of the test method and the clinical utility of the test in a resource limited setting (y-axis). In these figures, therefore, an ideal biomarker and test method will be in the upper right hand quadrant, with a high predictive power and low resource requirements. Figure 3 shows the most promising opportunities for diagnosing active TB cases, while Figure 4 depicts opportunities for TB drug susceptibility testing.

Figure 3. Future Approaches for Diagnosing Active Cases of TB and Their Utility in Resource Limited Settings.

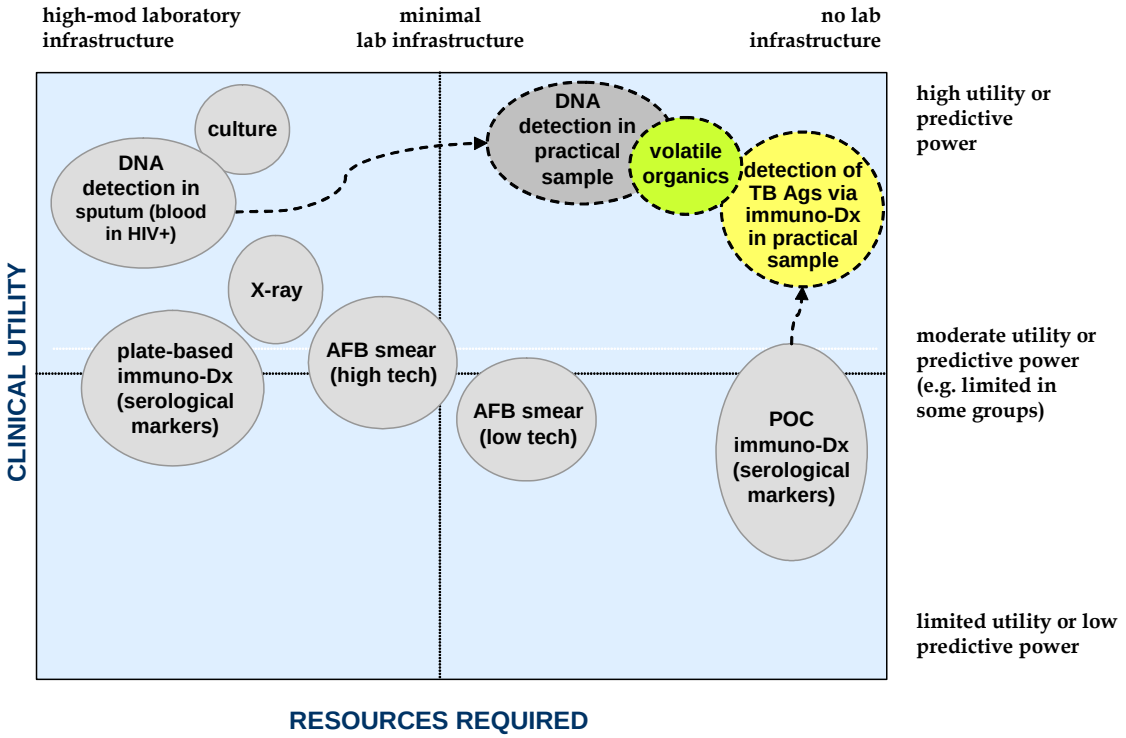
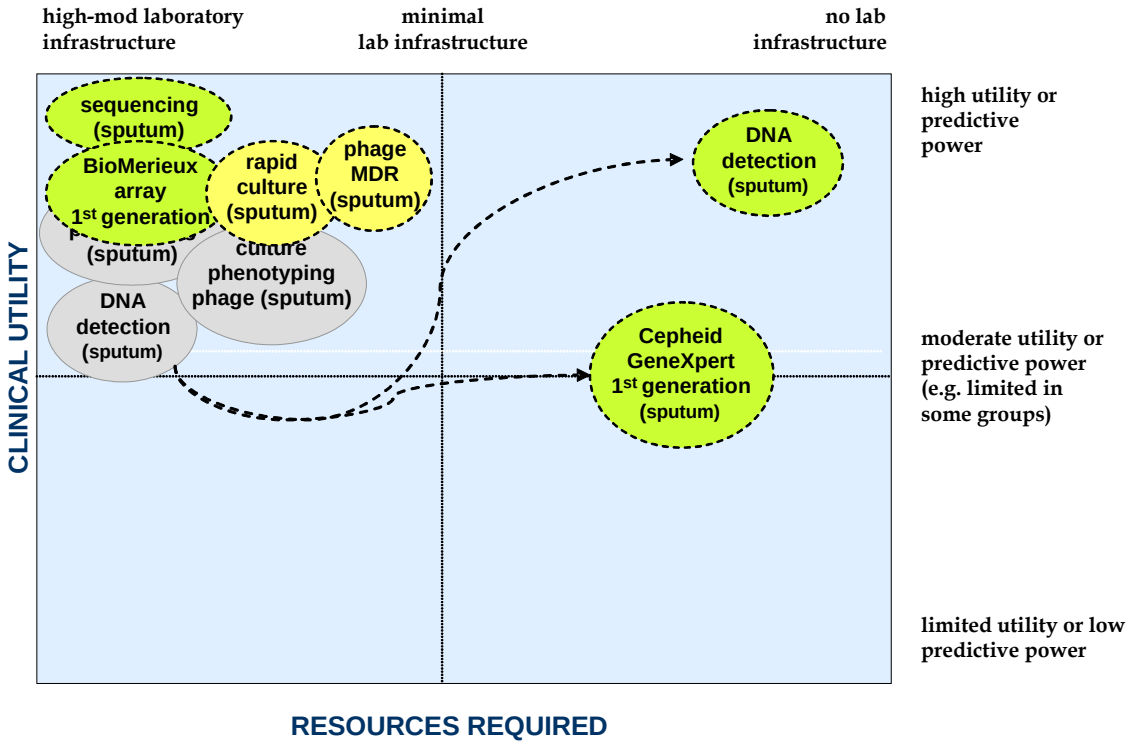


Figure 4. Future Approaches for Diagnosing Drug Susceptibility of TB and Their Utility in Resource Limited Settings.



For the resource-limited settings, it is difficult to see how a set of tests based upon existing technical approaches will serve the needs of a remote testing site. Although nucleic acid tests hold significant promise, they suffer from one main problem. They do not meet the "Easy, Rapid and Simple to interpret (ERS)" criteria. Nevertheless, nucleic acid testing (NAT) approaches are probably worth pursuing as a near term diagnostic approach for TB. Several companies are attempting to solve the ERS problem (Cepheid, Investigen, Handylab, and others; shown in combination as "Simplified NATs" in Figure 3).

There are also opportunities for the systematic evaluation of multi-analyte serology assays that use a POC format (Figure 3). Interestingly, in the last 12 months, several companies have reported promising sensitivities in immunodiagnostic assays that detect *M. tuberculosis* antigens, and this may be a very productive path forward for POC immunodiagnostics. Ultimately a combination approach may give the best performance.

As discussed previously, there are a number of new biomarkers under evaluation. It is worth considering funding a commercial clinical program to systematically investigate these putative diagnostic tools in a setting where a study group equipped with a set of clinical samples and screening tools could process many markers simultaneously and correlate them with the clinical data, both individually and in combination with other known and new markers. In this way, the necessary and sufficient biomarker sets would be identified. This is not trivial, but potentially very rewarding.

Current specimen banks are not adequate, and additional collections will be needed to bolster the existing WHO/TDR bank. In addition, we recommend support for a separate infrastructure/network, with trained personnel, which will be needed to conduct clinical validation trials that are product specific in the field at sites with access to appropriate populations. We would argue that this is roughly a 75:25 proposition with the need to have test-independent samples (with adequate coverage of sample type) for initial rounds of validation, and then switching to a field based clinical validation once the test format is established. Ideally the specimen bank should be large enough (specimens from ~2,000 individuals; at least 200 samples should be available for each intervention point with matched endemic controls) to allow two independent testing cycles to occur prior to field based testing.

It is clear that sputum as a sample type is not going away any time soon for TB diagnostics, particularly for drug susceptibility testing. Consequently, it is recommended that new approaches be brought to bear on collecting and processing sputum. The methods currently under investigation in the U.S. are costly and would be very nearly impossible to deploy in low resource settings. Panning approaches (magnetic bead pull down, etc.) which result in a more effective isolation of MTb from sputum samples may be useful.

One of the most exciting areas of development in the diagnosis of active cases of TB is the detection of volatile organics in the breath. If the clinical validation of this approach demonstrates useful sensitivity and specificity, it is strongly recommended that a field deployable technology be developed for this class of biomarkers (Figure 3).

At this time, the availability of the *M. tuberculosis* genomic sequence offers considerable opportunity for exploitation. We recommend a systematic review of the *M. tuberculosis* sequence databases using

bioinformatics tools that can identify potentially immunogenic sequences, to be followed by a commercial evaluation of panels of potential immunodiagnostic reagents that might be incorporated into diagnostic products. To enhance the FIND's efforts, we recommend a bioinformatics analysis of the genome sequence for secreted proteins. Monoclonal antibodies could then be developed for these proteins. The fundamental assessment of MTb immunogenic sequences at the genome scale is a worthwhile low risk project and will quickly reveal that 1) all the dominant antigens are known from previous work, or 2) some of the genes are predicted to function differently than previously thought and are worth studying further.

For drug susceptibility testing in resource limited environments, simpler methods are required, as illustrated by the paucity of spheres in the right hand portion of Figure 4. It is unlikely that rapid culture methods or sequencing will be practical in the near or medium term. The best bet appears to be the development of simple SNP detection methods. These may utilize a variety of approaches, including amplification using point-mutation-specific primers, or differential hybridization. Several technology platforms are already under development (see the Technology Document), and new approaches are emerging as well.

We propose the development of training programs, which cover the entire process including specimen acquisition, processing, shipment (if necessary), conducting the test, interpretation and communication of results. Establishing infrastructure to conduct proficiency testing (similar at some level to the current proficiency tests conducted by the College of American Pathologists, or CAP) will rapidly illustrate gaps in the system to provide effective diagnostics. TB diagnostics is particularly challenging due to inadequacies of, and in some cases total lack of, "gold standards" for reference. In the medium term, reference materials could be established and distributed. This would have an enormous impact.

Because of the uncertainty that any of the known biomarkers will be able to provide the required predictive power under the constraints of resource limited sites, it is recommended that the best tools available be used for new biomarker discovery (see Discovery Technology report).

These efforts will require a funded, systematic and coordinated program that assembles and synthesizes information from aggregate studies.

Summary of Recommendations:

Case detection of active TB in HIV +/- patients

- **Sensitivity/specificity: 85%/97%**
- **Time requirement <1h**
    - **Path1: Detection of (i) bacterial antigens or ii) host response)**
        - **Blood, Urine and Saliva**
        - **No laboratory resource setting**

- **Path2: Nucleic Acid**
    - **Blood and urine, nasopharyngeal swab**
    - **Minimal laboratory infrastructure**
- **Path 3: VOCs**
    - **Breath metabolites**
    - **Minimal laboratory infrastructure**

Case detection of multiple drug resistant TB

- **Sensitivity/specificity: ???%/99%**
- **Time requirement <1h**
    - **Path 4: Drug resistant TB – Nucleic acid limited subset of mutations**
        - **Sputum (or culture)**
        - **Minimal laboratory infrastructure**

The specificity and sensitivity targets used were reported in the RAND analysis. Specialists in the field mentioned many times that a first generation rapid test that can replace the sputum smear and provide the same sensitivity as the smear would be a significant improvement. This is partly due to the need for multiple visits (3 smear tests) to diagnose TB which leads to a very high patient drop-our rate (~50%). Consequently a test that can identify >96% smear positive patients and even 50% smear negative patients would have an significant impact on treatment rates.

Other Unmet Needs in TB Testing:

With improved access to therapies there is a real need for quantitative tests which can be used to monitor therapeutic efficacy at the two and three month window and that would enable the declaration of a "cured patient". Ideally the base line would be established at the time of diagnosis (Personal communication, Midori Kato-Maeda).

In addition, several, researchers have identified the need to have a test method that could enable assessment of the effectiveness of new vaccines that will be entering the market over the next few years.

With the advent of increased drug resistant TB it is increasingly necessary to transport samples to regional centers for more thorough evaluations, consequently effective sample preservation methods will need to be developed.

In many developing countries, individuals with active TB disease are co-infected with other pathogens. There is a need to gain a better understanding of the influence of microbial cohabitation in host tissues (e.g., presence of MTb with other pathogenic or normal host flora) (Ed Liu, Singapore, personal communication)

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