New tools and emerging technologies for the diagnosis of tuberculosis: Part II. Active tuberculosis and drug resistance

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Tuberculosis (TB) is one of the world’s most important infectious causes of morbidity and mortality among adults. Between 8 and 9 million develop TB disease, and approximately 2 million die from TB each year. Despite this enormous global burden, case detection rates are low, posing major hurdles for TB control. Conventional TB diagnosis continues to rely on smear microscopy, culture and chest radiography. These tests have known limitations. Conventional tests for detection of drug resistance are slow, tedious and difficult to perform in field conditions. This second half of a two-part review series on new tools for TB diagnosis describes recent advances and emerging technologies in the diagnosis of active disease, and detection of drug resistance. For diagnosis, new tools include newer versions of nucleic acid amplification tests, immune-based assays, skin patch test and rapid culture systems. For drug resistance, new tools include line-probe assays, bacteriophage-based assays, molecular beacons and microscopic observation drug susceptibility assay. Although the ideal test for TB is still not in sight, substantial progress has been made in the past decade. With the resurgence of interest in the development of new tools for TB control, it is likely that the next decade will see greater progress and tangible benefits. However, the challenge will be to ensure that new tools undergo rigorous evaluations in field conditions, and also to make sure that benefits of promising new tools actually reach the populations in developing countries that need them most. Latent TB is discussed in Part I; 413–422 of this issue.

Tuberculosis (TB) is one of the world’s most important infectious causes of morbidity and mortality among adults, infecting over 2 billion people. Between 8 and 9 million people develop TB, and approximately 2 million die from TB each year [1,2]. Despite this enormous global burden, case detection rates are low, posing major hurdles for TB control. Conventional TB diagnosis continues to rely on sputum smear microscopy, mycobacterial culture and chest radiography. These tests have several limitations [3–5]. Conventional tests for detection of drug resistance are slow, complicated, expensive and difficult to perform in field conditions [6]. The long-standing need for new, rapid, accurate and convenient tests for TB diagnosis and drug resistance is currently being addressed by several agencies and groups [101–105]. In this second half of a two-part review series on new tools for TB diagnosis, the authors describe recent advances and emerging technologies in the diagnosis of active disease, and detection of drug resistance.

Diagnostics for active tuberculosis

Nucleic acid amplification tests

Nucleic acid amplification tests (NATs), also called direct amplification tests, are designed to amplify nucleic acid regions specific to the Mycobacterium tuberculosis complex. These tests can be used directly on clinical specimens (e.g., sputum). NATs are available as commercial kits. They are also widely used as in-house assays. Commercial kits include the...
Amplified™ M. tuberculosis Direct Test® (MTD; Gen-Probe Inc., CA, USA [106]), the Amplicor® M. tuberculosis (MTB) tests (Roche Molecular Diagnostics, CA, USA [107]), and the BD ProbeTec™ ET assay (BD Biosciences, MD, USA [108]). The Amplicor and MTD tests are currently US FDA approved. In-house tests are laboratory-developed PCR assays, and they vary widely in their protocols and laboratory methods. In-house NATs tend to be used predominantly in research settings and in developing countries where commercial NATs are expensive.

The literature on NAT has been extensively reviewed [7–9]. Several recent meta-analyses have synthesized the evidence on accuracy, and the results demonstrate that NATs have high specificity for both pulmonary and extrapulmonary TB (but data on extrapulmonary TB are relatively limited) [10–14]. High specificity and positive-predictive value confer clear advantages in terms of the test's ability to rule-in TB. A positive NAT in a patient with a high pretest probability is fairly confirmatory of TB, particularly in those with positive smears. Therefore, NAT can be used to confirm that a sputum smear with acid-fast bacilli is due to M. tuberculosis [7]. However, this approach is largely unnecessary in high-burden countries where TB is treated mainly on the basis of positive sputum smears.

In contrast to specificity, sensitivity of NAT is lower and highly variable across studies. In many studies, sensitivity estimates have been lower in paucibacillary forms of TB (extrapulmonary TB and smear-negative pulmonary disease). Sensitivity of NAT has been highest in smear-positive specimens. Therefore, a negative test does not rule-out the diagnosis of TB. Therefore, NAT should not be performed if the smears are negative and if the index of clinical suspicion is low. A negative NAT in a patient with a high index of clinical suspicion should prompt continued work-up.

In general, the accuracy of in-house PCR tests has been more heterogeneous than commercial kits. Recently, Flores and colleagues published a meta-regression analysis on factors associated with heterogeneity in in-house PCR accuracy [10]. The use of IS6110 as an amplification target and the use of nested PCR were associated with higher accuracy [10]. However, the substantial heterogeneity in both sensitivity and specificity of the in-house NAT rendered clinically useful estimates of test accuracy difficult. On the other hand, commercial assays are standardized and appear to produce results that are more consistent.

In summary, available NATs have not realised their early promise. In addition to concerns about accuracy and reliability, their high costs and requirement for laboratory infrastructure reduce their applicability in resource-limited settings. Therefore, efforts are underway to simplify testing protocols and increase their accuracy. Loop-mediated isothermal amplification (LAMP) is one such development.

**Loop-mediated isothermal amplification**

LAMP is a novel, rapid and simplified NAT platform developed by Eiken Chemical Co., Ltd (Tokyo, Japan) [109]. The platform is not specific to TB and has been used to detect other organisms. Its major advantages are speed, simplicity and the lack of requirement for a thermal cycler, thus facilitating high throughput. The technology uses four different primers specifically designed to recognize six distinct regions on the target gene, and the reaction process proceeds at a constant temperature (isothermal) using strand displacement reaction (FIGURE 1) [109]. Amplification and detection of the gene products can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with
New tools for tuberculosis diagnosis

Immunological tests, including serological assays

Immune-based tests for the detection of antibodies, antigens and immune complexes have been attempted for decades, and their performance has been extensively reviewed [16–20]. These assays generally detect humoral immune response, as opposed to T-cell-based assays that detect cellular immunity (e.g., interferon-γ assays). Dozens of commercial kits are available, which are mostly focused on antibody detection. Despite the long history of serological tests and persistent attempts at improving them, no assay is currently accurate enough to replace microscopy and culture. Despite the evidence on lack of accuracy, serological tests continue to be marketed and used, mostly in developing countries.

Several authors have attempted to analyze the reasons for the apparent failure of serological tests [16,20–22]. A major challenge with immunological diagnosis of TB is that TB presents a wide spectrum ranging from exposure, through latent TB infection (LTBI) and active disease, to severe disease [20,22]. Furthermore, several other factors can affect the performance of immune-based tests: BCG vaccination, exposure to nontuberculous mycobacteria (NTM), M. tuberculosis strain and HIV co-infection. A good immunological test must distinguish between the various states of TB, and also distinguish between TB and other mycobacterial exposures. Early versions of serological assays used crude, nonspecific antigenic mixtures [20]. This led to loss of specificity, and the inability to distinguish TB from LTBI and NTM [20]. The development of well-defined, purified recombinant proteins specific to M. tuberculosis partially overcome this problem.

Antigen recognition in TB is broad and highly variable, and varies across the infection/disease spectrum [16,20–22]. M. tuberculosis expresses different genes and proteins during different stages of the disease [16,20–22]. For example, proteins expressed during latency may differ from those expressed during active disease. Even within active disease, proteins expressed in cavitary disease may differ from those expressed in noncavitary disease. Therefore, any test that detects immune responses must include antigens that are expressed during the various stages of disease progression. However, many serological assays use single antigens (e.g., 38-kDa protein) that may not be recognized by the host immune system during all stages. The development of cocktails of multiple specific antigens may partially address this problem [16,20–22].

Currently available serological assays are based on identifying markers positively associated with a particular state (e.g., active disease). This approach may be limited because antibody profiles associated with inactive TB may differ from those associated with active disease [21,22]. It has been suggested that each TB state is characterized by bacterial antigen signatures that resemble barcodes [21,22]. In other words, certain combinations of the presence and absence of antigen-specific immune responses may indicate specific disease states. Therefore, it may be necessary to study negative as well as positive responses to a panel of antigens. A pattern of positive and negative responses (analogous to a barcode) may then be used to distinguish between various stages of TB infection and disease [21,22]. Whether this approach has practical, clinical applicability has not been determined to date.

In addition to these approaches, efforts are also underway to develop immune-based assays that focus on antigen rather than antibody detection. For example, antigen-capture enzyme-linked immunosorbent assays for the detection of lipoarabinomannan in sputum [23] and urine [24] samples have demonstrated promise in early trials. Further work is necessary to determine their utility.

Overall, the lessons learnt from previous failures should facilitate the development of a new generation of immune-based tests, including ones that detect antigens and/or circulating immune complexes rather than antibodies, which will be sensitive and specific, and will be able to distinguish between latent and active TB. Such assays, if shown to be accurate, will have an impact on TB case finding, especially if they can be packaged as point-of-care strip tests that may be used in primary care settings.

MPB64 skin patch test for active tuberculosis

MPB64 is an immunogenic antigen specific to M. tuberculosis complex [25]. It is secreted by M. tuberculosis, Mycobacterium bovis, and some strains of M. bovis BCG. In 1998, Nakamura and colleagues demonstrated that a skin patch with MPB64 antigen (FIGURE 2A) was capable of eliciting a distinctive response (FIGURE 2B) in individuals with active, but not latent, TB [26]. In studies conducted in Japan and the Philippines, the MPB64 patch test was able to distinguish active TB from LTBI with 98% sensitivity and 100% specificity [26]. In an another study in the Philippines, Nakamura and coworkers demonstrated similar results, with a sensitivity of 88% and specificity of 100% for active TB [27]. However, the exact biological mechanism behind the skin response in these studies is still unclear. The skin patch test is currently being developed into a commercial test by Sequella, Inc., (MD, USA) [110]. Using recombinant rMPT64, the test is being evaluated in trials to establish the accuracy, dose ranges and other operational characteristics (e.g., ability to read results in dark-skinned individuals) [28]. Since the patch test is simple, noninvasive and does not require a laboratory or highly skilled personnel, it has the potential to make an impact, especially if it is shown to be accurate, unaffected by anergy (e.g., due to HIV) and capable of distinguishing between LTBI and active disease in high-burden countries.
**Rapid culture systems: TK Medium**

TK Medium® (Salubris, Inc., MA, USA) is a novel colorimetric system that indicates growth of mycobacteria by changing its color \[111\]. Metabolic activity of growing mycobacteria changes the color of the culture medium, and this enables an early positive identification before bacterial colonies appear. As illustrated in FIGURE 3, the test can distinguish between mycobacteria and contamination. TK Medium also enables susceptibility testing for drug resistance, and can allow for differentiation between \(M.\) \textit{tuberculosis} and NTM. Although TK Medium promises to be a practical, low-cost, simple test, published evidence on this test is limited \[29,30\]. The test is not currently FDA approved. Data from a multicentric study in Turkey demonstrated that TK Medium’s sensitivity for mycobacterial detection was comparable to that of the Lowenstein–Jensen (LJ) medium \[30\]. The average time to detection with TK Medium was 2 weeks, as compared with 4 weeks with the conventional LJ medium. Larger multicentric studies are ongoing, and they should help to define the accuracy of this new rapid culture system. In addition to accuracy, it is particularly important to study practical issues, such as contamination, quality control and training requirements, under field conditions.

**Rapid detection of drug resistance**

**Line-probe assays**

Line-probe assays are a family of novel DNA strip-based tests that use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance (FIGURE 4). Commercially available line-probe assays include the INNO-LiPA® Rif. TB kit (Innogenetics NV, Gent, Belgium) \[112\], and the GenoType® MTBDR assay (Hain Life-science GmbH, Nehren, Germany) \[113\]. These kits are not currently FDA approved for use in the USA, but are CE marked. Line-probe assays are designed to identify \(M.\) \textit{tuberculosis} complex and simultaneously detect mutations associated with drug resistance.

In a recent meta-analysis, Morgan and colleagues synthesized the evidence on line-probe assay from several studies on its accuracy for rifampicin resistance \[31\]. This meta-analysis demonstrated that line-probe assay has high sensitivity and...
specificity when culture isolates are used. The majority of studies had sensitivity of 95% or greater, and nearly all were 100% specific. However, the results are less accurate when the test is directly applied on clinical specimens (e.g., sputum). There is a paucity of data on the application of this test directly to clinical specimens. If further studies indicate that line-probe assays consistently and accurately detect rifampicin resistance when applied directly to clinical specimens, it could be a useful test in select patient populations in which drug resistance is strongly suspected. The GenoType MTBDR assay also appears to be promising, but published data are limited [32,33].

In general, line-probe assays are expensive and require sophisticated laboratory infrastructure. Their role and utility in low-income, high-burden countries will be limited unless cost and technical issues are addressed.

**Bacteriophage-based assays**

Mycobacteriophage-based tests have been evaluated for diagnosis of TB [34], as well as drug susceptibility testing [35]. The technology uses bacteriophages to infect live *M. tuberculosis* and detect the bacilli using either phage-amplification method or detection of light. In the first method, the underlying principle is amplification of phages after their infection of *M. tuberculosis*, followed by detection of progeny phages as plaques on a lawn of *Mycobacterium smegmatis* (FIGURE 5) [36]. In the second method, the principle is detection of light (using luminometry or photography) produced by luciferase reporter phages after their infection of live *M. tuberculosis* [37]. Amplification-based assays are commercially available, whereas luciferase reporter phage tests are under commercial development (see 'Bronx box®'). In general, phage assays have a turnaround time of 2–3 days, and require a laboratory infrastructure similar to that required for performing mycobacterial cultures.
Phage amplification assays are available as commercial kits. For diagnosis, the FASTPlaque-TB™ (Biotec Laboratories Ltd, Ipswich, UK) assay can be directly used on sputum specimens [114]. A variant, the FASTPlaque-TB-MDRi™ kit is designed to detect rifampicin resistance in culture isolates. A new version of this kit, FASTPlaque-TB-Response™, has been developed for direct use on sputum specimens [114]. Drug resistance is diagnosed when *M. tuberculosis* is detected in samples that contain the drug (e.g., rifampicin). The FAST-Plaque-TB kits are not currently FDA approved, but are CE marked for use in Europe.

The accuracy of phage assays (amplification as well as luciferase phage assays) for detection of rifampicin resistance has been synthesized in a recent meta-analysis [35]. The results demonstrated that, when performed on culture isolates, phage assays have relatively high accuracy. A total of 11 out of 19 (58%) studies included in the review reported sensitivity and specificity estimates of at least 95%. Specificity estimates were slightly lower and more variable than sensitivity; five out of 19 (26%) studies reported specificity under 90%. Only two studies performed phage assays directly on sputum specimens, with inconsistent results. Therefore, current evidence is mostly restricted to the use of phage assays for the detection of rifampicin resistance in culture isolates. When applied to isolates, these assays have relatively high sensitivity and specificity. However, the requirement for primary culture isolation somewhat reduces the rapidity of this assay. When applied directly to clinical specimens, phage assays appear to have lower sensitivity, presumably due to the lower bacillary load [34].

If phage-based assays can be directly used on clinical specimens, and if they are shown to be accurate, they have the potential to improve the diagnosis of drug-resistant TB [35]. Efforts are ongoing to improve their performance characteristics for the direct detection of rifampicin resistance in sputum specimens [38]. Preliminary results on the improved, direct phage assay demonstrate promise, but require further validation and field testing in high-burden settings [39].

**Bronx box®**

A luciferase reporter phage-based test called the Bronx box is currently being developed as a commercial kit by Sequella, Inc. [110]. In contrast to the research version of the Bronx box that uses a photographic plate to detect light produced by luciferase reporter phages, the commercial version is a semiautomated, digital version (FIGURE 6) [110]. Published data on the research version of the Bronx box are limited, but show promise [40,41]. No data exist on the accuracy of the commercial version, but field studies are underway.

**Molecular beacon assays**

Molecular beacons are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure (FIGURE 7) [115]. The loop contains a probe sequence that is complementary to a target sequence of interest (e.g., mutations-containing sequence), and the stem is formed by the annealing of complementary arm sequences that are located on either side of the probe sequence. A fluorophore moiety is linked to the end of one arm, and a nonfluorescent quencher moiety is linked to the end of the other arm. As illustrated in FIGURE 7, when the target sequence is absent, the probe cannot fluoresce, because the stem places the fluorophore very close to the quencher. When the target sequence is present, the probe and the target hybridize, and the beacon undergoes a spontaneous conformational change that forces the fluorophore and the quencher to dissociate and move away from each other, causing fluorescence that can be detected in a real-time PCR assay [115]. Beacons are highly specific, and can discriminate target sequences that differ from one another by a single nucleotide substitution. They can also be used for diseases other than TB [115].
Recently, novel tests that use beacons for the rapid detection of mutations associated with drug resistance have been evaluated [42–46]. These studies have shown that these tests have high sensitivity (89–98%) and specificity (99–100%) for the detection of mutations associated with rifampicin resistance [42–45]. Sensitivity for the detection of isoniazid resistance appears to be relatively lower [43,44]. Although the test has been used in public health settings [46], beacon assays are not currently FDA approved for clinical use, and they are not commercially available. Simplification of the technology and commercial development is needed to enhance the applicability of this novel tool. Currently, technological requirements and cost of the beacon assays limit their applicability in developing countries.

**Microscopic observation drug-susceptibility assay**

Microscopic observation drug-susceptibility assay (MODS) is a novel assay that uses an inverted light microscope and Middlebrook 7H9 broth culture to rapidly detect mycobacterial growth [47,48]. MODS uses simple light microscopy to detect early growth of *M. tuberculosis* as strings and tangles (FIGURE 8) of bacterial cells in the broth medium with or without antimicrobial drugs (for drug susceptibility testing) [47,48]. In a recent, large study from Peru, MODS detected 94% of 1908 positive sputum cultures, whereas the conventional LJ culture detected only 87% [48]. MODS also had a shorter time to culture positivity (average of 8 days) compared with LJ culture. The results obtained by direct susceptibility testing for isoniazid and rifampin using MODS were available at the same time as TB detection, and had high agreement with the results obtained by indirect colorimetric methods.

Overall, MODS appears to be a promising, novel, inexpensive tool that can rapidly detect TB and drug resistance directly from sputum specimens. Multicentric studies are ongoing to evaluate this assay, which may have applicability in high-burden, resource-limited settings.

**Expert commentary & five-year view**

Despite the enormous global burden of TB, case detection rates continue to be low, jeopardizing global TB control efforts, particularly in areas with high HIV prevalence. Presently, TB diagnosis relies on old tools with known limitations. The long-felt need for new tools is currently being addressed by several global health agencies, nonprofit groups, industry, funding agencies, public–private partnerships and academic institutions [101–105].

For diagnosis of active TB, several new tools are in the pipeline, including newer immune-based assays, simplified NAT, skin patch test and rapid, simplified culture systems. Some of these tests may become commercially available within the next 5–10 years. While existing serological assays have not been shown to be highly accurate, there is a definite need to continue development and refinement of newer versions. Similarly, the known limitations of existing NAT assays should be overcome with newer, simplified versions.

With respect to detection of drug resistance, several accurate molecular tests (e.g., line-probe assay, molecular beacon and NAT) are already available. Efforts are needed to reduce their cost and simplify them for resource-limited settings. Novel, relatively simple phenotypic tests (e.g., MODS, TK Medium, FASTPlaque-TB-Response) are also under development, and it is important to evaluate their performance against molecular and conventional tests. These simpler phenotypic tests may have a greater impact in resource-limited settings than molecular tools.

One concern with the development of new tools is that they may involve expensive technologies beyond the reach of developing countries affected by the dual TB/HIV epidemics. High-tech tools are unlikely to make an impact in these countries, even if they are accurate. By contrast, simpler, developing world-friendly formats, such as skin tests (e.g., skin patch test), rapid, point-of-care immunodiagnosics (e.g., lateral flow assays), innovative use of microscopy (e.g., MODS) and rapid, simplified cultures (e.g., TK Medium), might have greater applicability.

Another concern with new tool development is that these tests often perform well in early controlled trials, but fail in routine practice [49]. Lack of rigor in TB diagnostic studies is an important concern that must be addressed [49]. All of the emerging technologies should initially undergo rigorous clinical trials, and, if found to be accurate, undergo demonstration projects in the field, preferably in settings with high TB/HIV incidence, before their introduction into practice. Promising tests should also undergo review by regulatory agencies prior to marketing [49].

In conclusion, although the ideal tests for TB diagnosis and drug resistance are still not in sight, substantial progress has been made in the past decade. With the resurgence of interest
in the development of new tools for TB, the next decade should bring tangible benefits [50]. However, the challenge will be to ensure that new tools undergo rigorous evaluations in field conditions, and also to make sure that benefits of promising new tools reach the populations that need it most, but can least afford them. Agencies such as such the Stop TB Partnership, Foundation for Innovative New Diagnostics, TB Diagnostics Initiative of the Special Programme for Research and Training in Tropical Diseases, WHO, are well placed to address these challenges [51]. Funding and international support for the new Global Plan to Stop TB, 2006–2015 will also enhance the development and implementation of new tools for TB control [116].

Conflict of interest statement
None declared.

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Papers of special note have been highlighted as:
• of interest
  •• of considerable interest
• Good overview on the need for new tools for tuberculosis (TB) diagnosis.
• Comprehensive recent review on all aspects of TB diagnosis.
• Meta-analysis on in-house PCR for pulmonary TB.
• Meta-analysis on nucleic acid amplification test (NATs) for tuberculous pleuritis.
• Meta-analysis on NAT for TB meningitis.
Overview of commercially available NAT.


• Field evaluation of the microscopic observation drug-susceptibility assay test for drug resistance.


Websites

101 Stop TB Working Group on New Diagnostics www.stoptb.org/wg/new_diagnostics

102 Foundation for Innovative New Diagnostics www.finnovations.org

103 Tuberculosis Diagnostics Initiative, Special Programme for Research and Training in Tropical Diseases, WHO www.who.int/tdr/diseases/tb/tbdi.htm

104 Aeras Global TB Vaccine Foundation www.aeras.org

105 Global Alliance for Tuberculosis Drug Development www.tb alliance.org

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