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Nucleic acid testing for tuberculosis at the point-of-care in high-burden countries

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Abstract

Early diagnosis of tuberculosis (TB) facilitates appropriate treatment initiation and can limit the spread of this highly contagious disease. However, commonly used TB diagnostic methods are slow, often insensitive, cumbersome and inaccessible to most patients in TB endemic countries that lack necessary resources. This review discusses nucleic acid amplification technologies, which are being developed for rapid near patient TB diagnosis, that are in the market or undergoing clinical evaluation. They are based on PCR or isothermal methods and are implemented as manual assays or partially/fully integrated instrument systems, with associated tradeoffs between clinical performance, cost, robustness, quality assurance and usability in remote settings by minimally trained personnel. Unmet needs prevail for the identification of drug-resistant TB and for TB diagnosis in HIV-positive and pediatric patients.

Keywords

CPA; diagnosis; integrated system; LAMP; *Mycobacterium tuberculosis*; near patient; nucleic acid amplification; pulmonary; sample preparation

Tuberculosis overview

Tuberculosis (TB) [1] is the leading cause of death from a curable infectious disease, with an estimated 12 million prevalent and 8.8 million incident cases in 2010, resulting in 1.45 million deaths [101]. *Mycobacterium tuberculosis* (*M.tb*), the causative agent, is readily transmitted through aerosols. Approximately one-third of the world's population is infected with latent TB [2], which can reactivate if a patient's immune system is weakened, most commonly as pulmonary TB [1]. Treating TB is lengthy due to the pathogen's persistence and propensity for drug resistance [3]. The currently recommended treatment of drug-susceptible TB involves 6 months of combination therapy with first-line oral anti-TB drugs,

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with direct adherence monitoring to increase efficacy and limit drug resistance [1]. Multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB are emerging global health threats [4], compounded by rapidly spreading, highly virulent TB strains [5]. MDR-TB, defined as resistance to rifampicin and isoniazid, two common first-line anti-TB drugs, accounted for approximately 5% of TB cases in 2010, but has reached alarming proportions in certain regions of eastern Europe and Asia [101]. XDR-TB is defined as MDR-TB with additional resistance to a fluoroquinolone and one of the second-line injectable drugs. Recently, totally drug-resistant strains of TB have emerged, which are resistant to all drugs currently used for treatment [6]. Treating drug-resistant TB is extremely difficult, lengthy and expensive, with high mortality rates. TB is also the most common opportunistic infection among HIV/AIDS patients, and the leading cause of AIDS-related deaths worldwide [7].

Controlling the TB epidemic is complicated by the long turn-around time for diagnosis and drug susceptibility testing (DST) [8]. The current paradigm relies on symptomatic patients reporting to healthcare facilities; therefore, TB diagnosis occurs long after onset of infectiousness. New technologies are needed to rapidly identify individuals with active infectious TB in the field, to improve treatment, curb transmission and reduce morbidity and mortality [7,9]. Active pulmonary TB is most commonly diagnosed through sputum smear microscopy [1], which requires multiple sputum specimens, tedious processing and has low sensitivity. Culture is the current gold standard for TB diagnosis and DST, but typically requires 2–8 weeks to generate results [7,10], involves significant biohazard, and therefore necessitates a biosafety level 3 laboratory and trained personnel. Immunoassays have so far shown limited performance [11], and serological assays are not recommended for diagnosis of active TB [102]. Nucleic acid amplification testing (NAAT) enables sensitive and specific TB diagnosis [12,13]. NAAT systems with rapid turn-around time facilitate testing and treatment initiation in the same visit, which avoids loss to follow-up. In addition, NAAT can identify mutations in the *M.tb* genome associated with drug resistance [14].

Testing sites & system requirements

Approximately 80% of all new TB cases arise in 22 high burden countries [101], which vary widely in socioeconomic level, TB prevalence and healthcare infrastructure (Table 1). Despite global laboratory strengthening efforts [101] and rapid economic growth in countries such as India and China, most high-burden countries have an insufficient network of laboratories to effectively serve the affected population (Figure 1 & Table 1) [101,103]. Until recently, TB NAAT was performed exclusively in central reference laboratories. Since 2011, with the WHO-endorsed global roll-out of the *M.tb*/rifampicin resistance (MTB/RIF) assay performed on the GeneXpert® system (Cepheid, CA, USA) [13,15,16], TB NAAT has advanced to district and sub-district level laboratories of high burden countries [104]. The GeneXpert MTB/RIF is an integrated, automated, closed system, contamination-controlled NAAT platform, which enables TB diagnosis and rifampin resistance testing in <2 h performed by low-skilled users. However, the GeneXpert is expensive compared with smear microscopy [16,17], and requires regular maintenance, uninterrupted electrical line power, plus a temperature-controlled operating environment. Therefore, the GeneXpert is considered unsuitable for more remote settings [18,105] that serve the majority of affected patients (Figure 1) [103].

Sites that perform TB diagnostic testing in high burden countries include reference and referral laboratories with moderate to advanced laboratory infrastructure, sites with minimal laboratory infrastructure, such as microscopy centers, and peripheral-level sites with no laboratory infrastructure, such as community-level primary care (Figure 1) [19]. The discussion herein focuses on microscopy laboratories with minimal infrastructure, and peripheral-level primary care. These settings require diagnostic tests that are “so simple and

accurate as to render the likelihood of erroneous results by the user negligible”, the prerequisite for obtaining waived status under the Clinical Laboratory Improvement Amendments in the USA.

As settings become more remote, laboratory infrastructure and biosafety provisions decline and logistical challenges greatly increase (Figure 2), which leads to stringent product requirements (Table 2) [19–21] that are often mutually exclusive. Minimal specifications for a point-of-care (POC) TB test were articulated through a 2009 expert meeting hosted by Medecines San Frontiers and the Treatment Action Group (Table 2) [106]. Target product profiles (TPPs) specific to TB NAAT are currently under development by various aid and donor organizations, and are expected to provide clearer quantitative guidelines and possibly more stringent or additional requirements to ensure acceptable performance and sustainable implementation. Recent discussions amongst key stakeholders in high-burden countries such as India [22,23] may result in country-specific criteria. Additional requirements have been articulated related to external quality assurance, remote connectivity and electronic result reporting [24–26]. However, no definitive TPP for a POC NAAT is available to date, and a single TPP that fits the diverse spectrum of targeted countries and healthcare levels is probably unattainable. Such uncertainties are challenging for developers, and complicate device performance assessment required for adoption.

TB NAAT process overview

Sample collection, preprocessing & alternate sample types

Pulmonary TB is diagnosed from sputum, a viscous and heterogeneous matrix, which is difficult to collect and manipulate. In peripheral primary care settings, sputum is not routinely collected, and is often of poor quality, consisting more of saliva than being a lower respiratory tract specimen. Obtaining an appropriate specimen is critical, since even the best assay performed on inadequate specimens will generate false-negative results, which lowers the clinical sensitivity and negative-predictive value. For culture-based diagnostics that require live mycobacteria, sputum specimens are typically liquefied and decontaminated using *N*-acetyl-L-cysteine and/or sodium hydroxide, and such treated samples are often also used for laboratory-based NAAT [27,28]. However, sputum collection and manipulation puts healthcare workers and others at risk of infection [29], and for NAAT testing in remote settings without biosafety precautions it is necessary to kill all pathogens including mycobacteria [15]. TB diagnosis is very difficult in HIV-positive patients who produce sputum with low mycobacterial load or cannot produce sputum due to having an extrapulmonary infection, and in pediatric patients who often cannot expectorate an adequate sputum specimen or have extrapulmonary disease. Alternate specimens for NAAT-based TB diagnosis in these populations include pleural and gastric aspirates, induced sputum, cerebrospinal fluid and urine [16,30,31]. There is an urgent need for NAAT methods that can diagnose TB from other noninvasive sample types instead of sputum, especially for POC use.

Nucleic acid sample preparation

Nucleic acid sample preparation involves cumbersome and lengthy multistep processes that are prone to error if not automated. Sample preparation is a major hurdle in NAAT for TB diagnosis, particularly for POC applications. For TB diagnosis, sample preparation can be performed by concentrating and purifying the intact mycobacteria from sputum, followed by lysis to liberate the genomic material [28,32], or through lysis of mycobacteria in the sputum sample, followed by DNA purification through solid phase extraction [33,34]. Mycobacteria in liquefied sputum samples can be concentrated through centrifugation, followed by resuspending the pellet in buffer [27,28]. However, this sample preparation process does not

purify the DNA itself, and remaining polymerase inhibitors can impede subsequent amplification [35]. Mycobacteria can also be purified through filtration and washing, as implemented in the GeneXpert cartridge [13], or can be captured on functionalized beads [36,37]. Lysis of mycobacteria is difficult, due to their thick waxy cell wall. Mechanical disruption methods such as bead beating [38] or sonication [39] are more effective than chemical, enzymatic or heat lysis approaches [40].

Amplification

Most TB diagnostic NAAT methods utilize PCR with real-time fluorescence monitoring, which requires precision thermocycling and optical detection systems that add complexity and cost to the instrumentation. Isothermal nucleic acid amplification technologies require a constant incubation temperature, and therefore can be implemented with simplified instrumentation. Isothermal NAAT methods for detecting *M.tb* include loop-mediated amplification (LAMP; Eiken, Tokyo, Japan) [32,41] and cross-priming amplification (CPA; Ustar Biotechnologies, Hangzhou, China) [42], which use multiple primer sets and a strand-displacing polymerase to amplify DNA at 60–65°C. Helicase-dependent amplification (BioHelix, MA, USA) [43,44] and recombinase polymerase amplification (RPA; TwistDx, Cambridge, UK) [9,45] are ‘PCR-like’ in terms of primer design, but require a strand-displacing DNA polymerase, an additional enzyme to facilitate primer and probe binding (DNA helicase and recombinase, respectively), and single-strand DNA-binding proteins for DNA amplification. These and other isothermal amplification methods differ in amplification speed and performance [46].

Detection

PCR-based TB diagnostic systems generally use real-time detection, but simple end point detection may be more appropriate for POC applications in peripheral low-resource settings. LAMP is often implemented with end point detection based upon visual tube inspection via fluorescence enhancement after UV light excitation [32]. However, this monoplex readout cannot accommodate an internal amplification control [9]. Nucleic acid lateral flow enables specific amplicon detection, is amenable to low levels of multiplexing, and has been coupled to PCR [47], helicase-dependent amplification [48], RPA [45], LAMP and CPA [42].

Systems for TB diagnosis

Cepheid GeneXpert MTB/RIF

This easy-to-use and fully integrated NAAT system represents a significant advance in enabling accurate TB diagnosis in decentralized laboratory settings [13,15,16]. The total assay time is 90 min, and a four-module GeneXpert can process 16–20 specimens per 8 h working day with random access capability [16]. The Foundation for Innovative New Diagnostics (FIND) has negotiated preferential pricing for high burden countries [16,17,107], currently approximately US\$17,500 for the four-module instrument plus laptop computer, plus US\$1600–1800 annually for calibration. The cost per cartridge has been discounted from a list price of approximately US\$60 to US\$17, and through recent additional UNITAID support the price was further lowered to <US\$10 [108]. These associated costs are significantly higher than for smear microscopy, but comparable to or lower than for culture-based TB diagnosis and DST [16,17]. The GeneXpert also requires stable and uninterrupted line power, and a temperature-controlled facility to maintain operating temperatures at 30°C, and enable cartridge storage at 2–28°C [104]. Internet linkage is recommended to enable remote device performance monitoring and result recording. The cartridges are relatively bulky and have a limited guaranteed shelf life of 6–9 months. The required external computer is susceptible to theft.

For test execution, the sputum sample is treated for 15 min with a liquefying and bactericidal reagent containing sodium hydroxide and isopropanol [15], and then 2 ml of the treated sample is transferred into a cartridge (Figure 3) [49] that enables nucleic acid sample preparation and hemi-nested PCR amplification with realtime detection [50], with all reagents on board. For the MTB/RIF assay, mycobacteria are captured on a filter, washed and then lysed via a sonic horn. DNA liberated into a buffer is then mixed with lyophilized nested PCR reagents, followed by an initial round of amplification in the PCR reaction chamber. Next, amplified mastermix is pumped into a chamber containing additional lyophilized reagents prior to the second round of PCR amplification. Real-time detection is facilitated by six molecular beacons [50], of which five tile the rifampicin resistance determining region of the *M.tb rpoB* gene, whereas the sixth is used to detect the internal amplification control, enabling quality control of the extraction, amplification and detection processes. Based on multiple large international retrospective and prospective evaluation studies, from a single sputum sample the GeneXpert MTB/RIF assay has a clinical specificity of approximately 99% for culture-negative samples, and a clinical sensitivity of approximately 98–100% for smear- and culture-positive samples [13,16,51]. For smear-negative culture-positive samples the sensitivity was approximately 73–78% in the FIND-sponsored demonstration studies [13,51] and approximately 57–83% in additional studies [16]. Rifampicin resistance can be detected with sensitivity and specificity of 94–98% and approximately 98%, respectively, relative to phenotypic DST [13,51]. However, more false-positive rifampicin resistance results were observed later on [16], and in populations with low MDR-TB prevalence, the positive-predictive value was found to be <70%. Recent studies have shown improved diagnosis of pulmonary TB in pediatric cases and HIV-infected cohorts, compared with smear microscopy [11,51,52].

Systems under evaluation

At least four NAAT-based technologies for TB diagnosis targeted predominantly at low-resource microscopy laboratories are currently in evaluation studies. Two of these systems, a LAMP-based method developed by FIND and Eiken and a CPA-based method developed by Ustar Biotechnologies, involve manual sample preparation, isothermal amplification and visual end point detection. The other two technologies, developed by Molbio Diagnostics (Goa, India) and by Epistem (Manchester, UK), are PCR-based, partially automated platforms that feature novel thermocycling and optical designs to reduce cost, power requirements and improve robustness, relative to conventional real-time PCR-based systems. Although the Molbio and Epistem systems are not yet described in a peer-reviewed journal, information is included herein based on personal communications.

Loopamp® tuberculosis complex detection system

Eiken and FIND have partnered to develop a LAMP-based procedure for TB diagnosis that could replace smear microscopy, released in May 2011. At the time of this report, precise details concerning the kit components were unavailable and the kit was not yet endorsed by the WHO. The system involves the Loopamp PURE DNA Extraction Kit, the Loopamp Tuberculosis Complex Detection Reagent kit, and the PureLAMP™ Heater for thermal lysis and isothermal amplification with end point fluorescence detection. The assay process (Figure 4) [53] can be completed in approximately 1 h.

An evaluation of this system on clinical samples [53] found that starting from raw sputum, the clinical sensitivity was 98.2% for smear- and culture-positive samples, and 55.6 % for smear-negative/ culture-positive samples. The lower sensitivity for smear-negative specimens, compared with PCR-based methods, was explained by the smaller sample input volume. The specificity (percentage of correctly identified smear- and culture-negative samples) was 96.2%, again slightly lower than for PCR-based methods. Starting from

pretreated sputum resulted in poorer performance, but in POC settings the test will probably use raw sputum samples. The assay has recently been evaluated by FIND in multiple test sites, and performance data is expected to be presented to the WHO Scientific and Technical Advisory Group (WHO-STAG) in 2012. No details on costs are currently available.

NATeasy™ TB diagnostic system

Ustar Biotechnologies developed a TB diagnostic kit that includes reagents and disposables for manual sample preparation, CPA-based isothermal amplification of IS6110 [42], and nucleic acid lateral flow end point detection. For the complete test, based on personal communications, the user has to provide a heat block, vortexer, centrifuge, pipettor and tips, although Ustar is also developing a syringe-driven extraction device. The test starts with 1 ml of liquefied sputum, which is heated to lyse the mycobacteria. Lysed sample is then transferred into the DNA purification device, for standard solid-phase extraction using a DNA affinity membrane, with a series of wash steps, followed by elution. For *M.tb* detection, the DNA extract is added to glassified CPA reagents, and incubated at 63°C for 1 h. CPA amplicons are visually detected via the XCP Nucleic Acid Detection Device (Figure 5), an enclosed lateral flow detection cassette that provides highly sensitive, easy to interpret results while limiting amplicon contamination at the test site [42]. The total assay requires approximately 90 min, and has the ability to batch process. The recommended long-term storage conditions are –20°C for amplification reagents and 2–8°C for lysis reagents, although ambient temperature is sufficient for transport and short-term storage (<2 months). Ustar are addressing storage constraints by developing thermostable reagents and formulations.

In the inaugural evaluation, this CPA assay coupled with the XCP Device was able to detect TB extracted from sputum using a standard nucleic acid sample preparation approach with clinical sensitivity of 96.9 and 87.5% for smear-positive/culture-positive and smear-negative/culture-positive samples, respectively [42]. With culture-negative specimens the specificity was observed to be 98.8%. Screening of 13 non-tuberculous mycobacterial species showed that the assay is 100% specific for TB. To simplify the DNA extraction process, Ustar has since implemented the assay in conjunction with the DNA purification device described previously. The cost per test is estimated to be approximately US\$15.

Truelab Mycobacterium tuberculosis detection system

Molbio Diagnostics Private Ltd. (a collaborative venture between Bigtech Private Ltd. and the Tulip Group, both India) has developed the Truelab Uno, a low-cost, semi-automated, and quantitative NAAT platform for TB diagnosis in 1 h in remote healthcare settings. Sputum is produced into a collection cup, which contains dried reagents to liquefy the sample and kill bacteria. A 1 ml sample is then transferred to the Trueprep™-MAG system, which performs paramagnetic bead-based DNA extraction in a semi-automated fashion and is powered by either battery or line power (Figure 6A). The DNA extract is then manually transferred into a reaction cartridge containing lyophilized PCR reagents, which is inserted into the Truelab Uno (Figure 6B) for real-time PCR analysis with two-channel excitation and photo-detection. The instrument houses an optical detection system, an internal rechargeable battery that can run the system for up to 8 h, and an Android-type wireless device for data entry, platform control and processing, test analyses and result dissemination. As opposed to other PCR platforms, the reaction cartridge contains the thermocycling component that is controlled and powered via the instrument. Integrated quality control ensures appropriate equipment operation, reagent quality and adequate specimen extraction, whereas data transmission rapidly informs control programs of case detection rates and potential issues at test sites. Cost estimates are <US\$6000 for both instruments and US\$10–12 for the disposables.

Genedrive® *Mycobacterium tuberculosis* iD®

Epistem (Manchester, UK) has developed the Genedrive (Figure 7A) for rapid and user-friendly TB diagnosis from sputum or urine. The Genedrive is a lightweight (560 g), portable, high-performance bench-top instrument that enables rapid PCR amplification with real-time detection. Epistem's *M.tb* iD Test-kit, which enables *M.tb* complex detection and rifampicin resistance screening in conjunction with the Genedrive instrument, is currently undergoing clinical evaluation in India. The Genedrive technology uses a novel sample preparation approach, innovative low-power thermal cycling, plus simplified precision optics to enable cost-effective PCR-based NAAT at the POC in <45 min.

Cell lysis and DNA extraction is performed using a composite paper-based approach from raw or liquefied sputum. Three 20- μ l aliquots of the extract are transferred to the test cartridge before it is inserted into the Genedrive for analysis. The test cartridge (Figure 7B) contains lyophilized reagents for PCR amplification in three separate reactions. The instrument performs rapid PCR amplification with probe-based real-time fluorescence detection and high resolution melt curve analysis to verify target amplicon production, and to enable genotyping of the *rpoB* rifampicin resistance core region, within 30 min. The system features an integrated microprocessor and a touch-screen user interface for assay execution and result reporting (Figure 7C). Based on personal communications, the assay has been shown to detect 30 CFU/ml *M.tb* spiked into raw sputum samples, and preliminary testing demonstrated TB detection with sensitivity >90–95%, and specificity >95%. The Genedrive can be operated on 12V DC. The instrument price is currently quoted at US \$4000, or under US\$2000 with volume discounting. The cartridge cost is yet to be finalized, but US\$10–17 is the anticipated price.

Key performance characteristics of the GeneXpert MTB/RIF and the four new TB NAAT systems described herein are summarized in Table 3.

Other integrated platforms

Other PCR-based fully integrated *in vitro* diagnostic (IVD) NAAT systems are commercially available or in development, including the FilmArray (Idaho Technologies, UT, USA) [54], Liat™ Analyzer (IQum, MA, USA) [55], Apollo (Biocartis, Eindhoven, The Netherlands), Enigma® ML (Enigma Diagnostics, Salisbury, UK) and NAT Analyzer (Alere Technologies Clondrag, Jena, Germany). No TB diagnostics are currently offered on these platforms, although TB NAAT can probably be accommodated. Other partially or fully integrated platforms based on isothermal amplification have been reported [56–58]. Alere recently announced the iNAT system in development for POC infectious disease diagnosis based on isothermal amplification, following the acquisition of TwistDx, the company that developed RPA, and Ionian Technologies (CA, USA), the company that developed the nicking enzyme amplification reaction (NEAR) [59].

Expert commentary & five-year view

NAAT-based TB diagnosis is moving from central reference laboratories toward peripheral healthcare settings. The GeneXpert MTB/RIF system has set the current standard, but may be challenged in coming years by less expensive manual or semi-automated systems in development or undergoing clinical evaluation. Several technologies may succeed in different niches of the global TB diagnostic market; however, clearly defined optimal and minimally acceptable TPPs are required to guide technology development and enable performance assessment [9,17,24,26,60,105]. Since the 'ideal' product profile (Table 2) is probably unattainable, decisions about necessary tradeoffs have to be informed through input from all involved stakeholders [20,22,23], by modeling the potential epidemiological [8,61] and economic [26,62] impact of new diagnostic technologies, and by considering how

to best integrate new tests into treatment algorithms [24,63] to make a sustainable impact on patient care and global health.

Providing 100% access to rapid TB diagnosis with suitable clinical performance can save many lives through reduced transmission as a result of active case finding and appropriate treatment [8,61]. Existing TB NAAT systems are reasonably rapid and have clinical performance that is significantly better than smear microscopy; they therefore enable better case detection of pulmonary TB and timely treatment initiation. Major existing challenges for TB NAAT include reducing the instrumentation and consumables cost and enabling use by minimally trained personnel in remote low-resource settings without electricity or refrigeration. To date, the GeneXpert is the only fully integrated sample-in to answer-out platform, but it is not truly a low-resource POC system, since it requires uninterrupted line power, air conditioning to ensure operating temperatures $\sim 30^{\circ}\text{C}$, security provisions for the external computer, and ideally internet access to upload test data. Microscopy sites in low-resource settings vary significantly in terms of basic infrastructure and staffing, and many of them do not adequately meet all of the first three criteria. Effective roll-out of the GeneXpert therefore requires widespread facility upgrades as part of ongoing laboratory strengthening efforts [101], which although desirable may be a challenge for many countries, and thus will impede timely technology implementation. The partially integrated instruments developed by Molbio and Epistem require less infrastructure, have lower instrument costs and a smaller instrument footprint, can be battery powered, and do not require an external computer. The remote connectivity of the Molbio TrueLab Uno[®] is a key advance in permitting centralized performance monitoring of testing in remote areas. However, neither of these systems is as easy to use as the GeneXpert, since both platforms require separate sample preparation prior to inserting the final assay mixture into the real-time PCR device.

The manual LoopAmp (Eiken) and NATEasy (Ustar) TB assays have been developed to permit NAAT testing in more resource-limited microscopy laboratories with infrastructural constraints that prevent implementation of the GeneXpert system. Both manual assays are designed to require only basic instrumentation, and through batch processing can achieve throughput levels similar to smear microscopy. Although no precise cost estimates are available at the moment, the consumables cost for the manual tests will probably be lower, and capital equipment costs will be significantly lower than for the integrated/partially integrated systems. However, the Eiken and Ustar tests involve many manually performed steps, therefore are less user-friendly, and require more initial training. Effective quality assurance and proficiency monitoring will be challenging to implement, and along with the necessary extended training will increase the overall system cost. In addition, both manual systems require extra instrumentation that may not be readily available. Three of the four new systems currently require refrigerated reagent storage, which will significantly hamper implementation. All four new systems involve multiple separate consumables, which creates challenges in terms of supply chain management and storage. Overall, none of the four new platforms herein described address all of the minimal system requirements for POC TB diagnosis [106]. These systems can be implemented in microscopy centers, but not in primary care settings without laboratory infrastructure. Although these new systems are expected to reach a larger portion of the affected patient population, they will not be able to provide 100% access to TB diagnosis.

Additional unmet needs exist for improved TB diagnosis of HIV-positive and pediatric patients, and for *M.tb* drug resistance testing. Several existing platforms perform TB diagnosis and rifampin resistance testing simultaneously, but given the variability in MDR-TB prevalence it may be preferable to implement genotypic drug resistance testing as a separate follow-up test. Rifampin resistance testing is comparatively simple since most resistance mutations are located within a short region of a single gene [64]. Resistance

genotyping for other drugs is considerably more complicated, and new resistance alleles are constantly emerging [65]. Systems enabling higher levels of multiplexing are needed for diagnosis of XDR-TB. Ideal TB NAAT platforms should be adaptable to accommodate new genotypic drug resistance testing assays and tests for related endemic infectious diseases such as HIV infant diagnosis and HIV viral load testing.

Confusion about applicable regulatory frameworks and/or lack of regulatory systems in high burden countries can impede the development of new TB NAATs [24]. Regulatory approval is typically required before international aid organizations and governmental institutions will endorse a new test. Recently, the WHO has increased its role in the regulation of IVDs [109]. Quality assurance is a major concern for POC testing in high burden low-resource settings [25,26,63], since shortcomings compromise patient care and jeopardize support from governments and aid organizations. The WHO and CDC are now facilitating quality management for diagnostic testing in developing countries without existing programs [66]. Quality assurance needs to be considered in developing new IVDs. The manual kit by Eiken described herein has no internal control and minimal quality assurance provisions. The manual kit from Ustar and the instrumented platforms by Molbio and Epistem incorporate internal quality controls. The MolBio and Epistem systems enable electronic results recording and better provisions for quality assurance.

The global market size for TB diagnostics is substantial [103], with an estimated 15 million tests required annually in India alone, which carries approximately 20% of the global TB burden [67]. However, leading global IVD companies traditionally consider diagnostic testing targeted at developing economies unprofitable, a paradigm recently challenged by Alere [68]. In the coming decade, other large IVD companies may follow suit and devote more effort to global health. Many countries with high TB burden, especially India and China, have rapidly emerging economies and increasing technical expertise [22,67]. New diagnostic platforms are being developed in China (Ustar), India (Molbio/Bigtech/ Tulip group), or through international partnerships, for example between Epistem and Xcelris (India), which facilitates faster market penetration and true assessment in a local test environment. Development and commercialization of TB diagnostics in emerging economies with increasing private markets may be facilitated through stronger partnerships between the public and private sector.

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Key issues

- For more effective case detection, appropriate treatment initiation, and reduced transmission of tuberculosis (TB) in low-resource settings of high burden countries, the current method of smear microscopy needs to be replaced with a rapid yet higher-performing diagnostic test for pulmonary TB infection. Novel TB nucleic acid amplification testing (NAAT) systems, appropriate for use in peripheral settings, can facilitate such a shift.
- Providing 100% access to rapid TB diagnosis in peripheral healthcare settings is the primary goal. For TB NAATs, the necessary reduction in cost and complexity of instruments and disposables has to be balanced against appropriate clinical performance and ease of use. Key technical challenges include integrated sample preparation, ease of use, and long-term reagent stability at elevated temperatures.
- Improved TB diagnosis in patients with HIV comorbidities and children requires NAAT approaches with suitable performance from alternate minimally invasive sample types. Emerging highly virulent drug-resistant *Mycobacterium tuberculosis* strains may undermine TB control efforts. Technologies enabling higher levels of multiplexing are needed to detect an ever-increasing number of resistance alleles.
- The regulatory process for introducing new TB diagnostics into TB control programs is highly variable, country specific and often unclear. It is anticipated that the WHO will play a greater role through the PQ Dx process. The Stop TB partnership has developed a process for introducing new TB diagnostics which has been applied by Foundation for Innovative New Diagnostics in bringing donor-financed diagnostic technologies to the market.
- New TB NAAT platforms need to incorporate provisions for quality assurance, such as inclusion of an internal amplification control, external controls, electronic performance monitoring and results recording, plus wireless connectivity to facilitate external quality monitoring and rapid paperless notification of case rates to a centralized TB control program.
- Rapid development and commercialization of TB diagnostics can be facilitated through stronger partnerships between IVD developers and TB control programs, academia and industry, and between small and large companies. Agencies such as Foundation for Innovative New Diagnostics or the TB Clinical Diagnostics Research Consortium (NIH, USA) can play a key role by providing access to sample repositories and by facilitating clinical validation of new technologies.
- Increased public and private sector funding is needed to develop and deploy new TB diagnostics. The potential global market for TB diagnostics is substantial, especially in countries with emerging economies and high TB burden, such as India and China. New TB diagnostic technologies will probably be developed by or in close partnership with these countries, with increasingly country-financed models, and a stronger role of private sector funding.

Figure 1. Healthcare access pyramid for tuberculosis control in the developing world
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Figure 2. Levels of laboratory infrastructure in high tuberculosis-burden countries

(A) Tuberculosis testing in a district hospital with biosafety precautions entailing a simple dead air box. (B) Open sample processing at a crowded bench in a peripheral laboratory. (C) Community-level healthcare provider and (D) vehicle for ambulatory patient care. (E) Storage of laboratory supplies and (F) bio-hazardous waste disposal exemplify logistical challenges of clinical diagnostics in low-resource settings.

Photographs provided courtesy of Gerard Cangelosi, Tanya Ferguson and PATH.

Image (C) is reprinted with permission from [19].

Figure 3. Schematic representation of the Cepheid GeneXpert cartridge

Fluid transfer within the cartridge is controlled via a syringe plunger and a rotating valve. Sample preparation is executed in the bottom of the cartridge, whereas PCR amplification with real-time detection occurs in the rectangular PCR tube protruding from the back of the cartridge. Most of the cartridge body is used for reagent storage. Modified with permission from [49].

Figure 4. Process for tuberculosis diagnosis using the Eiken Loopamp Tuberculosis Complex Detection Reagent Kit, in conjunction with the Loopamp PURE DNA Extraction Kit

During sample preparation, a 40 μ l volume of either raw or processed sputum sample is inserted into the heating tube, and mycobacteria are killed and heat lysed at 95°C for 5 min in the PureLAMP heater. The sample is transferred into an adsorbent tube, wherein contaminants are removed from the sample (precise technical specifications were not available for this review). DNA-containing filtrate (30 μ l) is then transferred into a reaction tube containing dried LAMP TB assay mastermix. Sample preparation requires approximately 10 min, followed by isothermal amplification for 40 min, with either real-time detection using the Loopamp LF-160 or through visual end point detection using the PureLAMP Heater, which includes a UV lamp.

LAMP: Loop-mediated isothermal amplification.

Modified with permission from [53].

Figure 5. The XCP Nucleic Acid Detection Device (Ustar)

(A) Schematic cross-section, showing the amplicon cartridge containing a plastic bulb with lateral flow running buffer, and a 200 μ l tube containing the reaction mix, inserted into the detection chamber holding the lateral flow test-strip. The detection chamber is then closed using a levered lid, which pushes the amplicon cartridge down into the detection chamber body, where the plastic bulb containing the nucleic acid lateral flow running buffer and the reaction tube are pierced as the lid snaps shut. The two fluids combine and then run up the lateral flow test strip. Results can be visually interpreted after 5–10 min. (B) The device on the left shows a negative reaction with the test line T remaining blank while the control line C indicates appropriate flow and test reagent reactivity for internal quality control of the lateral flow strip. In the device on the right, the test line is apparent in addition to the control line, indicating a positive cross-priming amplification test result.

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Figure 6. The Molbio TrueLab *Mycobacterium tuberculosis* detection system

(A) The Trueprep™-MAG and (B) the Truelab™ Uno analyzer. The red-capped container is a sputum sample cup containing lyophilized reagents to liquefy the sputum. After DNA extraction 5 µl is transferred to the disposable reaction cartridge, which is then entered into the machine for processing and analysis.

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Figure 7. Key components of Epistem's Genedrive[®] *Mycobacterium tuberculosis* iD[®] system (A) The Genedrive instrument, with tripartite port at the front for the insertion of the test cartridge. (B) The Genedrive test cartridge, with three protruding clear reaction tubes that are inserted into the instrument port prior to test execution. (C) The user interface displays test results for *Mycobacterium tuberculosis* identification and rifampin drug resistance testing in an easily interpretable and clinically actionable form. Images reprinted with permission from Epistem (UK).

Table 1

Key 2010 indicator data for selected high TB-burden countries and the USA for comparison.

Country	Population [†]	TB prevalence [‡]	Smear laboratories [§]	Culture laboratories [¶]	GNI/capita in US\$ [#]	Health expenditure/ capita in US dollars (% private) ^{††}
India	1225	3.10 (256)	1	0.1	1270	54 (86)
China	1341	1.50 (108)	0.2	2.7	4270	221 (79)
Brazil	195	0.09 (47)	2.0	6.9	9390	990 (58)
Russian Federation	143	0.19 (136)	2.8	13.9	9900	525 (83)
South Africa	50	0.40 (798)	0.5	1.5	6090	649 (30)
Bangladesh	149	0.61 (411)	0.7	0.1	700	23 (97)
Pakistan	174	0.63 (364)	0.7	0.4	1050	22 (82)
Indonesia	240	0.69 (289)	2.9	0.9	2500	77 (75)
Philippines	93	0.47 (502)	2.1	1.4	2060	77 (84)
Nigeria	158	0.32 (199)	0.9	0.2	1230	63 (95)
Ethiopia	83	0.33 (394)	2.8	0.4	390	16 (80)
USA	309	0.01 (4)	NA	NA	47,340	8362 (25)

Data for 2010:

[†]Population in millions.

[‡]Total number of cases in millions and in parentheses cases per 100,000 population.

[§]Number of smear microscopy laboratories per 100,000 population.

[¶]Number of laboratories performing culture-based TB diagnosis per 5 million population.

[#]GNI per capita, converted to US dollars using the Atlas method.

^{††}The % of private health expenditure includes any direct outlay by households related to health.

GNI: Gross national income; NA: Not available; TB: Tuberculosis.

Sources: Population, TB prevalence, Smear laboratories, Culture laboratories: WHO global TB control report, 2011 [101], except US population; GNI/capita, Health expenditure/capita and US population: World Bank statistical data [111].

Table 2

Requirements and example target product profiles for tuberculosis nucleic acid amplification testing systems used in remote microscopy centers and primary care settings of high-burden countries.

Characteristics of testing environment	Parameter	Minimal POC TB diagnostics requirements [106] [†]	Optimal POC TB diagnostic requirements [‡]
Many patients in remote locations have no access to TB diagnostic methods, or to smear microscopy only. Active case finding with adequate clinical performance needed to initiate treatment and curb transmission	Clinical sensitivity	95% SSM+/C+; 60–80% SSM–/C+ [‡]	95% SSM+/C+; 60–80% SSM–/C+ [‡]
	Clinical specificity	95% SSM–/C–	>99% SSM–/C–
	Analytic sensitivity	Not specified	<250 CFU/ml
	Analytic specificity	Not specified	Not cross-reactive with NTMs and other relevant pathogens
Prevalence of MDR/XDR-TB varies by area, but increasing	Drug resistance screening	Not listed – focus on TB diagnosis only	Flexible platform, drug resistance testing optional as separate assays
Very limited financial resources – test needs to be affordable	Cost of all consumables per test	<US\$10 after scale-up	<US\$5 after scale-up
Purchase/maintenance of expensive instrumentation not possible	Cost of instrumentation	“Acceptable replacement cost”, maintenance-free	Capital instrument cost integrated into reagent cost, maintenance-free
Consumables have to be provided to many remote sites with limited storage space	Reagents/ consumables	Self-contained kit with all reagents, sample collection device and water if needed	All reagents in one consumable, sample collection device provided, small consumable size
Remote sites can only collect noninvasive sample types	Specimen type	Sputum OK but not ideal, aim for other noninvasive sample types	Sputum and other liquid specimens, noninvasive samples
Additional equipment often not available, may break down or disappear, rendering the system nonfunctional	Additional equipment	Not specified	No additional equipment required
	Electronics/data analysis	Not specified	Integrated, no external computer needed
Ambient operating temperatures often high and uncontrolled, cold storage/ cold chain for reagent supply generally not available	Reagent kit stability	24 months at 30°C, higher for shorter periods of time. Stable in high humidity	24 months at 40°C, 70% humidity, plus stability for 48 h at 50°C, and daily thermal cycling at 25°C for 4 days, to account for stress during transportation and storage
	Operating environment	Works in tropical conditions	Operational at 10°C to 45°C, system tolerates 70% humidity
Testing systems transported to remote sites using basic means	Portability/ruggedness	Fits in backpack, shock resistant	Fits in backpack, resistant to shock, vibration, water, dust
Low operator skill level, low-infrastructure test environment leads to high risk of operator errors	Number of operator steps	Three steps maximum	Integrated, sample-in – answer-out
	Type of operator steps/ integration	No need for precision pipetting, no time-sensitive processes	Simple sample addition only, fully integrated platform
Tests performed by large number of low-skilled minimally trained healthcare workers	Training and required skill level	Training in 1 day maximum, can be used by any healthcare worker	Training in <3 h, seventh grade level education or equivalent, ‘CLIA waivable’
Patients often lost to follow-up	Time-to-result	3 h maximum, desirable <15 min	<15 min
Required throughput varies from <10 per day at remote primary care settings to >100	Throughput	At least 20 tests per staff member per day	Modular design adapts to various throughput demands, if needed 64 tests per staff member per day

Characteristics of testing environment	Parameter	Minimal POC TB diagnostics requirements [106] [†]	Optimal POC TB diagnostic requirements [‡]
per day in some microscopy centers	Sample handling	Single sample processing (random access)	Single sample processing (random access), batch processing optional
Biosafety precautions minimal, high risk of infection	Biosafety	BSL1	BSL1, pathogen inactivated before sample introduction into device
Waste management challenging. Test site contamination with amplicons causes false positives	Waste management	Simple burning, sharps disposal, no glass, environmentally acceptable	Disposables volume kept to a minimum, sealed disposable, ideally amplicons destroyed after testing, biodegradable housing
Testing performed with minimal or no oversight – test results confounded by incorrect test execution, undetected reagent or equipment failure, use by unauthorized personnel	QC/EQA	Positive control in test kit, EQA/QC easier than for smear microscopy	Internal full-process positive control and negative control. EQA panel available to ensure operability at test site and to enable user proficiency testing, electronic tracking of reagent lots, user login, wireless connectivity links devices with central facility
Unreliable line power or no access to electricity	Power	Rechargeable battery	Rechargeable battery or solar power, AC or DC compatible
Results can be confusing, may not be read properly, have to be recorded for case notification	Result type	Simple yes/no/invalid answer	Simple yes/no/invalid answer
	Readout	Easy to read, unambiguous, readable for at least 1 h	Easy to read, unambiguous, electronic and printed, wireless transmission capable, including GPS

[†] Although the minimal POC TB diagnostic requirements listed here have been extensively vetted by many stakeholders, have been published [106] and frequently cited [9,17,60,105], the optimal POC TB diagnostic requirements included here are more hypothetical, based on the authors' opinions, personal communications with other stakeholders and opinions expressed in other publications [24–26]. It may not be possible for a system to meet all optimal product requirements.

[‡] According to [106], these clinical sensitivity values apply to adult pulmonary TB regardless of HIV status. Detection of extrapulmonary TB in adults preferred but not required; clinical sensitivity for children (including extrapulmonary TB, regardless of HIV status): 80% compared with culture of any specimen and 60% of probable TB cases (no gold standard).

BSL1: Biosafety Level 1; CFU: Colony forming units; CLIA: Clinical Laboratory Improvement Amendments; EQA: External quality assurance; MDR: Multidrug NTM:Non-tuberculous mycobacteria; POC: Point-of-care; QC: Quality control; SSM+/C+: Positive by sputum smear microscopy and culture; SSM–/C+: sputum smear microscopy, positive by culture; SSM–/C–: Negative by sputum smear microscopy and culture; TB: Tuberculosis; XDR: Extensively drug resistant.

Table 3

Performance comparison of selected tuberculosis nucleic acid amplification testing systems on the market and under development.

	GeneXpert MTB/RIF (Cepheid)	TrueLab™ <i>M.tb</i> detection (Molbio)	GeneDrive® <i>M.tb</i> iD (Epistem)	Loopamp TB detection (Eiken)	NATeas TB (Ustar)
Diagnostic capabilities	<i>M.tb</i> diagnosis and Rif resistance	<i>M.tb</i> diagnosis	<i>M.tb</i> diagnosis and Rif resistance	<i>M.tb</i> diagnosis	<i>M.tb</i> diagnosis
Input sample	2 ml of liquefied sputum (raw sputum ~1/3 of 2 ml volume)	Raw sputum (~1 ml)	Raw sputum (3 × 20 µl of sample extract)	Raw or processed sputum (40 µl into 960 µl lysis reagent)	Raw sputum (~1 ml)
Sample preparation	Mycobacteria in sputum filtered and washed, then lysis via sonication	Paramagnetic bead-based solid phase extraction	Paper-based DNA extraction method (limited details available)	Heat lysis of mycobacteria, then removal of inhibitors	Heat lysis of mycobacteria, then solid phase extraction of DNA
Amplification	PCR	PCR	PCR	LAMP	CPA
Detection	Real time, fluorescence	Real time, fluorescence	Real time, fluorescence	End point, fluorescence/turbidity	End point, nucleic acid lateral flow
Degree of automation/integration	Fully integrated sample preparation, amplification and detection	Semi-automated sample preparation, then automated amplification/detection	Manual sample preparation, then automated amplification/detection	Manual sample preparation, amplification and detection (closed tube readout)	Manual sample preparation, amplification and detection (NALF cartridge)
IAC	Yes	Yes	No	No	Yes
Electronic data transmission	Yes [†]	Yes	No	No	No
Disposables/cost	Liquefying reagent, MTB/RIF cartridge retail price: US\$60, discount price currently US\$17 (\$10)	Collection cup with reagents, DNA extraction kit, PCR reagent tubes US\$10–12 [‡]	<i>M.tb</i> iD® Test-kit: paper-based sample preparation; test cartridge US\$10–17 [‡]	DNA Extraction Kit and MTB Complex Detection Reagent Kit cost NA	DNA purification kit, CPA reagent tubes, XCP Nucleic Acid Detection Device; cost US\$15 per test
Endorsed by WHO	Yes	No [§]	No [§]	No [§]	No [§]
Dedicated instrumentation/cost	GeneXpert US\$17,500 (four-module)	TrueLab Mag/UNO (two instruments) <US\$6000 [¶]	GeneDrive <US\$4000	Optional: PureLAMP Heater + UV lamp, price NA	None
Additional instruments required	None	None	None	Alternatively: water bath/heating block, UV lamp	Water bath/heating block, vortexer, centrifuge
Electricity	Uninterrupted line power	Rechargeable battery	Rechargeable battery	Uninterrupted line power	Uninterrupted line power
Temperature control	Operating temperature <30°C	None	Refrigerated reagent storage	Refrigerated reagent storage	Refrigerated reagent storage [#]
Technical skills required ^{††}	Low	Low medium	Low medium	Medium	Medium
Time-to-result	<2 h	<1 h	<45 min	<1 h	<2 h
Throughput	16–20 tests per 8 h work shift for four-module instrument	12 tests per 8 h work shift	Not yet determined	Not yet determined	Not yet determined

	GeneXpert MTB/RIF (Cepheid)	TrueLab™ <i>M.tb</i> detection (Molbio)	GeneDrive® <i>M.tb</i> iD (Epistem)	Loopamp TB detection (Eiken)	NA Teasy TB (Ustar)
Sample handling	One sample per module, random access	Single sample per instrument	Single sample per instrument	Single sample, or batch processing	Single sample, or batch processing
Clinical sensitivity ^{††}	99.8% SSM+/C+ 72.5% SSM-/C+	Not yet determined/ published	Not yet determined/ published	98.5% SSM+/C+ 55.6 SSM-/C+	96.9% SSM+/C+ 87.5% SSM-/C+ §§
Clinical specificity ^{††}	99.2% SSM-/C-	Not yet determined/ published	Not yet determined/ published	96.2% SSM-/C-	98.8% SSM-/C- §§

[†]The data generated from the GeneXpert can be uploaded to a web-based server if connected to the internet.

[‡]Tentative cost per test, reflects nonsubsidized pricing unlike the volume-generated pricing associated with the Xpert MTB/RIF assay.

[§]Devices and assays are undergoing evaluation and demonstration at multiple test sites.

[¶]The price includes both the extraction system and PCR device.

[#]The Ustar reagents are thermostable for up to 1 week, permitting some transport without cold chain.

^{††}The technical skills required are described as low (1–3 days training of a non-expert user with seventh grade level education or equivalent) or medium (4–5 days training of a user with higher skill level).

^{‡‡}Performance is based on previously published data [13,42,53].

^{§§}Based on using a standard sample preparation approach, not the sample preparation method envisioned in the future test devices.

CPA: Cross-priming amplification; IAC: Internal amplification *M.tb* control; : *Mycobacterium* LAMP: *tuberculosis* Loop-mediated ; NA: Not available; amplification; NAAT: Nucleic acid amplification technique; NALF: Nucleic acid lateral flow; Rif: Rifamicin; SSM+/C+: Positive by sputum smear microscopy and SSM-/C+: Negative by sputum smear microscopy, positive by culture; SSM-/C-: Negative by sputum smear microscopy and culture.