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Emerging Technologies for Monitoring Drug-Resistant Tuberculosis at the Point-of-Care

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Abstract

Infectious diseases are the leading cause of death worldwide. Among them, tuberculosis (TB) remains a major threat to public health, exacerbated by the emergence of multiple drug-resistant (MDR) and extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (Mtb). MDR-Mtb strains are resistant to first-line anti-TB drugs such as isoniazid and rifampicin; whereas XDR-Mtb strains are resistant to additional drugs including at least to any fluoroquinolone and at least one of the second-line anti-TB injectable drugs such as kanamycin, capreomycin, or amikacin. Clinically, these strains have significantly impacted the management of TB in high-incidence developing countries, where systemic surveillance of TB drug resistance is lacking. For effective management of TB on-site, early detection of drug resistance is critical to initiate treatment, to reduce mortality, and to thwart drug-resistant TB transmission. In this review, we discuss the diagnostic challenges to detect drug-resistant TB at the point-of-care (POC). Moreover, we present the latest advances in nano/microscale technologies that can potentially detect TB drug resistance to improve on-site patient care.

Keywords

Tuberculosis; Drug resistance; Diagnostics; Nano/microscale technologies; Point-of Care

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Conflict of interest: Dr. Utkan Demirci is a founder of, and has an equity interest in, DxNow, a company that is developing microfluidic and imaging technologies for point-of-care diagnostic solutions. Dr. Demirci's interests were reviewed and are managed by the Brigham and Women's Hospital and Partners HealthCare in accordance with their conflict of interest policies.

1. Introduction

Infectious diseases account for an estimated 17 million deaths each year [1]. It is fueled by uncontrollable outbreaks, increasing drug resistance and poor disease management. For example, inappropriate use of antibiotics and poor patient adherence in hospital and community settings have led to a significant rise in the number of drug-resistant (DR) infectious strains of *Mycobacterium tuberculosis* (Mtb), *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacteriaceae* [2, 3]. Treating infections caused by multi drug-resistant (MDR) strains, also known as “superbugs”, is often difficult, and requires multiple drugs in addition to lengthy hospital stays, which significantly increases medical cost compared to treating infections caused by drug-susceptible strains [4]. Owing to limited drug options to treat superbugs, refractory and serious infections often result in high mortality and morbidity. Clearly, there is an urgent need for rapid point-of-care (POC) diagnostic tools to identify DR strains to ensure appropriate therapeutic intervention before they cause life threatening illness and further spread in the general population [5].

Globally, Mtb alone is estimated to cause 9.4 million new tuberculosis (TB) cases and 2 million TB-related deaths every year [6]. TB management is being deteriorated with the emergence of MDR and extensively-drug-resistant (XDR) strains due to inappropriate use of antibiotics, poor adherence to anti-TB drugs, and insufficient monitoring of drug resistance [7]. According to the World Health Organization (WHO), over 500,000 new cases of MDR-TB were detected worldwide in 2011 [8]. The overall prevalence of XDR-TB remains unknown, but XDR-TB cases have been detected in 84 countries [9]. For infected individuals, early detection of type of Mtb strain and administration of effective anti-TB drugs are essential for better clinical outcomes. Thus, reliable TB diagnostics that can detect active or latent TB as well as drug resistance at the POC are urgently needed to initiate therapy. However, current POC TB diagnostic tools often under-diagnose active TB infection, and are unable to predict the susceptibility of anti-TB drugs or to monitor treatment efficacy in resource-constrained settings [10].

At present, Mtb drug resistance testing employs either phenotypic or genotypic methods, which requires established laboratory infrastructure and well-trained staffs [10, 11]. Essentially, these technical constraints exclude the use of multiple-step and instrument-dependent diagnostic methods such as conventional polymerase chain reaction (PCR) and culture methods for drug resistance testing at the POC. Instead, smear microscopy, despite poor sensitivity and specificity, is widely used at the POC to acquire basic laboratory evidence for guiding clinical decision. Although a recently developed lateral flow assay based on the detection of lipoarabinomannan (LAM) is simple-to-use and inexpensive, it only provides clinical utility in diagnosis of HIV associated TB patients with CD4 cell count less than 50 cells per μL [12, 13]. Neither of these two POC TB diagnostic device provide the status of TB drug resistance, which can result in DR-TB undetected in resource-constrained countries. Recent endorsement of the Cepheid's GeneXpert assay by the WHO opens a new avenue for detection of TB infection and rifampicin resistance [14]. However, the high cost associated with GeneXpert instrumentation and its regular maintenance prevents its large-scale implementation in resource-constrained settings. In addition,

GeneXpert is limited to the detection of only rifampicin resistance and is not capable to diagnose MDR- or XDR-TB.

Clearly, there is an urgent need for developing new DR-TB diagnostic methods that are simple, accurate, inexpensive, rapid, and that can simultaneously detect multiple biomarkers at the POC for effective management of MDR/XDR-TB. These new diagnostic methods will be especially useful in resource-constrained settings where there is a lack of financial support, laboratory infrastructure and well-trained healthcare workers. Here, we review (i) advantages and disadvantages of current technologies for DR-TB diagnosis, (ii) challenges of developing TB drug resistance assays, (iii) the role of nano/microscale technologies in developing fully/partially integrated DR-TB diagnostic devices, and (iv) diagnostic gaps that needs to be bridged to deliver on-site TB patient care. The focus of this review is on developing novel on-chip nucleic acid amplification test (NAAT) and whole bacterial detection methods that are rapid, portable, accurate, and inexpensive. These innovative technologies can potentially provide rapid tools for diagnosis of DR-TB in resource-constrained settings. We envision these technologies reviewed herein for DR-TB can be translated to detect other DR bacterial, fungal or viral pathogens.

2. Current approaches for DR-TB diagnosis

Current DR-TB diagnostic assays in a laboratory setting include phenotypic assays and molecular genotypic methods. Table 1 summarizes current approaches to distinguish susceptible TB from DR-TB.

2.1 Phenotypic assays

2.1.1 Solid and liquid culture methods—In a conventional laboratory setting, DR-Mtb strains are identified with indirect drug susceptibility testing (DST) on solid agar medium or in liquid culture. This method involves isolation of the bacterium followed by DST in the presence of anti-TB drugs. Although DST on solid culture is relatively inexpensive (˜$30 per test) [15] and requires simple instrumentation, over 10-12 weeks are needed to obtain the result, due to the slow-growing nature of Mtb. This results in significant delay in diagnosis and increases the probability of transmission of DR strains in the population. With such long turnaround time, follow-up of TB patients from remote areas is problematic and therefore it is difficult to include TB patients within the framework of TB drug resistance surveillance. In comparison with solid culture methods, DST in liquid culture is more sensitive and requires shorter turnaround time (2-3 weeks). Despite these advantages, liquid culture systems still face several challenges for large-scale implementation, including (i) requirement for biosafety level 3 facilities, (ii) increased risk of contamination, (iii) extensive staff training, (iv) high level of quality control for microscopy, (v) rapid communication of results, and (vi) relatively high cost of reagents and instrumentation. Thus, these challenges render liquid DST culture systems unsuitable for POC testing.

2.1.2 Microscopic observation drug susceptibility (MODS)—The MODS assay is a direct DST test in which processed clinical samples are directly inoculated in drug-free and drug containing medium. This assay is based on visualization of characteristic cord-like formation of Mtb in liquid culture media when imaged under an inverted light microscope

[16]. Cord factor (trehalose 6,6′ dimycolate), a glycolipid that is present on Mtb cell wall promotes adhesion of bacterial cells and results in the formation of serpentine cords. In the presence of anti-TB drugs, drug susceptible Mtb strains do not form cord-like structures, whereas cord formation is unaffected with DR-Mtb strains. Compared to indirect DST on solid or liquid culture systems, this method can shorten the assay time to approximately 1 week. In addition, the MODS assay provides comparable sensitivity (92%) and specificity (96%) in reference to culture methods [17]. However, there are few drawbacks that prevent wide application of MODS at the POC, including the need for well-trained staff, laboratory infrastructure and an expensive inverted microscope (˜$5,000). A recent meta-analysis showed that MODS assay for rifampicin resistance had pooled estimates of 98.0% (95% CI 94.5-99.3) for sensitivity and 99.4% (95.7-99.9) for specificity, whereas for isoniazid resistance, pooled sensitivity was 97.7% (94.4-99.1) and pooled specificity was 95.8% (88.1-98.6) [18]. More clinical trials are required to validate this method in terms of accuracy for rapid detection of XDR-Mtb strains.

2.1.3 Phage amplification assays—Phage amplification assays are based on the detection of amplified progeny phages to reflect the viability of Mtb in sputum samples [19]. For identification of DR strains, amplified progeny phages are detected in the presence of anti-TB drugs. Biotec Labs, Ipswich, Suffolk, U.K. and Foundation of Innovative New Diagnostics (FIND) have developed *FastPlaque TB*™ and *FastPlaque-Response*™ assays for the detection of TB and rifampicin-resistant TB. The *FastPlaque-Response*™ assay relies on incubation of decontaminated sputum samples (*i.e.,* pretreated with n-acetyl-L-cysteine-sodium hydroxide) with or without rifampicin, followed by addition of bacteriophages that infect viable Mtb. Extracellular bacteriophages are inactivated by addition of a virucidal solution, whereas intracellular bacteriophages produce progeny bacteriophages, which in turn infect sensor cells (rapidly growing nonpathogenic host cells). After 48 hours, the amplified phages are then visualized as “plaques” (clear zones) in a lawn of host sensor cells. The appearance of plaques in the presence of rifampicin reflects the viable rifampicin-resistant Mtb [20], and the number of plaques correlates with the number of resistant Mtb cells. Compared to MODS and conventional culture methods, the phase amplification assays can be performed within 48 hours, while providing a comparable sensitivity of 95% and specificity of 97% for rifampicin resistance determination [21]. However, phage amplification assays suffer from the requirement of a high level of safety and quality control, risk of contamination, and difficulty in operation and data interpretation. Moreover, the application of phage amplification assays for identification of Mtb strains resistant to other anti-TB drugs is yet to be evaluated.

2.1.4 TK medium—TK MEDIUM® is a ready-to-use differential solid medium used for rapid determination of mycobacterial growth. *Mycobacterium* produces enzymes and metabolites that change the color of dye indicators present in TK medium from red to yellow, which can be used to indicate positive identification [22]. The color change usually takes 15 days, thus eliminating the need to wait for visualization of colonies that usually takes much longer time (3-6 weeks). TK medium can detect the contamination of fungi and Gram-negative bacteria by changing color from red to green. In addition, TK medium has also been utilized for DST for distinguishing susceptible strains from DR strains. In the

presence of anti-TB drugs, a color change from red to yellow indicates DR strains, whereas no change in color (red to red) indicates drug susceptible strains. A recent study, utilizing 16,303 clinical samples, showed that the TK Rapid Mycobacterial Culture System is a practical and reliable automated system that shortens the time required for both culture and susceptibility results [23]. Although this test is simple, easy to visualize and rapid, further clinical validation is needed.

2.2 Commercial molecular genotypic assays

NAATs has become an important analytical tool for diagnosis and detection of drug resistance in various pathogens [24]. Herein, we discuss three commercially available NAATs for TB: Innogentics's INNO-LiPA Rif. TB for diagnosing rifampicin-resistant TB, Hain Lifescience's Genotype MTBDRplus for diagnosing rifampicin and isoniazid-resistant TB, and Cepheid's GeneXpert MTB/RIF for diagnosis of rifampicin-resistant TB. These tests relies on detection of mutations in *rpoB* (encodes bacterial RNA polymerase), *inhA* (encodes enoyl-acyl protein reductase), and *katG* (encodes heme containing catalase-peroxidase enzyme) genes.

2.2.1 Line probe assays: INNO-LiPA Rif. TB and Genotype MTBDRplus—Line probe assay (LPA) is a strip test that identifies mutations in Mtb *rpoB*, *katG*, and *inhA* genes. In 2008, the WHO endorsed molecular LPA for TB drug resistance diagnosis. Currently, two companies have commercialized LPA for DR-TB, Innogentics, Belgium (INNO-LiPA Rif. TB) [25] and Hain LifeScience, Germany (Genotype MTBDRplus test) [26]. Both assays rely on reverse hybridization of PCR amplicons to complementary probes (sequences specific to wild type and common mutations in these three genes). Specifically biotinylated DNA amplicons are generated by PCR, and then captured to complementary DNA probes coated on a lateral flow strip. The alkaline phosphatase labeled streptavidin then binds to biotinylated DNA amplicons. Upon an enzymatic reaction, colorimetric bands develop that can be visually detected to indicate the presence of target DNA. Both tests are capable of detecting mutations in the *rpoB* gene conferring rifampicin resistance. In addition to rifampicin resistance, Genotype MTBDRplus can detect specific mutations in *katG* and *InhA* genes conferring isoniazid resistance and is thus applicable in MDR-TB diagnosis. Compared to standard culture methods, LPA is fast and only takes approximately 5 hours for positive TB identification directly from sputum or cultured samples. Both methods have sensitivity and specificity of more than 95% for the detection of rifampicin resistance in clinical isolates or clinical sputum samples [26, 27]. However, there is a need for trained personnel and sophisticated laboratory infrastructure to perform this assay. Another in-house LPA assay utilizing a reverse line blot hybridization (RLBH) principle has shown the capability of detecting XDR-TB strain, resistant to isoniazid, rifampicin, fluoroquinolones and second-line aminoglycosides with high sensitivity (>94%) [28]. However, the RLBH test is time-consuming and technologically complex, limiting its applicability at the POC.

2.2.2 Cepheid's GeneXpert MTB/RIF—GeneXpert MTB/RIF performs simultaneous detection of both susceptible and rifampicin-resistant strains of Mtb within 2 hours. Recently, the WHO has strongly endorsed this test for TB diagnostics at district or sub-district level hospitals. This assay relies on the PCR-based detection of Mtb *rpoB* gene and

mutations associated with rifampicin resistance [29]. Resistance to rifampicin can be detected with a sensitivity of 94% and specificity of 98%, which are comparable to MODS assay [30]. However, this test is not an ideal choice for POC applications, as the instrument itself costs about $17,620 and each cartridge costs around $10. Further, the system requires an uninterrupted electrical power source and a computer for programming, which may hinder its implementation and accessibility in frequently power shortage affected areas.

3. Recent advances in nano/microscale technologies for TB/DR-TB diagnosis

3.1 Nano and microscale-based NAATs

Nanotechnology offers unique optical, electrical and biocompatible properties that have been harnessed to improve sensitivity of geno/immunosensors [31, 32]. Integration of gold nanoparticles, magnetic nanoparticles or carbon nanotubes into microscale technologies has provided highly sensitive portable tools to rapidly diagnose infectious diseases at the POC [33-35]. Although many commercial NAAT systems have been developed for accurate, sensitive and specific detection of Mtb, they are generally bulky, costly, and have not yet been widely available at the POC [36]. To be applicable at POC settings, the device needs to be fully integrated to perform nucleic acid isolation, amplification and on-chip detection, leading to a simple sample-in-answer-out system. In this section, we present the latest advances in nano/microscale technologies for efficient on-chip nucleic acid isolation, amplification and detection for potential diagnosis of DR-Mtb at the POC.

Nucleic acid isolation is a prerequisite for most genosensors employing DNA detection for infectious disease diagnostics. Current methods for nucleic acid isolation are laborious, challenging, and time-consuming, and require centralized laboratory infrastructure. In comparison, microfluidic based nucleic acid extraction technologies offer unique advantages such as portability, low sample volume, low-cost and short assay time that are critical for POC diagnostics [35]. Most of these technologies rely on capture materials packed inside a microfluidic channel, allowing for efficient DNA capture and release for subsequent amplification/detection steps. For example, silica beads packed in glass microchannels enabled purification of DNA from whole blood [37]. In another study, nucleic acid was adsorbed in the presence of chaotrophic salt guanidine hydrochloride to high surface area glass micropillars, which were itched inside serpentine microchannels [38]. Low ionic strength buffer was then used to release the purified nucleic acid for downstream DNA amplification. Similarly, microposts [39], magnetic micro/nanoparticles [40], and plastic biochips [41] have been used in microfluidics for on-chip nucleic acid isolation.

In addition to nucleic acid isolation steps, integration of amplification and detection steps into a microscale instrument still poses significant challenges for POC testing. For example, on-chip PCR amplification often requires incompatible complex heating and electronic modules. Several studies have thus implemented alternative nucleic acid amplification strategies at a single temperature such as loop-mediated isothermal amplification (LAMP) [42], which can be easily integrated into a microchip. A recent study demonstrated that a self-heating device can be activated by mixing water with Mg-Fe alloy. This resulted in an

exothermic reaction and in turn provided a constant temperature for LAMP reaction [43]. Similarly, an electricity-free chemical device utilizing exothermic reaction of calcium oxide and water was used to provide a constant temperature for LAMP reaction [44]. The use of isothermal amplification significantly simplifies the development of fully integrated devices for TB or DR-TB diagnosis at the POC.

After the amplification step, DNA products are flowed through a microchip for singleplex or multiplex detection. Amplified DNA can be captured by complementary sequences coated on the surface of a planar microchip or beads/nanoparticles. The captured DNA amplicons can then be labeled via nucleic acid hybridization with a DNA probe conjugated with molecules such as enzymes, magnetic particle labels, gold nanoparticles to target gene for nucleic acid detection. These labels allow for the detection of signals resulting from colorimetric change [45], fluorescence labeling [46], nuclear magnetic resonance (NMR) [47], and giant magnetic resistance (GMR) [48]. These detection strategies are either integrated onto a microfluidic device or in an external benchtop instrument such as a fluorescence/colorimetric reader to perform detection and analysis.

The NAAT steps in nano/microscale devices demonstrated till date for TB diagnostics can be divided into two categories: (i) steps performed online (*i.e.*, inside of the chip/device), and (ii) steps performed offline (*i.e.*, outside of the chip or device). We consider partial/semi integration, if any of the steps are performed offline, which may hinder POC testing. Mtb gene detection employs sequential steps that are performed inside and outside of a microscale device include (i) sputum collection from a patient and decontamination, (mostly offline), (ii) Mtb concentration (mostly offline by centrifugation), (iii) lysis and nucleic acid extraction, (online/offline), (iv) nucleic acid amplification (online/offline), and (v) detection of amplified nucleic acid (mostly online) in singleplex or multiplex manner. Development of a fully integrated system from sample decontamination to detection of amplified genes has been a highly complex and challenging process. To date, no commercial method except for the GeneXpert MTB/RIF benchtop system enables fully integrated steps from sample-in to TB/DR-TB detection-out with sensitivities and specificity of over 90% for smear-positive patient samples, and over 85% for smear-negative patient samples [49]. Thus, there is a critical need for fully integrated portable systems that perform sample-in to detection-out at low-cost, with POC applications towards diagnosis in resource-constrained settings [10, 50-59]. Many technologies exist with varying levels of integration, ease-of-use, user intervention and implementation to POC settings [60].

3.1.1 Fully integrated devices for TB/DR-TB detection—Recently, a fully integrated thermoplastic microfluidic device providing sample-in-result-out based technology was demonstrated to detect DR-TB from clinical sputum samples [61]. DR-TB identification was enabled by the detection of single nucleotide variations in *rpoB* gene frequently associated with rifampicin-resistant Mtb utilizing a PCR/ligase system [61]. The main advantage of the system is the use of inexpensive fluidic cartridge to perform multistep reactions such as cell lysis, solid-phase micropillar DNA extraction, PCR/ligase reaction, and endpoint array fluorescence detection (Fig. 1). The array-based detection readout system uses an external benchtop instrument (dimensions: 0.3 meter × 0.3 meter × 0.3 meter) to provide simultaneous differentiation/detection of DR strains from drug-susceptible strains. This

device can detect as low as 50 Mtb cells/mL from sputum samples in less than 30 minutes with a sensitivity 100-fold higher than smear microscopy, offering rapid and highly sensitive DR-TB diagnosis at the POC. The only exception that may limit its POC use is the need for an external benchtop instrument to perform fluid manipulation and optical detection in a microfluidic device. Further, clinical validation of this device is needed to assess the capability of detecting DR-TB in a large number of sputum samples.

Another fully integrated microsystem where magnetic beads were actuated inside a capillary tube to perform DNA extraction, amplification and fluorescence detection of mycobacterial DNA has been demonstrated [62]. Capillary tubes contained reagents that were separated by oil such as amplification reagents, lysis buffer, elution buffer and wash buffer. With the use of an external magnet underneath, magnetic beads were used to control the capture and release of DNA from clinical samples. The released DNA was then amplified using LAMP inside capillary with an onboard temperature control module. The generated amplicons were then detected by on-chip fluorescence imaging. The accuracy of this assay was validated using 32 Mtb positive and 10 Mtb negative sputum samples. Out of 32 Mtb positive samples, 31 were accurately detected, whereas 1 sample showed negative result due to a low abundance of Mtb DNA. The approach takes less than 50 minutes to complete and requires less than 10 μL of sample for analysis.

3.1.2 Partially integrated devices for TB/DR-TB detection—Among partially/semi-integrated microsystems, a magnetic barcode label approach for Mtb gene detection using a portable NMR fluidic chip was demonstrated [63]. In this system, the sample-processing step (*i.e.*, extraction of mycobacterial DNA) was performed off-chip using a mechanical method (shaking with glass beads). Extracted DNA was then introduced into a fluidic chip for PCR amplification of target Mtb genes such as acyl-CoA dehydrogenase *fadE15* and *rpoB.* Polymeric beads (diameter of 1μm) immobilized with complementary sequences capture one end of the amplicon, and the other end is labeled with a magnetic nanoparticle (diameter of 30 nm) via complementary sequences, thus forming a sandwich structure (Fig. 2). Finally, an on-chip NMR module with inbuilt electronics measures the changes in relaxation of 1H NMR signal associated with a local magnetic field surrounding magnetic particles for quantification of target Mtb genes. This method showed a better detection sensitivity of ~10^3 colony forming unit (CFU) per mL of sputum compared to sputum microscopy. Additionally, the device accurately identifies single nucleotide mutations in the *rpoB* gene, showing its application in rifampicin-resistant TB detection. The assay requires less than 2.5 hours for sample-in to answer-out. However, isolation of DNA requires additional offline steps that hinder the use at POC settings. With that exception, this method provides high potential for TB diagnostics in resource-constrained settings, where cost, portability and sensitivity are critical parameters.

In another example, a pouch based cartridge employing a lateral flow device was developed for POC TB diagnosis [64]. With the exception of offline mechanical bacterial lysis and DNA extraction, the remaining steps such as amplification and detection were performed on-chip utilizing an electrolysis pump, heater and lateral flow device. Inside the device, the electrolysis pump controls the flow of DNA sample, and the built-in heater provides a constant temperature to amplify Mtb genomic DNA by LAMP reaction. After DNA

amplification, the amplicons were detected on a lateral flow device, where dye doped polystyrene microspheres were used for labeling. The microspheres carrying amplicons were captured by specific DNA probes on the test lateral flow strip, and accumulation of the microspheres can be visually detected to indicate the presence of target DNA. The device costs less than $6, and the assay can be completed within 70 minutes. With simple controls for sample flow and heating for DNA amplification, as well as easy visual detection, the device, when fully integrated with sample processing, would enable portable, inexpensive and rapid TB/DR-TB diagnosis at the POC.

Another partially integrated portable microfluidic chip has been developed to enable sample-in to TB detection-out [65]. In this device, the microfluidic chip contains separate chambers for DNA extraction and amplification/detection. One isolated chamber was used to extract Mtb DNA using a lysis buffer at 80ºC. The crude bacterial lysate containing Mtb DNA was expelled from the isolated chamber to an amplification/detection chamber via vapor pressure through a controlled screw valve. DNA amplification was carried out by LAMP at a constant temperature of 65ºC maintained in a water bath. After DNA amplification by LAMP, a large amount of byproduct pyrophosphate ions bind to manganous ions and results in quenching of fluorescence emission from calcein. The deprivation of manganous ions from calcein provided a visual readout (green color), indicative of the presence of target DNA. In a multiplexed detection format, the device enabled specific detection and identification of Mtb DNA from mixed culture strains including genetically engineered *Escherichia coli*. The assay can be performed within 60 minutes with high specificity and sensitivity (270 copies/μL). Although the sample preparation, amplification and detection were integrated into a microfluidic chip, the amplification temperature needs to be controlled by a lab-based water bath, which is less ideal for POC testing.

3.1.3 Potential translation of paper-based microfluidic devices to fully/partially integrated TB/DR-TB diagnostics—Paper microfluidic platforms have provided access to rapid prototyping of disposable, inexpensive, and miniaturized devices to perform micro total analysis systems (μTAS) for quantitative and semi-quantitative medical and environmental assays [66, 67]. Among them, microfluidic paper analytical devices (μPADs) [68] have shown numerous applications in biomedical applications [69]. μPADs were made by impregnating paper with hydrophobic materials to enable multiplexed diagnostic approaches. These devices are considered to be simple, inexpensive, light-weighted and biocompatible. They enable self-driven fluidic flow due to paper porosity and provide a white background for high contrast colorimetric detection. Further, a cell phone was utilized for colorimetric detection/quantification, eliminating the need for expensive and sophisticated laboratory infrastructure [68]. Recently, a “Gold on paper” device coupled with a mobile camera was reported for TB diagnosis (Fig. 3) [70]. The molecular assay principle herein relies on colorimetric changes in the solution when gold nanoprobes coated with complementary DNA sequence bind to Mtb DNA. The solution of gold nanoprobes exhibits a strong red color due to plasmonic characteristics of gold nanoprobes at 520 nm. In the absence of target DNA, salt causes aggregation of nanoprobes and exhibits a blue color. In contrast, target DNA, prevents aggregation of nanoprobes, and the solution remains red. With the same principle, the detection of single nucleotide mutations within the *rpoB* gene

for DR-TB detection was reported [71]. Aiming at POC use, this colorimetric gold nanoprobe approach was integrated with a paper microfluidic device, where color intensities were analyzed using a mobile phone [70]. The assay requires less than 2.5 hours to specifically detect the *rpoB* gene of Mtb with a detection limit down to 10 μg/mL. The DNA detection limit needs to be further improved to detect the low abundance of Mtb DNA in sputum. In addition, the paper microfluidic device needs to be integrated with an onboard PCR module before it can be used at the POC. Nevertheless, paper microfluidic devices offer many advantages including reduced cost, simplified procedure, increased speed, and data transmission via a mobile phone to centralized laboratories for epidemiological surveillance and professional analysis.

3.2 Telemedicine and mobile phone-based whole bacteria detection platforms

The past decade has witnessed the emergence of telemedicine and mobile health platforms targeting clinical applications at the POC settings, where skilled technicians and complex equipment are lacking [72-77]. Recently, a cell phone was employed with an inverted microscope to collect and transfer images featuring cord-like patterns of Mtb to a remote site for professional analysis [78]. The apparatus consisted of a commercial inverted light microscope (Nikon Eclipse TS100-F) with a 10x objective and 10x eye-piece coupled to a 4 megapixel digital camera (Olympus C-3030). The captured images of MODS cultures were first saved and stored on a SD memory card, and then, transferred via a mobile phone (Nokia 6110) to an adhoc web server for image analysis by a MODS expert. The system was validated with 50 images of MODS cultures containing Mtb, 20 images of MODS cultures of atypical mycobacteria and 5 culture-negative images. Mobile phone-transmitted imaging presented a 98.7% of concordance with conventional microscope imaging (n = 75 tests). Thus, this strategy demonstrated that a mobile phone-associated platform could be used in a remote setting without the need for skilled personnel to identify cord-like formation in the MODS assay. Although this platform facilitates the diagnosis of TB and DR-TB, the use of an inverted microscope (>$5,000) is still not practical in resource-constrained settings. To reduce the total cost of microscopy-based MODS assays, two inexpensive portable inverted microscopes were developed [79]. The first prototype consisted of a 60-100× magnification pocket microscope (Radioshack MM-100, $10) and a standard USB 320×240 pixels resolution webcam. A 0.5 W standard incandescent light bulb and a simple condenser lens were used for illumination. The images were captured and stored using a laptop. A second stand-alone prototype (Fig. 4) utilizes a 100× magnifier, a modified digital camera with a SD/MMC memory slot, a TV output, and a 5-inch LCD monitor. The magnifier consisted of a 10× objective (achromatic, NA 0.25) and 10× eyepiece (Huygenian), which were integrated with a 45° mirror. A digital camera (Genius DV600, working at resolution of 2048×1536 pixels) and a 12 V, 50 W Philips halogen dichroic lamp ($2) were mounted to the platform. Nine-day old MODS cultures were then tested using this prototype in manual and digital modes. In the manual mode, readings from a Nikon microscope and the prototype compared by the same technician, showed agreement of 96.6%. In the digital mode, the agreement was 94.1%. These results demonstrated that an expensive inverted light microscope is not necessary for imaging MODS results, and therefore an inexpensive prototype could be implemented as a TB tele-diagnostic tool in resource-constrained settings.

3.3 Other rapid technologies for detecting Mtb in sputum samples

In this section, we discuss devices/technologies that can rapidly detect intact Mtb or the activity of enzymes secreted/expressed by Mtb in sputum samples. In combination with solid or liquid culture DST methods, these technologies hold the potential for detection of DR-Mtb strains. For example, a recent study showed the detection of Mtb using β-lactamase (BlaC) specific fluorescent probes [80]. The chemical probe structure consists of a cephalosporin and a blue fluorescent molecule (Umbelliferone). In the presence of Mtb, BlaC triggers the cleavage of chemical probes, leading to the emission of fluorescence. The assay requires less than 10 minutes to determine the presence or absence of Mtb in sputum. As an effort to enable POC testing, a cellphone was utilized for fluorescence detection, which detected as low as 50 CFU/mL of Bacillus Calmette Guerin (BCG) spiked in sputum samples. In another study, Mtb was enriched using a POC device and subsequently detected using a microtip sensor (Fig. 5) [81]. A combination of streamlined flow, electrohydrodynamic flow, and dielectrophoresis was used for Mtb enrichment. Concentrated Mtb cells in close proximity to the microtip were captured by specific antibodies. Once Mtb cells were captured, the tip was withdrawn and dipped in fluorescence-labeled antibodies for immunosensing. The assay was completed within 25 minutes (15 minutes for liquefaction of sputum, 3 minutes for concentration, and 7 minutes for fluorescence labeling and signal processing). The detection limit was 200 CFU/mL using Mtb H37Ra spiked in sputum, and the device detected 23 out of 24 of these samples. The platform technology needs to be validated for accuracy with real sputum samples from TB patients.

For direct detection of Mtb, a study demonstrated enrichment of bacteria using magnetic nanoparticles and subsequent detection using a portable NMR [82]. In the first step, BCG was labeled with BCG specific magnetic nanoparticles (diameter of 16 nm). Bacteria labeled with superparamagnetic nanoparticles were then concentrated using a membrane filter integrated to a microfluidic chamber. After washing off unlabeled nanoparticles, only the bacteria labeled with magnetic particles were detected by monitoring changes in spin-spin relaxation time (T2) using microcoil (on-chip NMR) attached to the microfluidic chamber. The assay provides a detection limit of 20 CFU/mL of BCG in sputum samples and requires less than 30 min for completion of assay. These technologies promise to provide user-friendly, accurate, portable and rapid approaches for direct detection of Mtb in resource-constrained settings. A brief overview of nano/microscale approaches for diagnosis of drug-susceptible and DR-TB is summarized in Table 2.

3.4 Portable antimicrobial susceptibility testing (AST) for other DR pathogens

The AST microfluidic platforms provide access to monitor drug resistance of pathogens at the POC owing to simplicity, low-cost, and portability compared to conventional AST. For example, an autonomous microfluidic platform was developed to enable rapid determination of minimum inhibitory concentration (MIC) of antimicrobial agents against bacteria [83]. This platform used a degassed microfluidic device, with each chamber containing varying levels of dried antibiotics (Fig. 6). After sample addition, degas-driven flow enabled mixing of the bacterial suspension with dried antibiotics. Inside the chamber, growth of bacteria changed pH of suspension and resulted in a colorimetric change of pH indicator that can be

visualized by the naked eye. This platform provided MIC of various antibiotics against *Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae* and *Escherichia coli,* which was comparable to standard liquid broth dilution methods. The capability of this device was further expanded to detect a panel of pathogens at the POC in less than 4h [84]. In another platform, a portable paper device was used to monitor AST for *Salmonella typhimurium* and *Escherichia coli.* The device was fabricated using widely available materials such as paper, masking tape and PDMS membrane, and initial studies showed successful culture of bacteria on the device [85]. The growth of bacteria (*e.g., E.coli*) was then quantified with a fluorescent reporter enabling a detection limit of 10-100 CFU/mL. This portable-paper device was also used for susceptibility testing by measuring the size of bacterial inhibition areas in the presence of various drugs during bacterial culture [86]. Due to its portability, low-cost and easy-to-use interface, this paper platform enhances accessibility of AST in resource-limited settings. Due to simplicity and self-loading feature (*i.e.*, no requirement for additional external components such as pumps, electrical power, and detection modules), these platforms hold potential for initial screening and rapid identification of “superbugs” including MDR/XDR Mtb strains at the POC.

4. Unmet need for diagnosing DR-TB

Timely diagnosis of Mtb is needed to reduce treatment delay and dropout, and also to minimize the chances of generating DR-Mtb strains. This is because of the lengthy treatment regimens caused by poor patient adherence, leading to emergence of MDR and XDR strains and subsequent spread in population. Once drug resistance occurs, the choice of anti-TB drugs to initiate treatment is limited. Although new anti-TB drugs are under development, they are still in stage II/III clinical trials. Furthermore, diagnostic tools for closely monitoring anti-TB treatment are lacking. Traditional phenotypic assays are lengthy, laborious and infrastructure-dependent, and thus they are not practical to be implemented in decentralized laboratories for routine clinical use. This has created a global concern for the prevention and control of TB.

The challenges to develop rapid TB diagnostics that detect DR-Mtb strains are multifold. First, the lack of sufficient financial support, poor laboratory infrastructure, and shortage of well-trained operators in developing countries like India and China leads to improper management of TB cases and patient care. Second, there is lack of incentive to develop effective biomarkers, as opposed to drug development, that can be integrated into POC diagnostics. Third, novel diagnostic platforms that are rapid, simple, reliable and inexpensive need to be created for POC testing in resource-constrained settings. Although PCR and ELISA are widely used to develop TB diagnostics, these two platforms are still limited by assay cost, complexity and turnaround time. At the primary care in TB clinics, physicians often need to know the susceptibility of their patients to current TB drugs for optimal treatment, especially in DR-Mtb dominant regions. Thus, new diagnostic and treatment monitoring platforms that can yield reliable results in 30 minutes without reference to skilled operators or sophisticated instruments are urgently needed.

5. Concluding remarks

Infectious diseases such as TB remain a major threat to public health worldwide. The emergence of DR-Mtb strains demands early diagnosis and close monitoring of patients. However, the lack of drug resistance testing at the POC inevitably fuels the spread of DR strains. To strengthen patient care and establish surveillance networks, simple, inexpensive and rapid diagnostics are urgently needed to detect active TB cases and assess drug susceptibility. Emerging nano/micro-technologies due to significant advantages in portability and sensitivity have shown the trend toward creating fully-integrated diagnostic platforms for DR-TB detection at the POC. However, it should be noted that discovery of biomarkers is lagging behind, which is a significant barrier in translating the use of novel diagnostic platforms to clinic. Recently, next-generation sequencing (NGS) technologies, as an emerging high-throughput platform, have been widely used to identify new biomarkers and design novel molecular diagnostics tools. In November 2013, the US Food and Drug Administration (FDA) granted the first clearances of NGS based assays and high throughput DNA sequencing analyzers for molecular diagnosis of cystic fibrosis [87]. However, the application of NGS technologies for POC testing or treatment monitoring still faces many challenges, such as the requirement for multiple steps in the assay workflow including template preparation, sequencing and imaging, and high-throughput bioinformatics analysis [88]. Inherently, NGS technologies require expensive instruments and reagents, along with well-trained staff to perform sequencing and data interpretation. These restricting factors significantly limit NGS technologies for POC testing according to the ASSURED criteria (Affordable, Sensitive, Specific, User friendly, Reliable and robust, Equipment-free and Deliverable to those who need it) recommended by the WHO [89]. Nevertheless, NGS at a centralized facility have assisted researchers in identifying mutations conferring resistance to Mtb [90] and other infections. Future successes in developing reliable assays for POC detection of DR-TB could benefit from further refinement of nano/micro-technologies to reduce cost and increase clinical utility, as well as from biomarker discovery by high throughput sequencing technologies.

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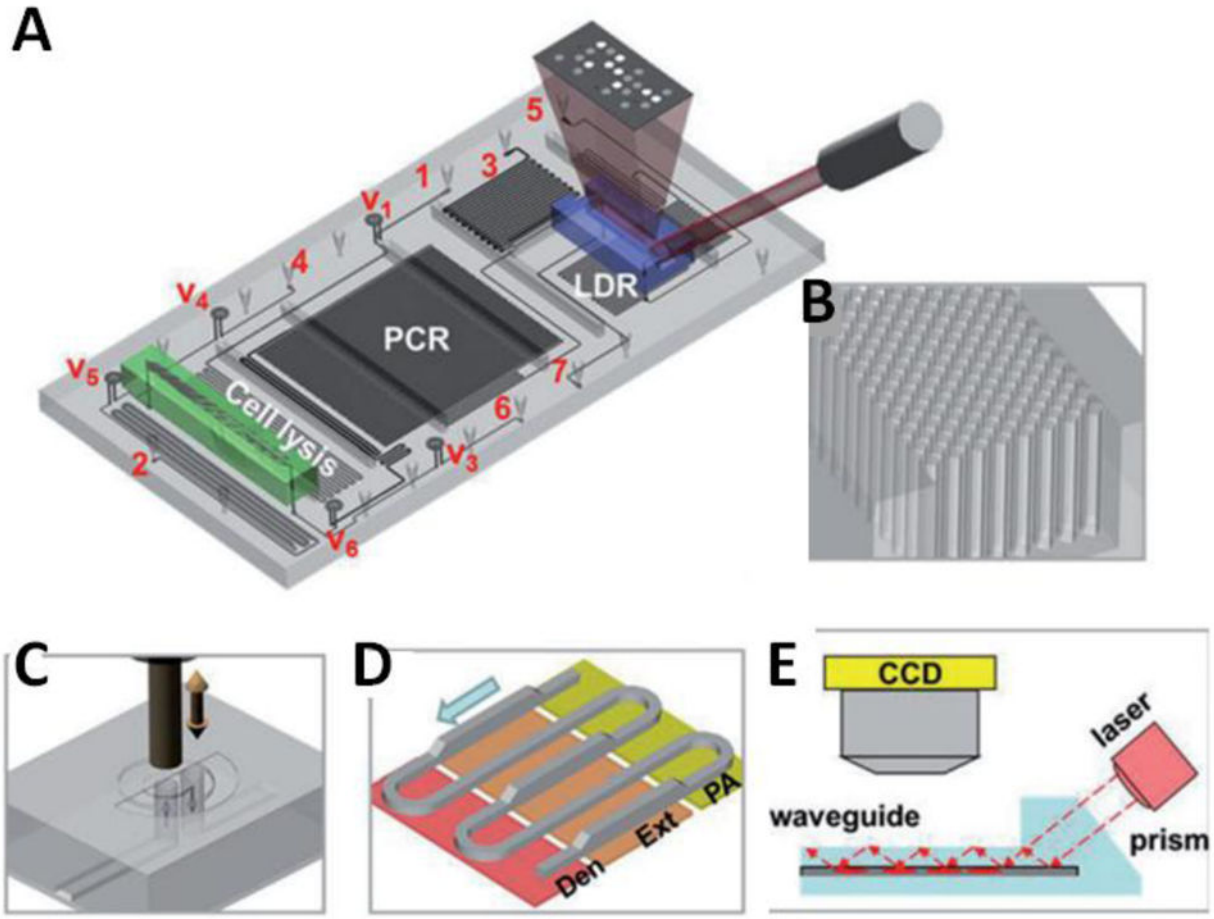

Fig. 1. A fully integrated microfluidic device for detection of DR-TB

(Adapted with permission from ref [61]). A) Schematic of a fluidic cartridge integrated with sample and reagent inlets/outlets (1-6), membrane valves (v1-v6) and serpentine reaction chambers that perform DNA extraction, PCR amplification, ligase detection reaction, and endpoint array-based fluorescence detection. B) A close-up view of polycarbonate pillars with a high-aspect ratio for solid-phase DNA extraction on-chip. C) A close-up view of membrane valve operation. D) Schematic of PCR reaction chamber with separate zones for denaturation, primer annealing and extension. E) Schematic of the detection module. It employs laser light that travels through a prism and waveguide for exciting fluorescent-labeled DNA probes hybridized to target DNA on the array surface, and the fluorescence signal is then detected using a CCD camera.

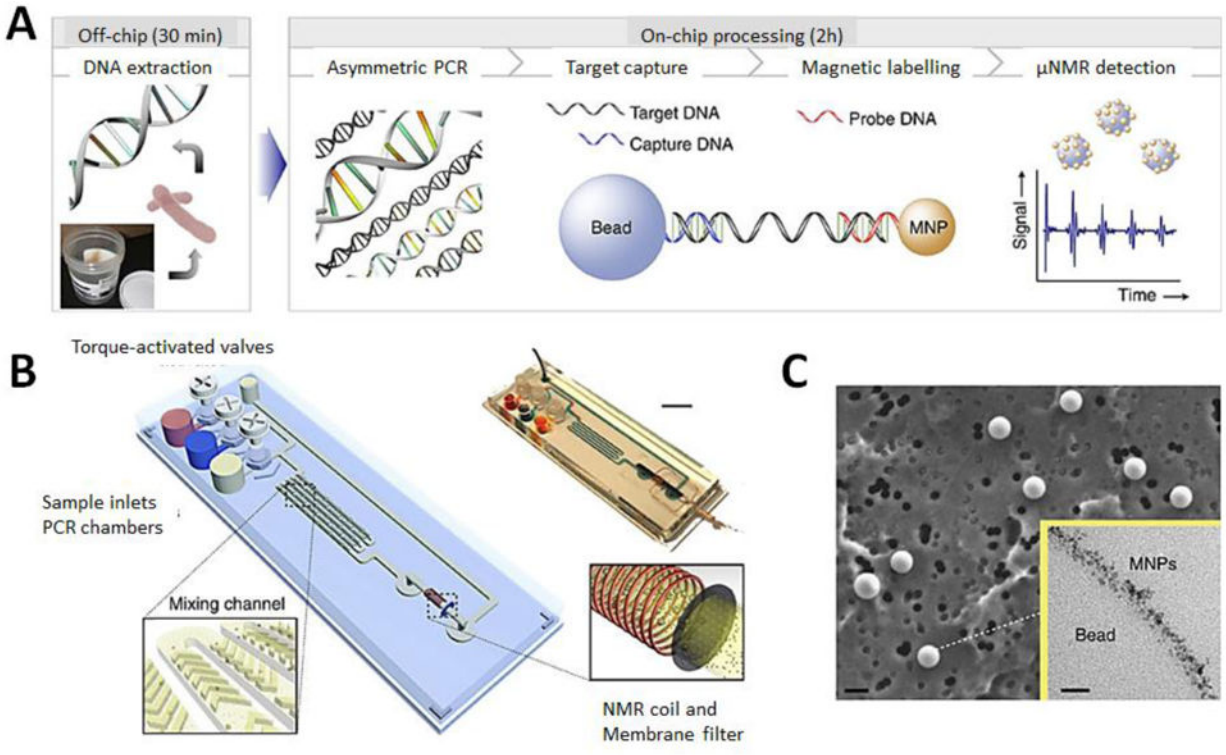

Fig. 2. µNMR fluidic chip that enables the detection of Mtb target DNA from sputum using a magnetic biobarcode approach

(Adapted with permission from ref [63], copyright 2011 Nature Publishing Group). A) Schematic of experimental procedures, which include off-chip DNA extraction from Mtb containing sputum sample and on-chip processing. Target DNA sequences first undergo asymmetric PCR amplification. The amplified DNA target sequences are then captured by microbeads coated with complementary DNA sequences. The captured target DNA sequences are then labeled with magnetic nanoparticle probes producing a bead-nanoparticle sandwich structure. Finally, the NMR signal decay of magnetic nanoparticles is measured using an onboard NMR device that correlates to the concentration of DNA target. B) Picture of a fluidic chip accommodated with fluidic reservoirs containing Mtb genomic DNA, beads with capture DNA and magnetic nanoparticle probes. Release of samples and reagents is enabled by a screw cap, followed by a mixing of reagents and membrane filtration. A membrane filter is used to remove the unlabeled magnetic nanoparticle probes by washing. Finally, the NMR signal decay from magnetic nanoparticles are detected by a NMR coil on board. C) A scanning electron microscopy (SEM) image of beads on a membrane filter and (inset) close up image of magnetic nanoparticles bound to beads.

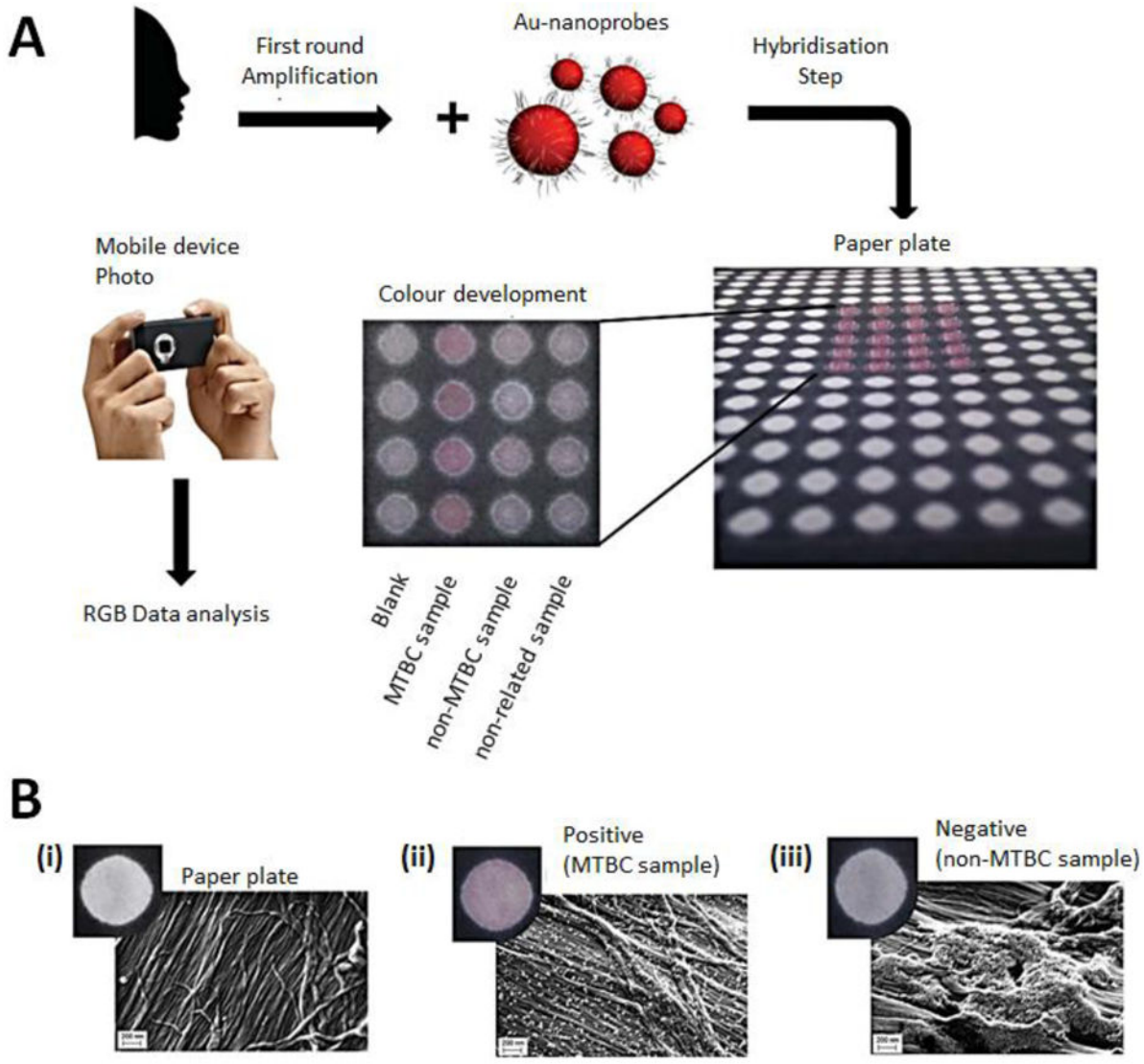

Fig. 3. Gold-nanoprobe based Mtb gene detection in a paper microfluidic device

(Reproduced with permission from ref [70]). A) Schematic representation of a colorimeteric assay utilizing a gold nanoprobe startegy in a paper microfluidic device for the detection of Mtb DNA. The assay procedure involves color change on paper hydrophilic spots upon aggregation of gold nanoprobes. In the absence of target DNA (non-Mtb sample), salt impregnated on paper induces nanoparticle aggregation and changes the color to blue, whereas the presence of target DNA (positive Mtb sample) specific to gold nanoprobes results in a red color (non-aggregated nanoparticles). The color change in the presence/absence of target DNA is then captured by a mobile phone camera for RGB analysis. B) SEM images of (i) paper hydrophilic spot, (ii) nanoprobes in the presence of target DNA (Mtb sample) showing a red color on paper, and (iii) salt-induced aggregation of nanoprobes in the absence of target DNA (non-Mtb) showing a blue color on paper.

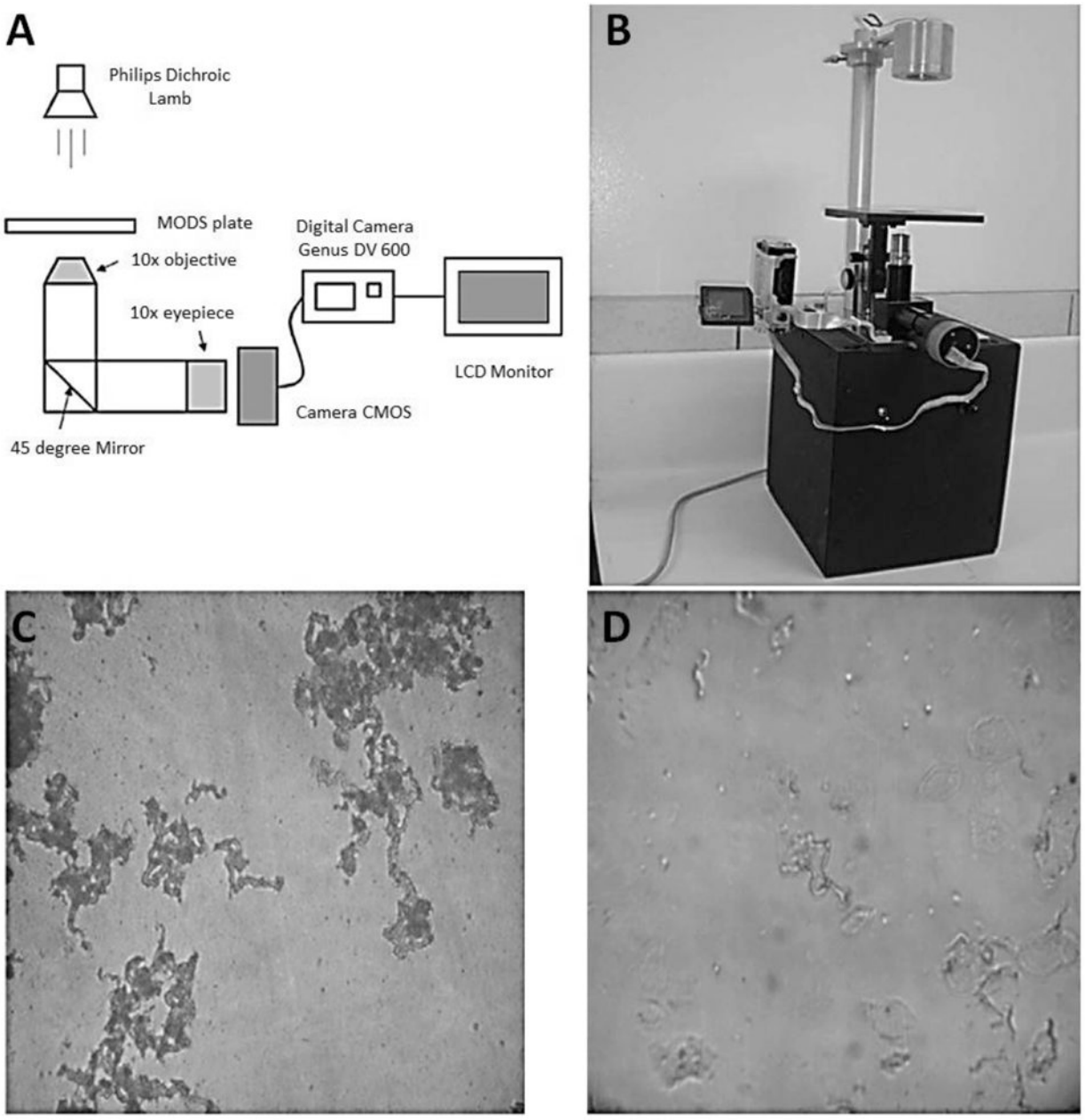

Fig. 4. A mobile platform for detection of whole Mtb bacteria

(Adapted with permission from ref [79]). A) Schematic of the principle of the mobile platform demonstrated as second prototype in ref [79]. The system comprised of a magnifier and a digital camera with an output to a digital screen. B) A photograph of second prototype system. C) A digital image of cording pattern of Mtb at day 9 in MODS, captured using a NIKON Eclipse TS100-F inverted microscope (100x) integrated with a 2 Megapixels Olympus CCD camera. D) A digital image of cording pattern of Mtb at day 9 in MODS, captured using second prototype consisting of a 100× magnifier, a modified digital camera with SD/MMC memory slot, a TV output, and a 5-inch LCD monitor.

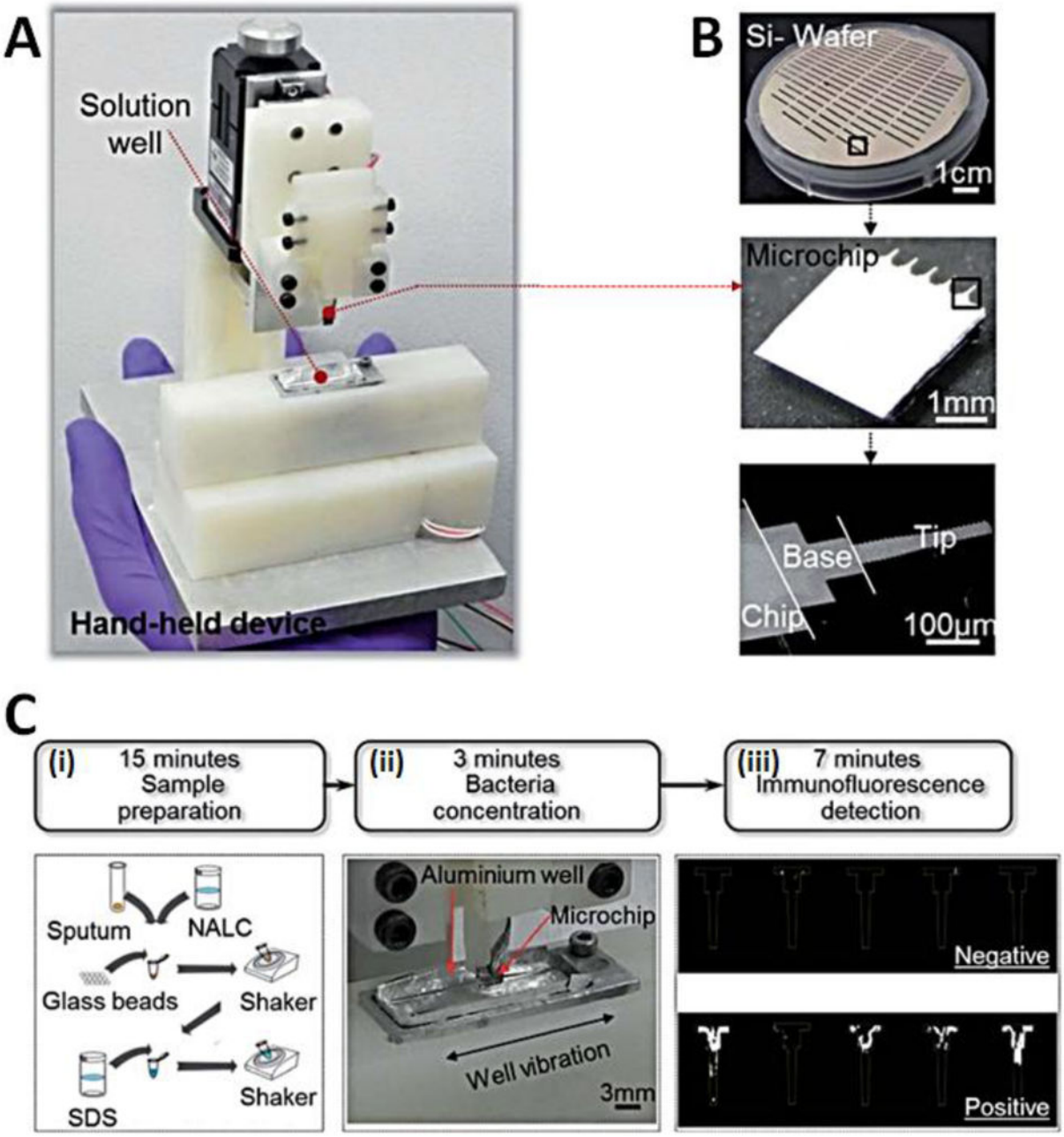

Fig. 5. Microtip-immunosensor platform for the detection of whole bacteria

(Reproduced with permission from ref [81]). A) Picture of hand-held device containing a microtip sensor (withdrawn from sample solution) and sample well. B) Picture of microtip where the immunoassay was performed. C) Assay procedure for BCG bacteria detection using a microtip immunosensor. The assay was performed in three steps: (i) Sputum liquefaction (15 minutes), (ii) Concentration of bacteria near the vicinity of antibody-coated tip using streamlined flow & electrohydrodynamic flow (3 minutes), and (iii) Labeling of captured bacteria on microtip with fluorescence antibodies and subsequent immunofluorescence detection (7 minutes).

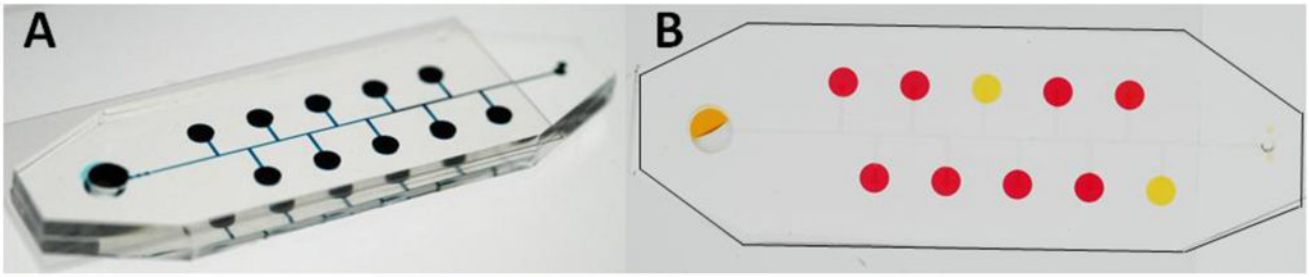

Fig. 6. Autonomous microfluidic device for the measurement of MIC of antibiotics against bacterial pathogens

(Figures are courtesy of Prof. Douglas B. Weibel). A) Image of microfluidic device integrated with a degas-driven flow. B) Image represent measurement of MIC of wild type and antibiotic resistant pathogen strains. Yellow spots indicate bacterial growth, whereas red spots indicate bacterial growth inhibition or no growth.

Table 1

Current approaches for detecting drug-susceptible and DR-TB

Tools/Technologies	Target detection methodology	Drug resistance testing? (Yes/No)	Sensitivity/specificity	Time of assay	Estimated cost per test	Potential POC application? (Yes/No) and Why?
Conventional solid/ liquid culture methods (indirect DST)	Accesses the growth inhibition of Mtb colonies in the presence/absence of anti-TB drugs	Yes, all anti-TB drugs	Standard method	8-12 weeks	~$30	**No**, slow process, requires laboratory infrastructure and a trained personnel
Microscope observation drug susceptibility (MODS) [18]	Visualization of cord like formation of Mtb in the presence/absence of anti-TB drugs	Yes, all anti-TB drugs	Rifampicin resistance: 98%/99% Isoniazid resistance: 98%/96%	9-11 days	$2; materials and supplies	**No**, limited by need for laboratory infrastructure, expensive microscope and long assay time
FastPlaque-Response bacteriophage assay [21]	Accesses the inhibition of plaque formation in the presence/absence of anti-TB drugs	Yes, rifampicin	Rifampicin resistance: 95%/97%	2 days	~$40	**No**, limited by need for biosafety cabinets, ease of contamination, and requires trained staff and continuous power supply
TK medium [23]	Visualization of change in the color of culture medium in the presence/ absence of anti-TB drugs	Yes, rifampicin and isoniazid	Drug susceptible: 96%/100%	2 weeks	Not available	**No**, limited by longer time of assay, and lack of clinical studies
Line probe assays by INNO-LiPA Rif TB[27]	Employs PCR and reverse hybridization on a lateral flow device to detect Mtb genes and mutations conferring resistance	Yes, rifampicin resistance	Rifampicin resistance: 100%/97%	5 hours	$45 per sample	**No**, expensive test, uses bulky thermocycler for PCR amplification
Line probe assays by Genotype MTBDRplus [26]	Same principle as above	Yes, rifampicin and isoniazid resistance	Rifampicin resistance: 98%/99% Isoniazid resistance: 84%/99%	5 hours	$50-110 per samples per run	**No**, same as above
Cepheid's GeneXpert MTB/RIF [30]	Automated system that performs Mtb *rpoB* gene and specific mutations detection. The instrument is integrated with sample preparation, PCR and detection	Yes, rifampicin resistance	Rifampicin resistance: 90%/94%	2 hours	$10-15 per cartridge $17,620 for cost of instrument	**No**, limited by expensive & bulky instrumentation, need for continuous power supply, regular maintenance

Table 2

Nano/microscale approaches for TB and DR-TB diagnosis

Tools/Technologies	Target detection methodology	Drug resistance testing? (Yes/No)	Sensitivity/specificity or LOD	Time of assay	Estimated cost per test	Potential POC application? (Yes/No) and Why?
Thermoplastic microfluidic device [61]	Fully integrated device, which performed cell lysis, DNA extraction, amplification and fluorescence detection in a thermoplastic microfluidic device.	Yes, rifampicin resistant strains	LOD: 50 Mtb/mL	30 minutes	Not reported	**Yes**, no operator intervention, capabilities of multiplexing & drug resistant detection.
Magnetic barcode label [63]	Partially integrated microfluidic device that employed a portable NMR to detect Mtb gene and associated mutations using magnetic biobarcode approach	Yes, rifampicin resistance	LOD: 10^3 Mtb/mL sputum	2.5 hours	$4300 for one time instrument $3 fluidic device	**Yes**, Limited user intervention, small device footprint
Capillary tube-DNA analysis [62]	The device employed capillary tubes with onboard reagents for Mtb DNA detection using magnetic particle capture and LAMP amplification	Not shown	LOD: 10 BCG cells	50 minutes	Not reported	**Yes**, Due to shorter time of assay, smaller device footprint
Pouch based cartridge [64]	Portable device containing lateral flow membrane to monitor Mtb DNA bands. DNA was amplified using onboard LAMP	Not shown, but possible	3000 copies of Mtb DNA-prototype 1 $6x10^5$ copies of Mtb DNA-prototype 2	Prototype 1: 20 min, Prototype 2: 70 min	$6 for device and reagents	**Yes**, due to device portability, low cost and accurate DNA detection
Portable microfluidic chip [65]	Device employed a microfluidic channel to perform DNA extraction, and LAMP amplification, and naked eye detection.	Not shown, but possible	LOD: 27-270 copies/μL of DNA	30-60 minutes	Not reported	**Yes**, uses simple device that performs nucleic acid extraction amplification and visual detection.
Paper microfluidics [70]	Device employed a paper hydrophilic spots to perform Mtb DNA detection using gold nanoparticle probes.Change in color of nanoparticles upon target presence was measured using mobile phone camera.	Not shown, but possible	LOD:10 μg/mL DNA	2 hour 30 minutes	Not reported	**Yes**, uses low cost flexible material, and mobile phone to transmit images to remote location for timely diagnosis.
MODS integrated with cellphone [78]	Digital camera was used to capture images of cord like formation of Mtb. Captured images were then transferred to cellphone for data transmission to web server for expert advice.	Not shown, but possible	Not reported	9-11 days for MODS	Not reported	**Yes**, test results can be transmitted to expert, particularly useful in remote location for timely patient care.
MODS integrated with digital camera (second prototype) [79]	Images of cord formation of Mtb were captured by CMOS sensor and visualized using digital camera.Images can be monitored by experts for timely diagnosis	Not shown, but possible	Not reported	9-11 days for MODS	$320 for the prototype device	**Yes**, uses widely available digital camera to capture

Tools/Technologies	Target detection methodology	Drug resistance testing? (Yes/No)	Sensitivity/specificity or LOD	Time of assay	Estimated cost per test	Potential POC application? (Yes/No) and Why?
						the images for expert analysis the images for expert analysis
Enzymatic activity detection [80]	BlaC enzyme expressed by Mtb triggered the release of fluorescent molecule from BlaC-specific fluorogenic probes	No	LOD: 50 CFU/mL BCG	10 minutes	Not reported	**Yes**, due to simplicity of assay and rapid assay procedure
Microtip sensor [81]	Mtb detected on a microtip sensor using immunofluorescence. Mtb concentrated using streamlined flow, electrodynamic flow for improved sensitivity	No	Sensitivity: 96%	25 minutes	Not reported	**Yes**, uses a portable device with rapid assay procedure that is ideal for resource constrained settings
Portable NMR-based bacterial detection [82]	Mtb in sputum sample was labeled by magnetic particles, concentrated by a filter membrane and detected by a portable NMR	No	LOD: 20 CFU/mL BCG	30 minutes	Not reported	**Yes,** small device foot print, accurate, and rapid testing

LOD – Limit of detection; CFU – Colony forming unit