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A rapid amplification/detection assay for analysis of *Mycobacterium tuberculosis* using an isothermal and silicon bio-photonic sensor complex

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ABSTRACT

Global tuberculosis (TB) control is hampered by cost and slow or insensitive diagnostic methods to be used for TB diagnosis in clinic. Thus, TB still remains a major global health problem. The failure to rapidly and accurately diagnose of TB has posed significant challenges with consequent secondary resistance and ongoing transmission. We developed a rapid *Mycobacterium tuberculosis* (MTB) amplification/detection method, called MTB isothermal solid-phase amplification/detection (MTB-ISAD), that couples isothermal solid-phase amplification and a silicon biophotonics-based detection sensor to allow the simultaneous amplification and detection of MTB in a label-free and real-time manner. We validated the clinical utility of the MTB-ISAD assay by detecting MTB nucleic acid in sputum samples from 42 patients. We showed the ability of the MTB-ISAD assay to detect MTB in 42 clinical specimens, confirming that the MTB-ISAD assay is fast (<20 min), highly sensitive, accurate ($>90\%$, 38/42), and cost-effective because it is a label-free method and does not involve thermal cycling. The MTB-ISAD assay has improved time-efficiency, affordability, and sensitivity compared with many existing methods. Therefore, it is potentially adaptable for better diagnosis across various clinical applications.

1. Introduction

Tuberculosis (TB) is an infectious disease of disadvantaged communities with high mortality and morbidity caused by several species collectively referred to as the *Mycobacterium tuberculosis* complex (MTBC). TB remains a major global health problem due to the continuous emergence and spread of TB strains, such as drug-resistant strains, and co-infection with other pathogens (WHO, 2013; De Lange, 2013; McNerney and Daley, 2011; Drobniewski et al., 2013; Koul et al., 2011; Zumla et al., 2013). The failure to rapidly and accurately diagnose TB, especially with resistant strains, and the incomplete adherence to prescribed drug regimens have posed significant challenges with consequent secondary resistance and ongoing transmission (Zumla et al., 2013; Lawn and Zumla, 2011; Shah et al., 2013; Casali et al., 2014). This has led to multi-drug resistant (MDR) and extremely-drug resistant (XDR) strains.

An ideal control strategy would include the early diagnosis of TB with the rapid detection of drug resistance. Current standard tests for TB diagnosis are the routine 'microscopy and culture', which have been the main diagnostic methods for decades. Although culture methods are the most sensitive and regarded as the reference standard, some communities cannot afford culture methods due to the time consuming process that are may take weeks to grow and susceptibility results another few weeks (Dheda et al., 2013; Lange and Mori, 2010). Thus, in many communities, smear microscopy continues to be primary method for TB diagnosis. While it offers a cheap and rapid diagnosis, smear microscopy is unable to provide resistance data and has a low sensitivity, detecting only 40–50% of culture positive TB (Inoue et al., 2011). Therefore, there is a great need for an affordable, robust and simple point-of-care (POC) testing with high accuracy in order to obviate the current laboratory challenges of global TB control.

To date, the problem of global TB control is that Primary Care lacks accurate diagnostics that are affordable, robust and portable enough to be used in clinics. This drives the paradigm of large central hospital laboratories with the consequent 'hospital centric' care seen in modern healthcare. This reliance on well funded large institutions has held back the development of solutions for use in

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primary care, where most of the patients are. It effectively denies care to communities without ready access to well funded institutions; this is particularly seen in developing countries. Even in well equipped hospitals there is a need to deliver diagnostics at the bedside, especially in the emergency department, to enable data driven decisions about admission, therapy and infection control. The current paradigm of sending samples to a central laboratory to be tested the next day delays management and adds to costs. Recognition of this imbalance has prompted research into devices that enable diagnostics at the point of care (POC). Currently available NAATs (Boehme et al., 2010; Liong et al., 2013; Cavusoglu et al., 2006) meet the requirements for the primary care but fail to replace smear microscopy because they are too expensive.

Recent advances in nucleic acid amplification tests (NAAT) enable a rapid and accurate detection of TB with high sensitivity and specificity (Boehme et al., 2010; Liong et al., 2013; Cavusoglu et al., 2006). Two NAAT-based technologies, the line probe assay (LPA) and GeneXpert MTB/RIF have improved clinical utility because they combined automated DNA extraction, PCR based amplification and specific probe assays with fluorescent or colorimetric detection into a single system (Boehme et al., 2010). Notably, the GeneXpert MTB/RIF assay, endorsed by World Health Organization (WHO) (WHO, 2013), detects MTB and *rpoB* gene mutations within 2 h, in a fully automated system. However, it is a relatively large instrument due to the inclusion of a thermal cycler for PCR. It also lacks sensitivity, detecting only 70% of smear-negative sample (Lin et al., 2012). While this is a step in the right direction, it is still far from the ideal POC test. In addition, despite its relatively higher sensitivity and specificity, compare to smear testing, NAAT tests are yet to replace microscopy because they are too expensive. Therefore, the WHO and Stop TB partnership have set a goal to develop a new generation technique that requires less equipment and is easy to use, fast, low-cost (i.e., label-free), accurate, and portable (WHO, 2013; De Lange, 2013). A simple POC test for TB could help prevent more than 15 million tuberculosis-related deaths by 2050 (Zumla et al., 2013; Dheda et al., 2013; Lange and Mori, 2010).

Here, we report MTB-ISAD assay to simultaneously amplify and detect the MTB with rapidity, affordability, simplicity, and accuracy in human clinical samples. We also demonstrated the clinical utility of the MTB-ISAD assay by detecting MTB nucleic acid in sputum samples from patients with TB. The technique is based on an isothermal solid-phase amplification/detection (ISAD) assay that combines solid-phase-based isothermal DNA amplification (recombinase polymerase amplification (RPA)) and silicon microring resonator (SMR)-based biophotonic sensors, which do not involve labeling (Shin et al., 2013a). Although the SMRs have been used for direct detection of nucleic acids based on the hybridization mechanism between the capture probe and the target (Bogaerts et al., 2012; Baaske and Vollmer, 2012; Shin et al., 2013b, 2013c; Iqbal et al., 2010), however, the MTB-ISAD technique is based on the amplification and the detection mechanism of nucleic acids that was increased the sensitivity of the nucleic acid detection by the combination. Using this strategy, MTB nucleic acid from human specimens is simultaneously amplified and detected by a grafted complementary primer on the SMR in a label-free and real-time manner. Furthermore, this proposed device is fabricated by CMOS (complementary metal oxide semiconductor) compatible and hence is ready for mass-production at low-cost ($10 per test including a disposable chip and reaction mixture) and the instrumentation cost is low due to the isothermal amplification process (no need of thermal cycler). Therefore, the expected cost of the fully integrated MTB-ISAD system including DNA extraction could be cheaper than that of the GeneXpert system.

2. Material and methods

2.1. MTB-ISAD device fabrication and operation

To design MTB-ISAD device, the modified protocol was used for the detailed structure and fabrication of silicon microring resonators (SMR) that have been previously described (Shin et al., 2013a, 2013b). The device (2.5 cm × 0.8 cm) structures such as microring structures, waveguides, and gratings, were patterned on a commercially available 200 mm SOI (Silicon-On-Insulator) wafer with a 220 nm thick top silicon layer and 2 μm thick buried oxide layer by 210 nm deep (ultraviolet) lithography. We measured the layer thickness after every lay by layer deposition as a process control for the sensor uniformity during the chip fabrication. An array of microring was designed to consist of four rings that are connected to a common input waveguide (through). Each ring had a dedicated output waveguide (drop). The insertion loss (IL) spectrum was measured using an EXFO IQS-12004B DWDM passive component test system (Bogaerts et al., 2012; Baaske and Vollmer, 2012).

To use the MTB-ISAD device as a rapid sensor of MTB detection, the modified protocol was used (Shin et al., 2013a). The primers (Table 1) for the amplification of the targets in this study were selected from the gene sequence encoding IS6110, an IS-like element of *M. tuberculosis* (NCBI accession number X17348) (Inoue et al., 2011). In order to enclose the microring sensor area [3 mm × 4 mm × 1 mm], an acrylic well [1.5 cm × 0.7 cm × 2 mm] for a microfluidic chamber was then pasted onto the chip. The acrylic wells with double-sided adhesive sheet were cut with a 60 W CO_2 laser cutter (Universal Laser System). Finally, in order to amplify the target using the modified RPA method, we prepared the assay solution that optimized for MTB detection. To start the reactions, 5 μL of MTB nucleic acids from sputum samples with patients (MTB or NTB or human gDNA) were mixed with the 10 μL reaction aliquot obtained from the 50 μL of reaction buffer containing 29.6 μL of rehydration buffer, 12.5 μL of nuclease-free water, 5 μL of other primers (10 μM) and, one dried enzyme pellet. The MTB-ISAD device was then placed on a thermoelectric cooler (TEC) with controller (Alpha Omega Instruments) to keep a constant temperature (37 °C) and the resonance spectrum of the device was immediately measured and used as a reference to obtain a baseline. The wavelength shift was monitored by every 5 min for up to 20 min in a label-free and real-time manner.

Table 1
Primer sequences of MTB (*NCBI accession number* X17348).

	Name	Sequence
MTB_IS6110	MTB_ISAD_forward primer	5′-NH_2-$(CH_2)_{12}$-ACCACCCGCGGCAAAGCCCGCAGGACCACGAT-3′
	MTB_ISAD_reverse primer	5′-TAGGCGAACCCTGCCCAGGTCGACACATAGGTGA-3′
	PCR_forward primer	5′-CAAAGCCCGCAGGACCACGA-3′
	PCR_reverse primer	5′-TGCCCAGGTCGACACATAGGTGA-3′