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Automated real-time detection of drug-resistant *Mycobacterium tuberculosis* on a lab-on-a-disc by Recombinase Polymerase Amplification

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ABSTRACT

With the emergence of multi- and extensive-drug (MDR/XDR) resistant *Mycobacterium tuberculosis* (*M. tb*), tuberculosis (TB) persists as one of the world's leading causes of death. Recently, isothermal DNA amplification methods received much attention due to their ease of translation onto portable point-of-care (POC) devices for TB diagnosis. In this study, we aimed to devise a simple yet robust detection method for *M. tb*. Amongst the numerous up-and-coming isothermal techniques, Recombinase Polymerase Amplification (RPA) was chosen for a real-time detection of TB with or without MDR. In our platform, real-time RPA (RT-RPA) was integrated on a lab-on-a-disc (LOAD) with on-board power to maintain temperature for DNA amplification. Sputa collected from healthy volunteers were spiked with respective target *M. tb* samples for testing. A limit of detection of 10^2 colony-forming unit per millilitre in 15 min was achieved, making early detection and differentiation of *M. tb* strains highly feasible in extreme POC settings. Our RT-RPA LOAD platform has also been successfully applied in the differentiation of MDR-TB from *H37Ra,* an attenuated TB strain. In summary, a quantitative RT-RPA on LOAD assay with a high level of sensitivity was developed as a foundation for further developments in medical bedside and POC diagnostics.

Introduction

The infectious disease tuberculosis (TB) was coined a leading cause of death alongside human immunodeficiency virus (HIV) in recent years by the World Health Organization (WHO) [1]. It is estimated that as much as 30% of the world population could be suffering from, or be a carrier of the disease. Bacterial culturing remains the standard method of diagnosis in medical laboratory and hospitals which are staffed with skilled personnel and meet the accredited laboratory safety requirements. Yet, due to the low turnover rate or long doubling time of *Mycobacterium tuberculosis* (*M. tb*), confirmation of TB infection requires a minimum duration of 4–6 weeks [2–4]. For developing countries, screening tests such as smear microscopy are favourable due to their low cost and swiftness of diagnosis. These, however, suffer in terms of objectivity, specificity and sensitivity [5–7].

It was reasonable that healthcare-providers and investigators look to molecular diagnostics to achieve such goals. In this respect, a number of isothermal DNA amplification methods were preferred over Polymerase Chain Reaction (PCR) variants in their speediness and liberation from the use of trademarked thermal cyclers. Even within the few emerging isothermal amplification methods, Recombinase Polymerase Amplification (RPA) stands out distinctively due to its simplicity and ambient operating temperature range (25–42 °C), and fast turn-around time (e.g. within 30 min) [8–10]. In contrast, the Loop-mediated Isothermal Amplification (LAMP) method demands a much stricter and strenuous primer design, as well as a requirement of higher

Abbreviations: cfu/mL, colony-forming unit per millilitre; LOAD, lab-on-a-disc; LAMP, loop-mediated isothermal amplification; MDR, multi-drug resistance; NAC, N-acetyl-L-cysteine; PCB, printed circuit board; PDMS, polydimethylsiloxane; POC, point-of-care; RT-RPA, real-time Recombinase Polymerase Amplification; MDR, multi-drug resistance; *M. tb*, *Mycobacterium tuberculosis*; XDR, extensive-drug resistance

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amplification temperature (usually around 60–70 °C) [11–13].

Since its emergence ten years ago, RPA has been known for its efficiency, specificity and cost-effectiveness in the nucleic acid amplification sector [14,15]. Areas of improvement in this technique had been made in various criteria, including but not limited to the nature of divalent ions in the buffer, the coupling of RPA to fluorescence detection methods, and the formulation of the enzyme mixture [16–18]. Indeed, RPA was employed in the detection of the *M. tb* genome with improved specificity and sensitivity over fluorescence microscopy [19]. The possibility of obtaining immediate results (within 20 min) by means of fluorescence has been established [20,21]. However, the rigorous mycolic acid cell wall of *M. tb* persists as an impediment in molecular diagnostics. The most stringent method recommended to this day composed of the digestion of the acids by strong alkali using 2% NaOH, sodium citrate and N-acetyl-L-cysteine (NAC) [22]. Even though these guidelines guaranteed improved yield of genomic DNA from the bacteria, the necessity of trained personnel lingered as a time-consuming step of most novel point-of-care (POC) testing strategies.

Furthermore, the escalating tendency of *M. tb* to develop multi- or even extensive-drug resistance (MDR/XDR) is becoming a major obstacle in the containment of TB spread. A resistance to the two core first-line anti-TB drugs isoniazid and rifampicin (used in combination therapy) is referred to as a case of MDR-TB. The XDR form of TB is, in addition to isoniazid and rifampicin, resistant to two or more of fluoroquinolones or other injectable second-line drugs. The treatment of both MDR- and XDR-TB requires longer duration and more expensive medications [23–25]. Mutations within the region of the RNA polymerase β subunit gene (*rpoB*) account for the majority of rifampicin-resistance cases; whereas a resistance towards isoniazid can be generally traced to alterations in the catalase peroxidase and InhA protein-coding genes (*katG* and *inhA*), amongst other protein-coding regions [26,27]. A delay or a complete failure in the identification of the specific strain of drug-resistant *M. tb* was found to be the cause of the unstoppable evolution of drug-resistant tendency [23–27]. Often, physicians would exhaust available first- and second-line drugs as the TB patients demonstrated a lack of improvement in symptoms. The increased exposure of *M. tb* to these drugs allowed them to evolve resistance to all of these otherwise effective medications. As these MDR-TB strains prevail, clinical practitioners called upon the reserved third-line therapeutics. It would be disastrous should these be exhausted, as we approach the forewarned prospect of having no effective antibiotics of use by 2050 [28,29].

In light of the need to devise a more robust and reliable detection method of drug-resistant strains of TB, the renewed “ASSURED” principles proposed by Drain et al. upon the existing WHO guidelines had been considered [30,31]. The set of 7 principles were originally proposed by the WHO as a guidance to deploy POC diagnostics in resource-limited settings [30]. The acronym Affordable, Sensitive, Specific, User-friendliness, Rapid, Equipment-free, and Delivered to those in need (ASSURED) have been reduced to six general directions (see (i)-(vi) below). It is believed that the improved classification of such principles could identify platforms of practical use. Therefore, in order to construct a system which allows (i) fast results output in a high through-put manner, (ii) a quick clinical decision, (iii) ease of use at point-of-care settings, (iv) affordability, (v) efficacy and (vi) cost-effectiveness; we look to high through-put integrated systems such as centrifugal microfluidic devices [32–34]. Lab-on-a-disc (LOAD) devices allow customisation of chambers to house separate components of the RPA reaction, assembled with on-board power to initiate and maintain reactions at desired temperatures. In this respect, the current project focuses on the automation of RPA on an integrated sample-to-answer LOAD for a real-time detection of drug-resistant *M. tb* strain.

Materials and methods

Bacterial strains

The *M. tb* (Zopf) Lehmann and Neumann *H37Ra,* an attenuated TB strain (ATCC 25177) was purchased from ATCC, USA. Bacterial cultures were maintained on Middlebrook 7H9 media supplemented with 10% Middlebrook OADC Enrichment (Becton Dickinson BBL, USA). Sputum specimens were donated by healthy individuals with consent. The concentration of bacteria was estimated on Middlebrook 7H11 agar plates in colony-forming unit per millilitres (cfu/mL). In order to mimic actual clinical samples, sputa donated from healthy subjects were spiked with *H37Ra* bacteria in varying concentrations for RT-RPA on LOAD experiments. TB bacterium DNA extraction was performed as previously reported [35]. The concentration of genomic DNA was measured by the NanoDrop-1000 spectrophotometer (Thermo Scientific, USA), with the average value of three readings taken.

A drug-resistant *M. tb* strain developed *in vitro* with antibiotics in a level-3 culture hood was kindly provided by Professor KW Tsui from The Chinese University of Hong Kong. PCR evaluations were performed prior to RT-RPA analysis for characterisation of drug-resistant genes. They were found to be resistant to isoniazid, a first-line drug for the treatment of tuberculosis [26,27]. The MDR bacteria were subjected to NAC/NaOH heat denaturation and genomic DNA extraction before benchtop experiments as described [35]. A mutation of serine to threonine in codon 315 of the *katG* gene, a most common mutation in rifampicin- and isoniazid-resistant strains of *M. tb,* was found to be present. Hereafter, MDR-TB will refer to the sample described.

Real-time RPA (RT-RPA) assay design

The TwistAmp™ Basic Kit (Improved Formulation) was purchased from TwistDx Ltd., UK. The rehydration buffer, forward and reverse primers (Table 1), magnesium acetate were mixed with lyophilised enzymes according to manufacturer's instructions (TwistDx Ltd., UK). Five μM of SYTO-9 green fluorescent nucleic acid stain (Thermo Fisher Scientific, USA) and 1 μL of bacterial DNA had been added in final reaction volumes of 50 μL. The real-time RPA reactions were incubated at 37 °C for 30 min using a Bio-Rad CFX96 Real-time PCR system (Bio-Rad Laboratories, Inc., USA). The lid temperature of CFX96 was manually set to 37 °C for all RPA reactions.

Oligonucleotides were designed to target the codon 315 of the *katG* gene and insertion sequence *IS6110* in accordance with the primer design considerations for the TwistAmp™ Basic Kit (Appendix to reaction kit manuals, TwistDx Ltd., UK). The underlined codon from the *katG* gene was changed from the original serine (ACG) to be specific for threonine (ACC) in the forward primer for MDR-TB in Table 1. Specificity of all primers was verified by using NCBI BLAST algorithm before RPA reactions. The Improved Formulation of the TwistAmp™ Basic Kit stated a possibility to utilise common PCR primers for RPA reactions, hence PCR primers from Eisenach et al. (1991) were used in addition to target the *IS6110* gene [36]. This insertion element is found exclusively within the members of the *M. tb* complex (MTBC), a genetically related group of Mycobacterium species that can cause TB in humans, which was often chosen as the target sequence for the detection of *M. tb* owing to its repetitive nature [12].

Restriction endonuclease digestion

The endonuclease *MnlI* and corresponding buffers were purchased from New England BioLabs, UK for the restriction of the target sequence *IS6110* in RPA reactions. The cutting site of the enzyme in *IS6110* was shown in Table 1. The reaction mixture consisted of 1 X CutSmart® Buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 μg/mL BSA, pH 7.9), 1 unit of *MnlI*, 1 ng of genomic DNA in a total volume of 10 μL as adjusted by autoclaved nuclease-free