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Rapid and visual detection of *Mycobacterium tuberculosis* complex using recombinase polymerase amplification combined with lateral flow strips

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ABSTRACT

To definitively diagnose active pulmonary Tuberculosis (TB), *Mycobacterium tuberculosis* complex (MTBC) bacilli must be identified within clinical specimens from patients. In this study, we introduced a rapid and visual detection method of MTBC using recombinase polymerase amplification (RPA) combined with lateral flow (LF) strips. The LF-RPA assay, read results with naked eyes, could detect as few as 5 genome copies of *M. tuberculosis* H37Rv (ATCC 27294) per reaction and had no cross-reactions with other control bacteria even using excessive amount of template DNA. The system could work well at a broad range of temperature 25–45 °C and reach detectable level even within 5 min. When testing a total of 137 clinical specimens, the sensitivity and specificity of the LF-RPA assay were 100% (95% CI: 95.94%–100%) and 97.92% (95% CI: 88.93%–99.95%), respectively, compared to culture identification method. Therefore, the LF-RPA system we have demonstrated is a rapid, simple, robust method for MTBC detection which, subject to the availability of a suitable sample extraction method, has the potentiality to diagnose TB at the point-of-care testing.

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1. Introduction

Tuberculosis (TB), an infectious disease caused by the bacillus *Mycobacterium tuberculosis*, remains a major global health problem. In 2015, there were an estimated 10.4 million new (incident) TB cases and 1.4 million TB deaths worldwide [1]. TB was one of the top 10 causes of death worldwide, ranking above HIV/AIDS as one of the leading causes of death from an infectious disease [1]. Making a prompt and confirmed diagnosis of active TB is vital both at the individual and population level, thus reducing morbidity, mortality and transmission.

For a definitive diagnosis of active pulmonary TB, *Mycobacterium tuberculosis* complex (MTBC) bacilli must be identified within clinical specimens from the patients. Sputum smear microscopy, being rapid, simple and low cost, is the primary TB detection method recommended by World Health Organization (WHO), but it has several drawbacks such as laborious for the technician and relatively insensitive case detection rate [1–5].

The culture identification method is more accurate and sensitive than smear microscopy [6]. Thus, *M. tuberculosis* culture is the current reference method and gold standard for clinical and research diagnosis of active TB. But it requires more developed laboratory capacity and can take up to 12 weeks to provide results [1]. Moreover, in some cases the bacilli are not successfully cultured, which results in diagnostic delay and even error [7].

The WHO-endorsed rapid molecular diagnostic test for detection of TB currently available is the Xpert MTB/RIF assay [1]. Although the test has much better accuracy than microscopy and culture methods, it has a lower sensitivity in smear-negative sputum samples and its sensitivity in extra-pulmonary TB samples is highly variable [8–10]. Furthermore, the installed

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instruments are underused in many high TB burden countries because of the cost of cartridges, instrumentation and maintenance [1].

Recently, recombinase polymerase amplification (RPA) combined with lateral flow (LF) strips (LF-RPA assay), being simple, robust, rapid, reliable, and suitable for point-of-care testing (POCT) diagnostics, was successfully used to detect several pathogens including bacteria [11,12], viruses [13–18], and parasites [19–22] et al. David S. Boyle et al., reported the application of real-time RPA to diagnose TB in sputum in 2014 [23]. Two assays for *M. tuberculosis* specific repetitive elements, IS*6110* and IS*1081*, were investigated, both confirming the ability to determine the existence of MTBC DNA with a high degree of sensitivity and specificity [23]. However, they require special fluorescence detection device and corresponding analysis software to show the results of RPA reaction in real-time, which limits their application in POCT diagnostics to a certain extent.

In present study, we have investigated the performance of RPA assay in combination with LF dipsticks for detecting *M. tuberculosis* DNA in clinical samples from patients suspected to have TB. The LF-RPA system showed a high degree of specificity with no cross-reactions with closely related bacilli and was able to detect as low as 5 genome equivalent copies per reaction. The system proved to be a simple, robust, reliable method with short processing time ($\leq$20 min) and variable incubation temperature (25–45 °C) and might be suitable for MTBC detection after further improvement and optimization.

2. Materials and methods

2.1. Primer and probe design

The primers and probes for our LF-RPA assay were designed according to the combined instruction manual of TwistAmp® DNA amplification kits (TwistDx Ltd., UK) based on the repetitive insert sequence IS*1081*, present in all MTBC species, of *M. tuberculosis* H37Rv genome (GenBank accession No.: NC_000962.3) [24,25]. Several initial primer combinations were screened by BLASTN and the NCBI nucleotide database, and then evaluated via actual testing to identify the optimal primers. The primers and probes for real-time RPA assay were selected as previously described [23]. All oligonucleotides were purchased from Beijing AuGCT DNA-SYN Biotechnology Co. Ltd. (Beijing, China) and listed in Table 1.

2.2. Clinical samples and bacterial strains

An evaluation of the LF-RPA system was carried out using a total of 137 clinical samples (one specimen from one person) randomly collected from Shenzhen Third People's Hospital and derived from patients with similar signs and symptoms consistent with pulmonary TB. All the samples, including 125 sputum and 12 respiratory washes, were residual and discarded specimens used for routine clinical diagnosis. Study investigators were blinded to the status of the above samples. The results were compared to that of culture identification test. Diagnostic parameters including sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using free statistical calculators “diagnostic test evaluation calculator” (https://www.medcalc.org/calc/diagnostic_test.php) and presented as a percent.

Modified Ziehl–Neelsen stain was performed according to the manufacturer's instructions (YZB/Yuezhu 0063-2013, Baso diagnostics, inc)(Zhuhai, Guangdong, China).

Culture was performed using BACTEC™ MGIT™ 960 Mycobacterial Detection System (BD Diagnostics, New Jersey, USA) with incubation at 37 °C for 42 days. Most of the positive results appeared on the 12–14 days and the fastest one came out on the second day. If the results were not present for 42 days, they were treated as culture negative. The positive cultures were screened for MTBC using the Mycobacterium Species Identification Detecting Kit (PCR-reverse dot blot)(Yaneng BIO, Guangdong, China) that could identify MTBC and 21 species common non-tuberculous mycobacteria (NTMs). Only both culture and MTBC identification positive isolates were identified as MTBC true positive. The culture positive but mycobacterium identification negative isolates were identified by additional biochemical testing.

Clinical isolates were isolated and identified by the clinical laboratory of Shenzhen Third People's Hospital and the reference strains were obtained through the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.3. DNA extraction

Total DNA of clinical isolates and standard strains was extracted using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. An earlier described DNA extraction program was performed to obtain the bacterial total DNA of clinical specimens [26]. DNA concentrations were quantified using the Thermo Scientific NanoDrop 2000 Spectrophotometers (Thermo Fisher Scientific, Wilmington, USA). All DNA extractions and lysed samples within 1× TE buffer (10 mM Tris, pH 8.0 with HCl, and 1 mM EDTA) were stored at −80 °C until use and avoid repeated freeze-thaw cycles.

2.4. RPA assay

Real-time RPA assay was performed according to the operating manual of TwistAmp® exo kit (TwistDx Ltd., UK) and the protocol previously described [23]. LightCycler® 480 II real-time PCR instrument (Roche Diagnostics International Ltd., Switzerland) was used as the isothermal heating and fluorescence detection device.

LF-RPA assay was performed according to the quick guide of TwistAmp® nfo kit (TwistDx Ltd., UK) with a little revision. Briefly, each reaction contained 29.5 μl of rehydration solution (supplied by the kit), 2.1 μl of forward primer (10 mM), 2.1 μl of reverse primer

Table 1
Oligonucleotide primers and probes for LF-RPA and real-time RPA assays.

Assay	Name	Sequence (5′-3′)
LF-RPA	IS1081 LF F	CCAAGCTGCGCCAGGGCAGCTATTTCCCGGAC
	IS1081 LF R	Biotin-TTGGCCATGATCGACACTTGCGACTTGGA
	IS1081 LF Pr	FITC-GAACGCGCACTGACCAGCGTGGTGGCGACCTG(H)TACCTGCTGGGAGTATC-P
Real-time RPA	IS1081a_F	CAGTAGTGGGCGGTCATCGCGTGATCCTTCGAAACGACC
	IS1081a_R	CTCGCCTGTGCGAGTTGGTCAGCCAGAAGCTG
	IS1081a_Pr	CGATAAGATGAGAAGAGGTCATTGCGTCATT(F)(H)C(Q)TCGATTGACTTTTGCT-Spacer C3

AbbreviationsF = Forward, R = Reverse, Pr=Probe, FITC = fluorescein isothiocyanate, H = tetrahydrofuran spacer, P = 3′ phosphate to block elongation, F = dT-FAM = thymidine nucleotide carrying fluorescein, and Q = thymidine nucleotide carrying black hole quencher 1.

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