Rapid Detection of *Mycobacterium tuberculosis* by Recombinase Polymerase Amplification

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Abstract

Improved access to effective tests for diagnosing tuberculosis (TB) has been designated a public health priority by the World Health Organisation. In high burden TB countries nucleic acid based TB tests have been restricted to centralised laboratories and specialised research settings. Requirements such as a constant electrical supply, air conditioning and skilled, computer literate operators prevent implementation of such tests in many settings. Isothermal DNA amplification technologies permit the use of simpler, less energy intensive detection platforms more suited to low resource settings that allow the accurate diagnosis of a disease within a short timeframe. Recombinase Polymerase Amplification (RPA) is a rapid, low temperature isothermal DNA amplification reaction. We report here RPA-based detection of *Mycobacterium tuberculosis* complex (MTC) DNA in ~20 minutes at 39°C. Assays for two MTC specific targets were investigated, IS6110 and IS1081. When testing purified MTC genomic DNA, limits of detection of 6.25 fg (IS6110) and 20 fg (IS1081) were consistently achieved. When testing a convenience sample of pulmonary specimens from suspected TB patients, RPA demonstrated superior accuracy to indirect fluorescence microscopy. Compared to culture, sensitivities for the IS1081 RPA and microscopy were 91.4% (95% CI: 85, 97.9) and 86.1% (95% CI: 78.1, 94.1) respectively (n = 71). Specificities were 100% and 88.6% (95% CI: 80.8, 96.1) respectively. For the IS6110 RPA and microscopy sensitivities of 87.5% (95% CI: 81.7, 93.2) and 70.8% (95% CI: 62.9, 78.7) were obtained (n = 90). Specificities were 95.4% (95% CI: 92.3, 98.1) and 88% (95% CI: 83.6, 92.4) respectively. The superior specificity of RPA for detecting tuberculosis was due to the reduced ability of fluorescence microscopy to distinguish Mtb complex from other acid fast bacteria. The rapid nature of the RPA assay and its low energy requirement compared to other amplification technologies suggest RPA-based TB assays could be of use for integration into a point-of-care test for use in resource constrained settings.

Citation: Boyle DS, McNerney R, Teng Low H, Leader BT, Pérez-Osorio AC, et al. (2014) Rapid Detection of *Mycobacterium tuberculosis* by Recombinase Polymerase Amplification. PLoS ONE 9(8): e103091. doi:10.1371/journal.pone.0103091

Competing Interests: JCM, DGB, OP, and MSF are employed by TwistDx, and have a stake in the success of its technology. TwistDx Limited is the developer and manufacturer of RPA technology and is part of the of the Alere group of companies. There are no further patents, products in development or marketed products to declare. This does not alter the authors’ adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors. DSB, BTL, APD, RM, HTL, and DGS have no financial interests in this technology.

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Introduction

Tuberculosis (TB) is an infectious disease that is very often difficult to diagnose. The World Health Organisation (WHO) estimates that during 2012 the global case detection rate for TB was 66%, suggesting that of the estimated 8.6 million incident cases that year almost 3 million cases were not diagnosed and notified [1]. Pulmonary TB, the most infectious form of the disease, is diagnosed by detecting *Mycobacterium tuberculosis* complex (MTBC) bacilli in samples of sputum expectorated by the patient. Smear microscopy, which is the primary test used for the diagnosis of pulmonary TB endemic countries [1], is a laborious and relatively insensitive test with a case detection rate of only 56 to 68% [2]. While microscopy can diagnose late stage TB disease, it performs poorly on specimens acquired from cases with HIV co-infection due to the reduced numbers of TB cells produced in a sputum specimen. Failure to detect and treat pulmonary disease in a timely manner results in onward transmission and the continuation of the epidemic which claimed an estimated 1.2 million lives during 2012 [1]. The WHO recently noted that in 2012 approximately three million cases of active TB went undiagnosed by country programs [1] and there is a pressing
need for improved diagnostic tools to supplant smear microscopy to facilitate rapid detection [3,4].

To this aim, the Xpert MTB/RIF (Cepheid, USA) was endorsed by WHO in 2010 to detect pulmonary disease in settings with a high incidence of TB/HIV co-infection or with high rates of drug resistance [5]. This polymerase chain reaction (PCR) based test is fully integrated into a modular system and can detect both TB infection and indicate the presence of resistance to one of the key anti-tuberculosis drugs, rifampicin. The key attribute of the GeneXpert system is its ease of use, as the majority of key processes are integrated and automated from DNA extraction to interpretation of a test result [6]. However, the GeneXpert requires consistent electricity and laboratory temperatures maintained under 30°C, which poses logistical challenges [7]. Furthermore, the cost of cartridges, instrumentation and maintenance threaten its sustainability in the longer term in high TB burden countries that currently rely on global donor assistance to provide this technology. Therefore, to increase access to timely and accurate diagnosis for more TB suspected patients, rapid, high performance TB diagnostic tests that can meet the logistical challenges of unsupervised use in limited infrastructure settings are required and at a price that is more comparable to smear microscopy [3,8–10]. Other technologies have been developed for TB diagnosis in microscopy centers in high burden countries and are currently undergoing evaluation. These include semi-modular systems (Epistem Genedrive and Molbio EASYNAT) or methods with manual specimen processing and test determination (Eiken LoopAMP TB and Ustar Biotechnologies Easy NAT) [9,11–15].

Recently, isothermal amplification assays utilizing loop mediated amplification (LAMP), helicase dependant amplification (HDA) and cross priming amplification (CPA) have been described for the diagnosis of pulmonary TB [12,16,17]. Unlike PCR, isothermal assays do not require precisely controlled thermal cycling and instead use only a uniform incubation temperature, typically 55–65°C, to permit DNA amplification and therefore may offer greater utility with more simplistic reactor designs or heat sources [12,18–20]. Recombinase Polymerase Amplification (RPA) has emerged as a novel, isothermal technology for use in molecular diagnosis of infectious disease [21]. Unlike many other isothermal technologies, RPA does not require elevated or precise temperatures, and may proceed at temperatures between 25°C and 42°C [21]. RPA replaces the thermal cycling needed for PCR with three core enzymes. The titular enzyme, a recombinase, binds to oligonucleotide primers to form recombination filaments that can recombine with homologous DNA (Figure 1). The second enzyme, a single-stranded DNA binding protein, binds to the strand of DNA displaced by the primer, stabilising the D-loop that has formed and preventing the dissociation of the primer. The third core enzyme is a strand-displacing DNA polymerase that copies the DNA template by adding bases onto the 3’ end of the primer, forcing open the DNA double helix as it progresses. As with PCR, the use of closely spaced opposing primers allows the exponential amplification of a defined region of DNA. RPA reactions typically run to completion in 5–15 minutes, depending on amplicon size and the template copy number [21]. This time to test result is significantly more rapid than the other isothermal MTBC assays which note incubation times in the range of 45–60 minutes [12,13,16].

The highly specific detection of RPA products is achieved via the use of custom designed oligonucleotide probes which recognize a complementary region within either strand of the RPA amplicon. The probe DNA is also inserted via a recombinase mediated event. RPA probes are constructed with an abasic nucleotide analogue within the probe sequence. This abasic site is

recognized and cleaved by several types of endo- or exonucleases, but only when the probe has bound to its complementary sequence. Different nucleases are specifically added to the core RPA reaction mixture for use in a variety of detection formats, including fluorescence detection in real time or endpoint detection via a lateral flow strip [21,22]. Reverse transcriptase, can be also included in RPA reactions to facilitate RPA from RNA targets such as Middle East Respiratory Syndrome Coronavirus or Rift Valley Fever [23,24]. Recently RPA was been demonstrated to be highly sensitive for the detection of HIV proviral DNA [22] and in this work we have investigated the use of RPA to detect *Mycobacterium tuberculosis* DNA from patients presenting with suspected pulmonary TB.

**Materials and Methods**

**RPA assay design**

Oligonucleotide primers and probes used to develop the RPA assays were purchased from Eurogentec Ltd (Southampton, UK) and Biocarch Technologies (Novato, CA) respectively. Preliminary screening of primer and probe combinations used the Twist Amp Exo kits according to the manufacturer’s instructions (TwistDx Ltd., UK) in final reaction volumes of 50 µL. The RPA reactions were incubated at 39°C using a combined heating and fluorescence detection device (Twista, TwistDx, UK) [22]. Samples were mixed prior to amplification and also at six minutes during incubation. The output of the RPA reaction was monitored in real time using Twista Studio Software (TwistDx, UK) with fluorescence measurements taken every 20 seconds for a total of 20 minutes.

Once optimal primer and probe sequences were identified, lyophilized pellets of the TwistAmp Exo reactions were prepared that also contained the primers and probe. For 50 µL reactions, 4 µL of 280 mM MgOAc, 37.5 µL TwistAmp Primer-In-Pellet Resuspension Buffer (PIRB, TwistDx Ltd) and 8.5 µL of sample/water were used to rehydrate the pellets. Double amounts of reagent were used for 100 µL reactions. Sample incubation conditions remained the same as used for primer screening.

**DNA Samples**

Cultures of *M. tuberculosis* (H37Rv) and *M. bovis* BCG-Bulgaria (BB-NCIPD Ltd., Bulgaria) were maintained on Lowenstein Jensen slants supplemented with glycerol (Media for Mycobacteria, UK) and Middlebrook 7H9 media supplemented with 10% ADC (BD BBL, USA). DNA was extracted using the standardised RFLP protocol and quantified using the Qubit platform (Invitrogen, Life Technologies Ltd. UK) [25]. Samples of purified DNA from non tuberculous mycobacteria (NTM) were supplied by the Mycobacteriology Unit (Institute of Tropical Medicine, Antwerp, Belgium [Table 1]). DNA concentrations were quantified using the Qubit platform (Invitrogen, Life Technologies Ltd. UK) and 500 pg (~114,000 genomes) samples in a volume of 1 µL were added to the RPA reactions. Using the molecular weight per genome as determined from the whole genome sequence of the BCG Tokyo 172 strain (Accession #: 224771496) we calculate that 5 fg of BCG DNA correlates to an estimated genome equivalent (GE) of one cell [26]. A second panel of fully characterized NTM strains and other bacteria including common respiratory pathogens were accessed from the reference collection of the Washington State Public Health Laboratory (Table 1). Crude genomic DNA extracts were prepared as described previously [27].
Clinical samples

An evaluation of the RPA assay was performed using a panel of sputum and respiratory specimens derived from suspected cases of pulmonary TB obtained from the Washington State Public Health Laboratories. Samples tested by RPA were remains of specimens collected for routine diagnosis and were not representative of the population attending the clinic having been selected to provide a panel with an unusually high proportion of positive samples. Study investigators were blinded to the status of the samples. The panel comprised 121 specimens including induced and expectorated sputum (n = 119) and respiratory washes (bronchial and tracheal, n = 2) collected from a total of 101 TB suspect cases (no more than two specimens per individual were tested). Specimens were processed via digestion and decontamination using the N-acetyl L-cysteine NaOH-Na citrate method [28]. For each specimen, cells were pelleted by centrifugation at 6000 \( g \) and the supernatant was discarded. Each pellet was then resuspended in 1.5 mL of sterile phosphate-buffered saline (PBS, pH 6.8). Smears were prepared from the decontaminated specimens to determine the presence acid fast bacteria using Auramine O and fluorescence microscopy. A specimen was considered smear positive if greater than 1 AFB was present per 10 observed microscopic fields. The smear positive specimens were scored from + to 4+ based on the number of observed AFB positive cells present [28].

Culture was performed using MGIT modified Middlebrook 7H-9 liquid medium in an automated BACTEC 460TB System (Becton, Dickinson and Company, New Jersey, USA) with incubation at 37°C for up to 1 month. A 0.5 mL inoculum of processed specimen was used per mycobacterial culture. All positive cultures were then screened for MTBC using the Mycobacterium Tuberculosis Complex Culture Identification Test (Gen-Probe, San Diego, CA) according to the manufacturer’s instructions. The MTBC negative culture positive isolates were speciated by additional biochemical testing. A 250 \( \mu \)L aliquot of each processed specimen was used for DNA extraction. A previously described extraction process using glass beads and heat lysis was used, the lysed samples were then stored at \(-20^\circ C\) until use [27]. The specimens screened by RPA TB assays were prepared as described earlier. Each RPA reaction contained 5 \( \mu \)L of specimen extract added with rehydration buffer, MgOAc and molecular grade water to create a final reaction volume of 50 \( \mu \)L. Scoring of positive RPA reactions incubated in the Twista RPA reactor was indicated by an increase in FAM fluorescence by greater than 300 mV from six to twenty minutes incubation.

The clinical materials used in this study were discarded remains of specimen collected for routine diagnostic examination, all data was blinded with no links to enable identification of patients. As such, the study was exempt from ethical approval in accordance with US Code of Federal Regulations 45 CFR 46.101. Work in

![The RPA Cycle](http://www.twistdx.co.uk/our_technology/)
Table 1. Strains used for specificity testing.

| Strain              | Source                                                                 |
|---------------------|------------------------------------------------------------------------|
| M. abscessus ATCC 19977 | M. mageritense 981294                                                  |
| M. aurum ATCC 23366* | E. coli ATCC 25922                                                    |
| M. avium 961091*     | B. pertussis                                                          |
| M. avium ATCC 25291  | M. araba ATCC 927                                                      |
| M. brisbanense       | M. marinum1717                                                        |
| M. catarrhalis       | M. nebraskense                                                        |
| M. cheloneae         | M. neoaurum 40853                                                     |
| M. cheloneae 41426   | M. nonchromogenicum 970454*                                           |
| M. chitae ATCC 19628*| M. nonchromogenicum ATCC 19530                                        |
| M. dierhoferi ATCC 19340* | S. pneumoniae ATCC 13077                                           |
| M. fortuitum ATCC 6841 | M. peregrinum                                                      |
| M. gordonae ATCC 14470 | M. peregrinum 10086*                                                |
| M. gordonae10284     | M. poriferae ATCC 35087                                               |
| M. heidelbergense 981295* | S. pyogenes ATCC 12370                                           |
| M. immunogen 981291* | S. salivar                                                        |
| M. intracellularle 32665* | S. pneumoniae ATCC49619                                           |
| M. intracellularle ATCC 13950 | M. smeegmatis mc2155*                                                 |
| M. intracellularle ATCC 35761 | M. zulgi ATCC 35799                                                  |
| M. kansasii          | M. szulgi 4981*                                                       |
| M. kansasii ATCC 12478 | M. ulcerans 5147                                                      |
| M. kansasii MYC 4296  | M. xenop                                                          |
| M. lentiflum 960190*  | M. xenop 9741*                                                       |
| M. liflandi 40413*   | Yersinia pseudotuberculosis ATCC 23207                                |

Non-tuberculous mycobacterial (NTM) strains and other bacterial species used in the determination of the specificity of the IS6110 and IS1081 RPA assays. All tested negative by both RPA assays. Isolates marked * were supplied by the Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp, Belgium. All other isolates were from the Washington State Public Health Laboratory strain collection.

doi:10.1371/journal.pone.0103091.t001

Table 2. Oligonucleotide primers and probes.

| Oligonucleotide | Sequence (5’ – 3’)                                    |
|-----------------|-------------------------------------------------------|
| IS6110c_Forward | GATCTCGAGGTAGGCTGAGCAGTCGACAAAAAGCCAGCTTAG             |
| IS6110c_Reverse | CGTATCGGAGGCTCCGAGTACCTGTCGACG                      |
| IS6110c_Probe   | CGAACCGCTGCAAGCTGACGTCGACGATTCGGGCTGGACG          |
| IS1081a_Forward | CAGTAGTGGCCGATCGACGTCGACGTCGACG                      |
| IS1081a_Reverse | CGTCCCGTTCGATGCTGACGTCGACGTCGACG                      |
| IS1081a_Probe   | CGAAGATGAGAAAGAGGATGCTGACGATTCGGGCTGGACG          |

The oligonucleotides chosen for amplification and detection of IS6110 and IS1081 are shown in Table 2. F = dT-FAM, H = tetra hydrofuran and Q = dT-Black Hole Quencher 1.

doi:10.1371/journal.pone.0103091.t002
Figure 2. Real-time RPA amplification of IS\textsuperscript{1081} and IS\textsuperscript{6110}. Figure 2a shows the real time detection of IS\textsuperscript{1081} amplification from a dilution series of quantitated \textit{M. bovis} BCG DNA with a sensitivity as low as 0.04 pg of DNA per reaction. Figure 2b shows the real time detection of IS\textsuperscript{6110} amplification from a dilution series of quantitated \textit{M. bovis} BCG DNA with a sensitivity as low as 5.0 fg of DNA per reaction.

doi:10.1371/journal.pone.0103091.g002
Table 3. Limit of detection of IS6110 and IS1081 RPA.

| DNA amount | 0 | 0.1 | 0.25 | 0.5 | 1 | 2 | 5 | 10 | 25 | 50 | 100 | 500 | 1000 ng |
|------------|---|-----|------|-----|---|---|----|-----|-----|-----|------|------|--------|
| % positive | 0 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 | 0/8 |

Results shown are for purified DNA extracted from M. bovis BCG. DNA concentrations were measured prior to the preparation of serial dilutions for testing by RPA. The numbers of positive test results and the total number of tests run at each concentration of DNA are recorded.

Analytical sensitivity of RPA

The sensitivity of the RPA reactions were assessed by testing serial dilutions of genomic DNA extracted from M. bovis BCG. Multiple tests were performed at each concentration of DNA, with water used as a negative control (Table 3). An example of the output is shown in Figures 2a and 2b. The time to onset of a rise in fluorescence increased as the amount of BCG genomic DNA in the sample decreased. When using IS6110 primers, 6.25 fg per 100 µL reaction were required for reactions to be consistently positive, which is approximately equivalent to the DNA found in a single bacteria (n = 9). As shown in figure 2b, when testing low levels of DNA the time to onset of fluorescence was associated with the amount of DNA in the samples, with 11 min being required when testing 5 fg. When using IS1081 primers consistent positive results were obtained for all samples containing at least 20 fg BCG genomic DNA (Table 3). The time to onset of signal at the lowest concentration of 20 fg ranged from 7 to 9 minutes. These results suggested that either RPA assay is highly sensitive for the rapid amplification and detection of MTBC DNA, and that a 15 minute data collection period is sufficient to detect positive samples. Sensitivity was also assessed by testing replicate serial dilutions of a culture of BCG. Results (not shown) indicated that both RPA assays were capable of detecting a single colony forming unit of bacteria.

Specificity of RPA

To assess the specificity of the IS6110 and IS1081 RPA reactions for the identification of MTBC, samples of DNA from a
Table 4. Tuberculosis detection by indirect smear microscopy vs RPA IS6110.

|                          | Culture positive | Culture negative |
|--------------------------|------------------|------------------|
| Indirect smear microscopy positive | 34               | 5                |
| Indirect smear microscopy negative | 14               | 37               |
| RPA IS6110 positive       | 42               | 2                |
| RPA IS6110 negative       | 6                | 40               |

Testing pulmonary specimens (n = 90) by indirect smear microscopy and RPA IS6110 to detect tuberculosis, with comparison to liquid culture based test data. RPA IS6110 was more sensitive than indirect smear microscopy (87.5% (95% CI: 81.7, 93.2) vs 70.8% (95% CI: 62.9, 78.75)) and also more specific (95.4 (95% CI: 92.3, 98.1) vs 88% (95% CI: 83.6, 92.4)).

doi:10.1371/journal.pone.0103091.t004

Table 5. Tuberculosis detection by indirect smear microscopy vs RPA IS1081.

|                          | Culture positive | Culture negative |
|--------------------------|------------------|------------------|
| Indirect smear microscopy positive | 31               | 4                |
| Indirect smear microscopy negative | 5                | 31               |
| RPA IS1081 positive       | 32               | 0                |
| RPA IS1081 negative       | 4                | 35               |

Testing pulmonary specimens (n = 71) by indirect smear microscopy and RPA IS1081 to detect tuberculosis, with comparison to liquid culture based test data. RPA IS1081 was more sensitive than indirect smear microscopy (91.4% (95% CI: 85,98.9) vs 86.1% (95% CI: 78.1, 94.1)) and also more specific (100% vs 88.6% (95% CI: 80.8, 96.1)).

doi:10.1371/journal.pone.0103091.t005
could detect samples estimated to contain the genome equivalent of a single bacterial cell. The IS\textsubscript{1081} assay required slightly higher amounts of DNA 20 fg, which was not expected as it does not reflect the increased number of copies of this insertion element in the genome (six IS\textsubscript{1081} vs two IS\textsubscript{6110}). We suspect the lower sensitivity observed is due to reduced primer and probe binding efficiency of the oligonucleotides screened in this study. We also cannot rule out the possibility of localized secondary or tertiary DNA structures affecting the recombinase mediated insertion of the oligonucleotides despite the presence of high levels of ssDNA binding protein in RPA reactions. Nonetheless both assays demonstrated the potential of RPA as a diagnostic tool to detect tuberculosis in samples containing low numbers of bacteria. Both assays were shown to be highly specific for MTBC (100%) when challenged with acid fast bacterial species that do not cause tuberculosis or 18 other common commensal or respiratory bacterial pathogens.

When the performance RPA was assessed with clinically derived specimens, we found that both assays had high sensitivity (100%) with isolates that were smear and culture positive. For samples that were smear negative but culture positive 8/14 (57.1%) and 2/5 (40%) were identified as positives via the IS\textsubscript{6110} and IS\textsubscript{1081} assays, respectively. It should be noted a crude DNA extraction procedure was used and improved sample handling and DNA extraction may improve detection from complex sample matrices such as sputum [42]. Similarly whereas 0.5 ml inoculums were used for culture the volume taken for DNA extraction was only 0.25 ml, with just 5 µL being used for each RPA assay and there may be opportunity to improve sensitivity by testing larger sample volumes or by incorporating a sample concentration step. To fulfil the full potential of RPA for point-of-care diagnostics, an integrated platform to perform sample preparation and run the assay must be developed.

Further work is required to optimize specificity of RPA for TB diagnosis from clinical samples. In particular the number of negative samples included in this study were insufficient to assess the clinical specificity and the number of culture-positive, smear-negative samples should be increased to better determine the clinical sensitivity. Ten of ten NTM positive specimens were correctly assigned by the IS\textsubscript{1081} assay as being MTBC negative, demonstrated a specificity of 100%. However, whereas the IS\textsubscript{6110} assay correctly assigned two NTM specimens as negative two false positives were observed for smear negative specimens that were culture positive for NTM. The positive predictive value (PPV) and negative predictive value (NPV) are not presented as the contrived signals. Note that in all instances, poor duplicates are often indicative that an assay is close to its limit of detection.

The comparatively low isothermal incubation temperature combined with rapid time to result allows RPA to be used with a battery powered device, making it amenable to health clinics in developing countries that do not have reliable electricity supply [44]. However, further studies are required to adapt sample extraction methodology for use at the point of care.

**Supporting Information**

**Figure S1 Benefits of RPA primer optimisation.** This figure shows a side-by-side comparison of duplicates of the optimal primer pairs chosen during each round of screening for IS\textsubscript{6110} with either 50 (dotted lines) or 500 (solid lines) copies of template. The primers chosen at the end of the first round of screening (purple, ‘Round 1’), can detect both 50 and 500 copies of template. The optimal primers identified by shuffling these primers upstream and downstream in single base increments (red, ‘Shuffled’) give faster detection times and result in higher levels of fluorescence. The primers chosen from lengthening and shortening the 3’ end of these shuffled primers (green, ‘Length’) show further improvement to detection time and fluorescent signals. Note that in all instances, poor duplicates are often indicative that an assay is close to its limit of detection.

**Table S1 Culture, smear and RPA test results derived from clinical specimens.** The scores for bacteria counted in the smear +ve specimens are shown in parenthesis, e.g. +ve (2+). Smear scores that were scancy are shown as +/−. Acronyms and other points. MTBC – Mycobacterium tuberculosis complex; NT – Not Tested; NTM = Non Tuberculous Mycobacteria. MTBC.

| Specimen Type | IS\textsubscript{1081} | IS\textsubscript{6110} |
|---------------|----------------|-----------------|
| +ve | 100% | 100% |
| −ve | 20% | 90% |

**Author Contributions**

The authors would like to acknowledge the efforts and assistance of Alla Otash and Kathy Lisle from the WA PHL, TB reference laboratory for providing specimens and key test data. We would also like to acknowledge the Mycobacteria Reference Laboratory at the Institute of Tropical Medicine for the supply of further NTM isolates.

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**Author Contributions**

Conceived and designed the experiments: DSB RM APO DGB OP MSF. Performed the experiments: DSB HTL BTI APO DMO JCM MSF. Analyzed the data: DSB RM MSF. Contributed reagents/materials/analysis tools: DSB RM APO MSF. Contributed to the writing of the manuscript: DSB RM MSF.
Rapid Detection of M. tuberculosis by RPA

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