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Thermal lysis and isothermal amplification of *Mycobacterium tuberculosis* H37Rv in one tube

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ABSTRACT

Tuberculosis (TB) is a leading cause of high mortality rates in developing countries. Sample preparation is one of the major challenges in developing an inexpensive point-of-care device for rapid and confirmed detection of tuberculosis. Existing chemical and mechanical lysis methods are unsuitable for field applications, as they require intermediate wash steps, manual intervention or separate lysis equipment. We report a one-step reaction protocol (65 °C and 60 min) for the H37Rv strain of *Mycobacterium tuberculosis* that (i) completely disinfects the mycobacteria culture, (ii) lyses the cells and (iii) performs helicase dependent amplification on the extracted DNA. Our assay combines multiple functions in a single step, uses a dry heat bath and does not require any intermediate user intervention, which makes it suitable for use by minimally trained health workers at the point of care.

1. Introduction

Tuberculosis (TB) is a communicable disease and a global health threat, especially in the developing countries. TB is caused by a bacterial pathogen *Mycobacterium tuberculosis*. Nucleic acid amplification tests (NAATs) for diagnosing TB are rapid, specific and sensitive (~86%) (Dinnes et al., 2007). But commercial NAAT platforms are not suitable for use in primary healthcare centres due to their high price, lack of robustness and need for trained personnel to operate. Therefore, the WHO has declared a need for nucleic acid-based tests on sputum samples that are suitable for use in microscopy centres (WHO, 2014).

Most of the NAAT platforms rely on the polymerase chain reaction (PCR) to amplify the nucleic acids. Unlike PCR, isothermal amplification techniques, such as, helicase dependent amplification (HDA), recombinase polymerase amplification (RPA), loop-mediated amplification (LAMP), etc. can amplify the nucleic acid at a single incubation temperature (Gill and Ghaemi, 2008). These techniques can be performed with low-cost heat sources, such as, hot plates, heat blocks and hand/toe warmers (Huang et al., 2013; Shetty et al., 2016).

Recently we reported rapid (~10 min) helicase dependent amplification of *M. tuberculosis* (H37Rv) DNA on a disposable paper substrate (Shetty et al., 2016). We performed off-chip sample preparation prior to amplifying the DNA on the paper chip. In most lab protocols, sample preparation involves two primary processes: (a) disinfection (i.e. making the pathogen in sputum/culture samples non-viable) or decontamination (i.e. suppressing the growth of other bacteria in the sample to ensure *M. tuberculosis* growth) and, (b) lysis (e.g. DNA extraction and purification). Disinfection (Best et al., 1988; Rikimaru et al., 2002) or decontamination (Helb et al., 2010) of mycobacteria is done chemically, while lysis is performed chemically, mechanically or thermally. As chemical lysis requires a number of centrifugation steps and manual washes, it is difficult to integrate into a point-of-care device. Existing mechanical lysis techniques (Ferguson et al., 2016) also require frequent manual intervention.

The advantage of thermal lysis is that it does not require incompatible chemicals, or separate equipment. Thermal lysis of *M. tuberculosis* at 80 °C (Doig et al., 2002; Warren et al., 2006) has been demonstrated. The high temperatures (95 °C) employed during PCR (Elbir et al., 2008) can also release DNA directly from cells. Some groups have amplified tuberculosis DNA by isothermal LAMP assays (Li et al., 2014; Rudeeaneksin et al., 2012) after performing DNA extraction separately. Another group (Bentaleb et al., 2016) demonstrated LAMP on sputum samples after chemically decontaminating the sputum is a separate step prior to amplification.

Here, we have combined the three steps of disinfection, lysis and helicase dependent amplification (HDA) of *M. tuberculosis* into a single heat incubation step performed at 65 °C. All experiments in this study were performed using the standard H37Rv laboratory strain of *M.*

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tuberculosis. The complete assay takes place in a single tube requiring no intermediate intervention. The helicase dependent amplification protocol amplified DNA in presence of cell debris. There was no colony growth from the thermal lysate, which confirmed complete disinfection. To the best of our knowledge, this is the first demonstration of complete disinfection, lysis and helicase dependent amplification of *M. tuberculosis* H37Rv strain achieved in a single heat incubation step at 65 °C. Our one-step protocol (a) renders the mycobacteria non-viable, (b) functions without any user intervention, (c) does not require any additional equipment, and (d) can be safely performed in low-resource settings with limited biosafety measures.

2. Materials and methods

2.1. Equipment and chemicals

We used a MJ Mini thermal cycler from Bio-Rad (India) for PCR and a temperature-controlled heat block from Trishul Equipment (India) for isothermal amplification. FastPrep 24 mechanical lysis instrument and kit were bought from MP Biomedicals (USA). PureLyse kit from Claremont BioSolutions (USA) was also used for mechanical lysis. The concentration of DNA was measured using a NanoDrop instrument (NanoPhotometer® P 300, IMPLEN) (Germany). The electrophoresis unit was bought from Genetix (India). PerkinElmer Geliance 1000 imaging system (India) was used for imaging the agarose gels.

M7H9 Middlebrook, albumin dextrose catalase (ADC) and Luria agar for growing the mycobacteria were bought from Himedia (India). Middlebrook 7H11 agar base, glycerol, and Tween 80 were purchased from Sigma Aldrich (India). Middlebrook oleic albumin dextrose catalase (OADC) enrichment medium was obtained from Beckton Dickinson (India). Chemical DNA extraction was performed with DNAeasy Blood and tissue kit, purchased from Qiagen (India). IsoAmp III enzyme mix for helicase dependent amplification was purchased from Biohelix Inc. (Beverly, USA). We got the amplification primers synthesized by Integrated DNA Technologies (India). Deoxyribonucleotide triphosphate mix (dNTP) and deoxyadenosine triphosphate (dATP) were purchased from New England Biolabs (India). Filter tips (ART barrier specialty pipette tips), ethidium bromide and DNA ladder (O'GeneRuler ultra low range 10–300 bp ladder) were obtained from Thermo Scientific (India). The *M. tuberculosis* strain (H37Rv) was kindly donated by the Foundation for Medical Research, Mumbai.

2.2. Growth of M. tuberculosis (H37Rv)

All work with *M. tuberculosis* H37Rv was performed in the biosafety level 3 (BSL-3) facility of the Foundation for Medical Research, Mumbai. *M. tuberculosis* was cultured on M7H9 Middlebrook liquid media. The final broth was prepared by mixing 0.52 g M7H9, 440 µl of glycerol, 150 µl of Tween 80 and 100 ml of water. The broth was then autoclaved at 121 °C for 20 min. 10% v/v filtered albumin dextrose catalase (ADC) was added into the media (i.e. 1 ml of ADC in 9 ml of M7H9 liquid media) after it cooled down to 40 °C. Then 1% inoculum (i.e. 0.1 ml inoculum in 10 ml liquid media approximating to 10^7–10^8 CFU/ml) was added to the broth and kept in an incubator at 37 °C without any shaking to prevent aerosol formation in the incubator.

2.3. Chemical and mechanical lysis conditions

Chemical lysis was performed according to the protocol accompanying the Qiagen DNAeasy blood and tissue kit for gram-positive bacteria. Mechanical lysis was performed using the recommended protocols of the FastPrep instrument or the PureLyse kit, both of which are based on bead beating. However, we did not perform any separate disinfection of the bacteria before carrying out any of the lysis protocols. The detailed description of the chemical and mechanical lysis steps is given in the supplementary information.

2.4. Combined heat disinfection and thermal lysis conditions

To perform the combined disinfection and thermal lysis protocol, 100 µl of the live liquid culture (containing typically 10^7 CFU/ml) was transferred to a 200 µl thin-walled micro-centrifuge tube and kept on a heat block. We tested the protocol at different temperatures (95 °C and 65 °C) and for different durations (60 min and 30 min). The thermal lysate was directly used as the template for HDA without any purification.

2.5. Checking cell viability after heat disinfection

For checking the viability of the lysed cells, 10 µl of *M. tuberculosis* lysate was plated on M7H11 agar plates supplemented with oleic albumin dextrose catalase (OADC). The plates were incubated up to 8 weeks at 37 °C and monitored regularly for any signs of cell viability. Cell viability after thermal disinfection and lysis was checked by performing four separate experiments. Each experiment was performed with duplicate plates. Images of the bacterial plates were acquired after 3 or 4 weeks for comparison of the various lysates with the unlysed sample.

2.6. Helicase dependent amplification

After each lysis experiment, helicase dependent amplification was performed at 65 °C using 10 µl reaction volume. Either the lysate or the purified DNA (from chemical and mechanical lyses) was used as the template for HDA for initial optimization. An 84 bp region of the IS6110 gene was targeted using a previously reported primer set (Motré et al., 2011). The IsoAmp III enzyme mix includes T4 gene 32 protein, *exo*-klenow fragment of DNA polymerase I, *E. coli* UvrD helicase, and the accessory protein MutL. We used ~2.5 × concentration of the enzyme mix for all our reactions. The remaining reaction components were 1 × annealing buffer II, 4 mM $MgSO_4$, 40 mM NaCl, 0.4 mM of each dNTP, 4.65 mM additional dATP, forward and reverse primers (0.12 µM each), DNA template (either purified genomic DNA or whole bacterial cells) and DNase-free water. The amplified DNA was detected in a 4% agarose gel stained with ethidium bromide (0.5 µg/ml). The mean greyscale pixel intensities of the gel bands corresponding to pure DNA (obtained by chemical or mechanical lysis) and the one-step reaction were obtained using the image analysis software ImageJ.

2.7. Single step protocol combining disinfection, thermal lysis and HDA

For the combined thermal disinfection, lysis and amplification protocol (i.e. the schematic shown in Fig. 1), the cell culture was directly incubated along with the HDA reaction mixture. The HDA reaction mix was spiked with 2 µl of cultured H37Rv strain (10^7 CFU/ml) and heated at 65 °C for 60 min. This combined protocol is referred to as 'one-step' throughout the manuscript. We performed this experiment four times and each experiment was performed in duplicate.

3. Results and discussion

3.1. Validating the combined thermal disinfection and lysis protocol using H37Rv

All reported protocols for thermal disinfection (or lysis) of *M. tuberculosis* involve high (> 80 °C) temperatures to render the pathogen inactive. Therefore, we also added a positive lysis control by incubating the sample at 95 °C for 30 min. Fig. 2 shows the cell viability after thermal disinfection and lysis. Panels (a) and (c) confirm that *M. tuberculosis* H37Rv can be successfully inactivated at 65 °C with no colony growth up to 3 weeks. We further monitored the plates for up to