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Development and evaluation of a rapid multiplex-PCR based system for *Mycobacterium tuberculosis* diagnosis using sputum samples

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ABSTRACT

Global tuberculosis (TB) control and eradication is hampered by the unavailability of simple, rapid and affordable diagnostic tests deployable at low infrastructure microscopy centers. We have developed and evaluated the performance of a nucleic acid amplification test for detection of *Mycobacterium tuberculosis* (MTB), the NWU-TB test, in clinical sputum specimens from 306 patients with suspected pulmonary tuberculosis. The test involves sputum sample processing using a Lyser device within 7 min, followed by rapid multiplex-PCR on a fast thermal cycler within 25 min, and amplicon resolution on agarose gel electrophoresis. Samples were also examined for presence of MTB using smear microscopy, GeneXpert and MGIT culture. Results were assessed in comparison to a MGIT culture as gold standard. Of the 306 patients, 174 had a previous TB history or already on treatment, and 132 were TB naïve cases. The NWU-TB system was found to have an overall sensitivity and specificity of 80.8% (95% CI: 75–85.7) and 75.6% (95% CI: 64.9–84.4) respectively, in comparison to 85.3% (95% CI: 79.9–89.6) and 73.2% (95% CI: 62.2–82.4) respectively for GeneXpert; and 62.1% (95% CI: 55.3–68.4) and 56.1% (95% CI: 44.7–67) respectively for smear microscopy. The study has shown that the NWU-TB system allows detection of TB in less than two hours and can be utilized at low infrastructure sites to provide quick and accurate diagnosis at a very low cost.

1. Introduction

One hundred and thirty three years after Robert Koch recognized *Mycobacterium tuberculosis* (MTB) as the causative agent of tuberculosis (TB), it still poses an enormous global public health burden today (Koch, 1882). It is estimated that a third of the world's population, is infected with MTB, but most never develop active TB disease (Sudre et al., 1992). This is not the case, however, for persons living with human immunodeficiency virus (HIV) who contract TB infection. People with HIV-infection are 26 to 31 times more likely to become sick with TB in a given year compared to HIV-negative persons (World Health Organization, 2014). Worldwide, an estimated one-third of people living with HIV/AIDS are co-infected with TB. In 2013, there were an estimated 9.0 million new cases of TB (13% co-infected with HIV) and 1.4 million TB deaths globally (World Health Organization, 2014). In the same year, of the 27 countries that reported their multi-drug resistant tuberculosis (MDR-TB) data, the cases reached 450,000; of these, 170,000 people died (World Health Organization, 2014).

South Africa is home to the highest number (6.1 million individuals) of people living with HIV/AIDS (UNAIDS, 2013). This has led to an increase in the number of TB cases over the past decade, with the estimated TB incidence approaching 715/100,000 (World Health Organization, 2014). HIV-positive patients with smear-negative pulmonary TB (SN-PTB) are generally more severely immunosuppressed than those with smear-positive TB. Outcomes for them are poorer, especially when drug regimens do not contain rifampicin (Corbett et al., 2003). In a study of HIV-positive TB patients in Khayelitsha, South Africa, 49% of patients on TB treatment had negative smears on direct microscopy but their sputum cultures were positive (Coetzee et al., 2004). This makes smear microscopy, the most commonly used TB diagnostic test at low infrastructure sites, unreliable in Sub-Saharan Africa where HIV prevalence is high.

Global partnerships to improve disease diagnosis and appropriate treatment are a priority to prevent further global spread of TB. Complications due to HIV co-infection and emergence of drug resistant MTB strains seek to erode the gains that have been made so far to eradicate TB globally (Sharma and Mohan, 2006; Small and Pai, 2010). One critical impediment in the global fight against TB is the lack of rapid, affordable and effective diagnostics tests that can be performed at low-infrastructure sites, right at the doorsteps of most of the vulnerable communities (Urdea et al., 2006). Without better diagnostic tools for

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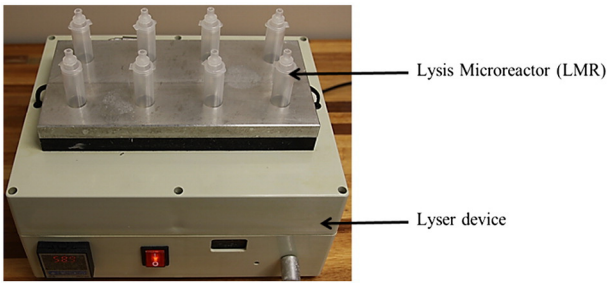

Fig. 1. Lyser device and lysis microreactor used during cell lysis step in the NWU-TB system.

TB/MDR-TB and effective strategies for their implementation, transmission will not be interrupted, mortality will not be checked, and TB will not be controlled especially in areas where HIV infection is prevalent.

Culture remains the gold standard for TB diagnosis despite its long turnaround time of between 2 and 6 weeks due to slow mycobacterial growth (Pai et al., 2004a). Ziehl-Neelsen sputum smear microscopy (SSM) is the most widely method at the primary level in Sub-Saharan Africa (SSA) due to its low installation and per sample costs. However it has poor and variable sensitivity (20–60%) especially in HIV/TB co-infected patients, and cannot distinguish MTB from other non-tuberculosis mycobacteria (NTM) (Pai et al., 2004b; Steingart et al., 2006). The costs to patients associated with multiple visits to clinics to provide specimens before treatment starts are oftentimes prohibitive and cause infectious individuals to drop out of the treatment program. Recently, GeneXpert, an automated user-friendly real-time polymerase chain reaction (PCR) assay able to simultaneously detect *M. tuberculosis* (MTB) and rifampicin resistance has been widely deployed and adopted in South Africa (Lawn et al., 2013; Nicol et al., 2011). It has a total assay time (TAT) of 2 h, high sensitivity and specificity in sputum samples but the test is expensive with a current unsubsidized cost of US$16 per test, which the majority of people in Africa cannot afford. Given the background of these concerns, current research must focus on simplifying test protocols, decreasing cost and improving accuracy. Moreover, for sustainable solutions to the TB/HIV burden to become a reality, Africa as the greatest bearer of that burden should be part of the whole supply chain equation, right from product development to patient use. Without building and supporting diagnostic tests, development initiatives in Africa to solve diseases particular to that region, the list of manageable diseases that continues to unnecessarily kill millions of people yearly, is sure to keep growing uncurbed.

Several studies have documented the risk of mycobacterial infection of people working in diagnostic and research laboratories (Garber et al., 2003; Grist and Emslie, 1985; Miller et al., 1987; Muller, 1988). In spite of current knowledge on precautions and safety guidelines in TB laboratories, in 2003 TB conversion among healthcare workers (HCWs) in New York ranged from 2 to 6.6% (Garber et al., 2003). Some reports even suggest underreporting of the cases due to the social stigma attached and slow disease progression, which may result in HCW retiring before becoming symptomatic (Collins and Kennedy, 1999; Pike, 1976, 1979). Manipulation of liquid clinical specimens involves generation of infectious aerosols and exposure to these aerosols represents one of the most serious hazards encountered in the laboratory (Miller et al., 1987; Muller, 1988). Any new TB diagnosis system must have a proven safety profile to the health workers that operate it.

In an effort to have a simple, affordable, rapid and safe TB diagnostic system, a simple DNA diagnostic system for MTB detection (herein after referred to as NWU-TB system) was developed, which can be readily applied to some other diseases. This molecular based system aims to possess the high accuracy associated with other molecular based diagnostic systems, but with the low cost structure of SSM. It is envisaged that such a system may replace SSM in SSA. However, before deployment of the NWU-TB system, there is a need to conduct a number of independent studies to evaluate its performance using clinical samples. In the present study we describe and evaluate the performance and biosafety of NWU-TB system in clinical sputum samples.

2. Materials and methods

Specimen collection, SSM, MGIT culture, and Xpert MTB/Rif were performed by independent staff at Orkney-Westvaal Hospital, North West Province, South Africa. The Orkney-Westvaal Hospital is wholly owned and privately operated by AngloGold Ashanti to treat its gold miners. The NWU-TB system development and testing was performed at the North-West University, Potchefstroom Campus, South Africa.

2.1. Study population and specimens

This was a single site, blinded, prospective study conducted between January 2013 and October 2014 to evaluate the performance of the NWU-TB system in patients with suspected pulmonary TB in comparison to standard tests. Sputum samples were obtained under the supervision of trained nurses from presumptive TB patients on routine visit to the hospital. Standard TB tests (SSM, MGIT culture, GeneXpert) were performed on all patients, however, due to the large sputum volume required to perform all three tests it was not possible to perform GeneXpert for some patients. Left-over sputum samples after standard TB tests were frozen at −20 °C and transferred to the North-West University every fortnight where they were stored under similar conditions until testing. To avoid bias, sputum samples from both TB treatment naive individuals and those at various anti-TB treatment stages were included in the study. Relevant patient information [including the TB patient history (for reinfection cases)] was only received at the data analysis stage. Sample storage time at the North-West University ranged from 2 weeks to 1 year before performing the indicator test.

HIV testing was performed as part of routine care for consenting patients according to standard guidelines (World Health Organization, 2004).

2.2. Reference standard tests

2.2.1. Sputum smear microscopy (SSM)

Two drops of NaOH-NALC decontaminated sample pellet were used for smear microscopy (ZN staining), according to standard protocol (World Health Organization, 1998). The grading of the AFB results was done in accordance with the WHO/International Union Against Tuberculosis and Lung Disease guidelines and scored as "0" for absence of AFB, scanty 1–9, 1+, 2+, or 3+ (World Health Organization, 1998).

2.2.2. MGIT culture

Samples were processed according to the manufacturers' guidelines for MGIT 960 cultures (Becton Dickinson and Company). Briefly, N-Acetyl-L-cysteine–1% sodium hydroxide (final concentration after mixing with the specimen) was used to decontaminate the sputum sample. Specimens were concentrated by centrifugation at 3000 ×*g*, and pellets were resuspended in 0.5 ml of sterile phosphate buffer. 0.5 ml of the processed samples was inoculated into pre-prepared mycobacterial growth indicator tubes (MGITs) (Becton Dickinson, Sparks, Maryland), and incubated at 37 °C for 6 weeks using the BACTEC

Table 1
Sequences of primers used in multiplex PCR.

Primer name	Sequence	fragment
Pab f	5′-ACC ACC GAG CGG TTC GCC TGA-3′	419 bp
Pab r	5′-GAT CTG CGG GTC GTC CCA GGT-3′	
IS1	5′-CCT GCG AGC GTA GGC GT-3′	123 bp
IS2	5′-CTC GTC CAG CGC CGC TTC GG-3′	
MPB1	5′-TCC GCT GCC AGT CGT CTT CC-3′	240 bp
MPB2	5′-GTC CTC GCG AGT CTA GGC CA-3′	

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