Journal of Microbiological Methods 116 (2015) 37–43

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

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

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ARTICLE INFO

Article history:
Received 8 May 2015
Received in revised form 15 June 2015
Accepted 16 June 2015
Available online 18 June 2015

Keywords:
Tuberculosis
Rapid diagnosis
PCR
NWU-TB system
Sputum

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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http://dx.doi.org/10.1016/j.mimet.2015.06.007

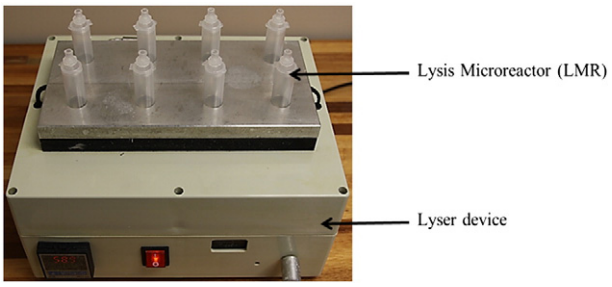

Fig. 1. Lyser device and lysis microreactor used during cell lysis step in the NWU-TB system.

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, 2013). 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′	

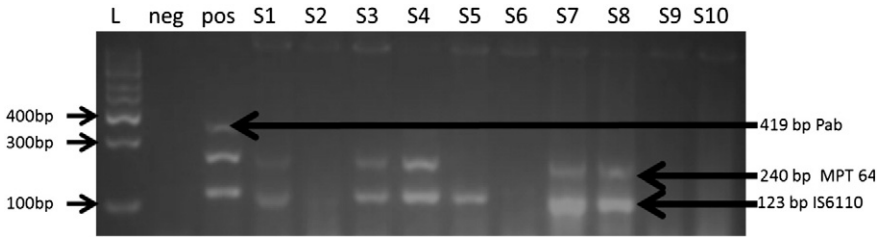

Fig. 2. Multiplex PCR amplification of IS6110 (123 bp), MPT64 (240 bp) and protein antigen b (419 bp). L — 100 bp marker; neg — negative control; pos — positive control; and S1 to S10 — patient sputum samples.

MGIT 960 instrument. The instrument automatically monitors and calls a positive result. Culture was considered negative after six weeks if the instrument did not detect mycobacterial growth except in cases where AFB smear was positive: then cultures were held for a total of 8 weeks. Qualitative confirmation of Mycobacterium tuberculosis complex (MTBC) in culture positive MGIT tubes was performed using the MGIT BD MGIT TBc Identification Test (TBc ID) (Becton, Dickinson & Company, Sparks, MD, USA) according to the manufacturers' instructions (BD Diagnostics, 2013).

2.2.3. GeneXpert

GeneXpert was performed according to the manufacturer's standard operating procedure (SOP) (Cepheid, USA). Briefly, in a 2:1 ratio, the sample reagent (Cepheid) was mixed with NaOH–NALC decontaminated sputum sample and incubated at room temperature for 15 min. Two milliliters of the mixture were transferred into an Xpert MTB/Rif cartridge and inserted into the instrument. The instrument automatically called a result.

2.2.4. NWU-TB

The NWU-TB system consists of three sequential steps; cell lysis within 7 min using a Lyser device, followed by Multiplex-PCR within 25 min and amplicon visualization on an agarose gel. Stored frozen sputum samples were thawed overnight at 4 °C, 250 μl of sample was mixed with an equal volume of lysis buffer [of 20 mM DTT (Dithiothreitol, Sigma-Aldrich, Inc., St. Louis, MO) and 20× TE (Tris–EDTA, Sigma-Aldrich, Inc., St. Louis, MO)] in a lysis microreactor (LMR) (Fig. 1). The LMR was placed on a preset (115 °C and 5000 rpm) Lyser device (Fig. 1) for 7 min. Bacterial cell lysis is concurrently achieved through chemical, thermal and mechanical means (Grobler et al., 2012). 4 μl of lysate was used in the subsequent Multiplex-PCR (M-PCR) step. Each M-PCR reaction in a final volume of 20 μl contained 0.4 mg/ml BSA (Ambion, Foster City, CA, USA), 200 mM of each dNTP, 3.5 mM MgSO4, 1 U KOD Hotstart DNA polymerase in appropriate 10× buffer (Novagen, San Diego, USA), and 200 nM of each of the previously described primers as shown in Table 1 (Kusum et al., 2011). The multiplex primers used target three separate genes in the M. tuberculosis complex genome which are IS6110, protein antigen b and MPB 64. M-PCR was performed on a Philisa rapid thermal cycler (Streck Inc., Omaha, NE) using the following protocol: Initial enzyme activation at 95 °C for 120 s, followed by 40 cycles of 95 °C for 10 s, 66 °C for 15 s and 72 °C for 6 s, and a final extension step at 72 °C for 120 s. PCR products were resolved by electrophoresis (Fig. 2) on a 2% agarose gel stained with gelred nucleic acid stain (Biotium, Hayward, CA, USA). Any specimen yielding a negative result in the NWU-TB system was re-tested if there was sufficient residual sputum and the latter result was considered in the analysis.

2.2.5. Biosafety evaluation of NWU-TB cell lysis step

MTB clinical isolates were inoculated into 12 MGIT tubes and incubated using the BACTEC-MGIT 960 automated culture system until the instrument automatically called a positive result, which occurred on day 12. This has been reported to correspond with approximately 10^6 CFU/ml (Castan et al., 2014). Tween-80 (0.05%) was added to all MGIT tubes before transferring the contents into separate 15 ml sterile conical tubes followed by three successive rounds of vortexing each lasting about 7 min to declump the mycobacteria.

Confirmed smear negative-culture negative samples from two individuals were pooled together and used as diluent in preparing 10 fold serial dilutions (10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml) in quintuples. For each dilution, the contents of 3 of the 5 tubes were subjected to lysis, and the other two tubes were not lysed (used as positive controls). Lysis was performed as per standard procedure in the NWU-TB system (described above). Pre-prepared MGIT tubes were inoculated with 500 μl of appropriate sample (dilutions described above) and incubated using the BACTEC-MGIT-960 for 42 days.

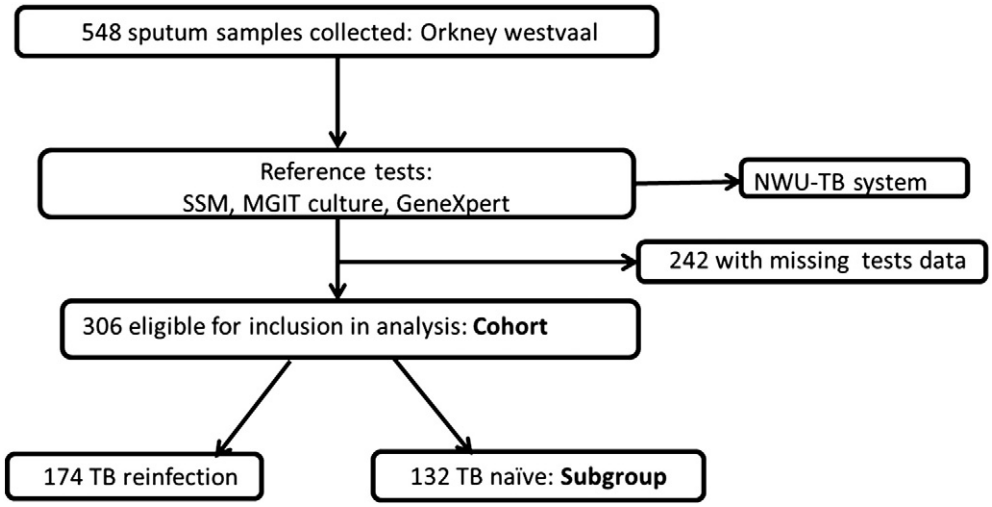

Fig. 3. Patient flow, showing the two arms for data analyses, namely the Cohort and Subgroup.

2.2.6. Statistical analysis

Accuracy measures (sensitivity, specificity, positive and negative predictive values) of the evaluated tests for *M. tuberculosis* detection were determined using MGIT culture as the gold standard. Only samples that had all smear, culture, Xpert MTB/Rif, and NWU-TB system results were included in the analysis. Statistical analyses were performed using STATA 11 (StataCorp, College Station, TX, USA). Due to the high frequency of TB re-infections (28.4%) in our cohort, a subgroup analysis (herein after referred to as subgroup) of patients with no known TB history and treatment TB naive was carried out.

2.2.7. Ethics

This study was approved by the North-West University Research Ethics Committee under ethics number: NWU-00005-11-A5. NWU-TB system results were not used in clinical decision making.

3. Results

3.1. Patient population

548 sputum samples were supplied to the North-West University research team by Orkney-Westvaal Hospital over the indicated period; however for various reasons such as inadequate sample volumes to perform GeneXpert test or culture contamination, only 306 samples had results for all four tests and were thus included in the analysis. After unblinding and considering TB patient history, two data analysis arms were decided upon. Of the 306 (referred to as cohort) samples, 132 met the subgroup inclusion criteria as highlighted in the statistical analysis section (Fig. 3).

General demographic and microbiological characteristics of the study population are shown in Table 2. The median age of the cohort and subgroup was 43.9 and 46.0 years respectively. The cohort is predominantly male (96.4%), which is above the 2:1 (male: female) gender inequality seen in adult TB. This is because the hospital caters only for miners, who are mainly males. Of the 160 patients tested for HIV in the cohort, 122 (73.7% prevalence) were positive, which is similar to 75.3% in the subgroup. Based on SSM and culture classification, smear positive-culture positives were the highest: 50% and 45.4% in the subgroup and cohort respectively.

3.2. Performance of NWU-TB in the individuals with no TB history (referred to as subgroup analysis)

When analyzed against MGIT culture as gold standard, the sensitivities of SSM, GeneXpert and NWU-TB were 62.9% [95% CI: 52.9, 72.1]; 83.3% [95% CI: 75.3, 90.3]; and 87.6% [95% CI: 79.8, 93.2], respectively (Table 3). NWU-TB was more sensitive than SSM ($p = 0.001$) and had comparable sensitivity to GeneXpert ($p = 0.135$). The specificity of both GeneXpert and NWU-TB was significantly lower than expected as reported in other studies.

Positive predictive values (PPV), negative predictive values (NPV), and Diagnostic odds ratio (DOR) of SSM, GeneXpert, and NWU-TB are provided in Table 3. In all the indicators NWU-TB is better than SSM and comparable to GeneXpert.

The sensitivity of NWU-TB in the paucibacillary smear negative-culture positive group was 76.1% [95% CI: 60.7, 88.9]. Interestingly, in the culture negative group, 13 samples had positive results with the other 3 tests contributing to the unexpected high sensitivities for both NWU-TB and GeneXpert in smear positive-culture negative and smear negative-culture negative groups (Table 4). This raises a number of questions as to the sensitivity of culture in a high TB prevalence setting (mining/prisons) and the impact on diagnostic test evaluation in such a cohort.

Table 2
Demographic information and microbiological characteristics of subgroup and cohort in the analysis arms.

	Subgroup	Cohort
No. of TB suspects	132	306
Median age in years (95% CI)	43.9 (42.4–45.4)	46.0 (39.0–51.0)
HIV prevalence (n) %	(64/85) 75.3	(122/160) 73.7
Sex		
Male n (%)	127 (95.5)	295 (96.4)
Female n (%)	5 (4.5)	11 (3.6)
Race		
Black n (%)	132 (100)	306 (100)
Previous TB history n (%)	0 (0)	174 (56.9)
Smear and culture grading		
$Smear^+$ $culture^+$ n (%)	66 (50.0)	139 (45.4)
$Smear^-$ $Culture^+$ n (%)	39 (29.5)	85 (27.7)
$Smear^+$ $Culture^-$ n (%)	20 (15.2)	36 (11.8)
$Smear^-$ $Culture^-$ n (%)	7 (5.3)	46 (15.0)

3.3. Performance of NWU-TB in cohort analysis

The performance of NWU-TB against MGIT culture performed on the same sputum samples is shown in Table 5 alongside outcomes for SSM and GeneXpert. The overall sensitivity in NWU-TB at 95% confidence interval was 80.8% (75%–85.7%). This was lower than GeneXpert with a sensitivity of 85.3% (79.9%–89.6%) ($p = 0.026$), but better than SSM 62.1% (55.3%–68.4%) ($p = 0.001$). Specificity of NWU-TB and GeneXpert was still lower than expected as in the subgroup analysis.

For the NWU-TB system, when stratified by HIV status, there was better sensitivity in the HIV negative group 86.8% (71.9%–95.6%) compared to the HIV positive group 78.7% (70.4%–85.6%), though not statistically significant ($p = 0.190$). Similar results with no statistical significance ($p = 0.519$) were also observed with GeneXpert 89.5% (75.2%–92.7%) in the HIV seronegative group compared to 86.1% (78.6–91.7%) in the HIV seropositive group.

3.4. Frequency of non-tuberculosis mycobacteria

Though this was not part of the initial study design, the high frequency of non-tuberculosis mycobacteria (NTM) (Table 6) in our cohort warrants reporting on.

3.5. Biosafety evaluation

No growth was observed in any of the lysed cultures, independent of the concentration after 42 days of MGIT culture (Fig. 4). There was growth in all positive controls and time to positivity was inversely proportional to starting mycobacteria concentration.

4. Discussion

Diagnostics tests are important decision support tools for physicians and nurses in deciding on the most appropriate drug regimens for various diseases. Toward this end, clinicians need rapid, accurate, affordable and accessible diagnostic tests before prescribing appropriate medication. Despite the recent upsurge in the number of MTB detection

Table 3
Performance of SSM, GeneXpert, and NWU-TB system against MGIT culture as gold standard.

Parameter	SSM	GeneXpert	NWU-TB
Sensitivity % (95% CI)	62.9 (52.9–72.1)	83.8 (75.3–90.3)	87.6 (79.8–93.2)
Specificity % (95% CI)	74.1 (53.7–88.9)	70.4 (49.8–86.2)	88.9 (70.8–97.6)
PPV % (95% CI)	90.4 (81.2–96.1)	91.7 (84.2–96.3)	96.8 (91–99.3)
NPV % (95% CI)	33.9 (22.1–47.4)	52.8 (35.5–69.6)	64.9 (47.5–79.8)
DOR	4.84 (1.91–12.2)	12.3 (4.7–32.1)	56.6 (15.6–201)

Table 4
Sensitivities of SSM, GeneXpert, and NWU-TB in various SSM and culture result groups.

Smear–culture grade	GeneXpert % (95% CI)	NWU-TB % (95% CI)
Smear-positive, culture-positive	96.7 (89.5–99.6)	93.9 (85.2–98.3)
Smear-negative, culture-positive	61.5 (44.6–76.6)	76.1 (60.7–88.9)
Smear-positive, culture-negative	15.0 (3.21–37.9)	0.0 (0.0–16.8)
Smear-negative, culture-negative	71.4 (29.4–96.3)	42.9 (9.9–81.6)

systems, improved or novel systems suitable for resource limited settings are still a high priority. Urdea et al. (2006) states that a rapid TB diagnostic test requiring no laboratory infrastructure, with at least 85% pooled sensitivity for smear-positive and smear-negatives cases, and 97% specificity, could save approximately 400,000 lives annually (Urdea et al., 2006). However, even the most promising diagnostic test will have only limited impact if it does not reach the patients who need it. A real-world evaluation of one such system under development, aiming to address those concerns and more especially the affordability aspect has been conducted.

The NWU-TB system performance in the both the subgroup and cohort analysis is comparable to that of GeneXpert, which is also a molecular based system. The observed sensitivity of GeneXpert in this study is within the range previously reported in similar studies conducted in other high HIV prevalence settings (Chang et al., 2012; Scott et al., 2011; Theron et al., 2011). However, the specificity is lower than that reported in literature: From our data we observed 13 samples in which all 3 of the other tests were positive except culture. Marlowe et al. (2011) also reported true positive results with the GeneXpert system in culture-negative clinical specimens. Furthermore, although, MGIT culture is considered the current gold standard for MTB diagnosis, it is not 100% sensitive itself, thus some of the MGIT culture negatives are likely to be true positives (McCarthy et al., 2012; Muchwa et al., 2012). Culture negative active TB has also been shown to be characterized by clinical disease and highly suggestive Chest X-ray (CXR) abnormalities (Maher, 2009).

The high prevalence of NTMs in our cohort is bound to have contributed to the poor specificity, as the NTM-positive participants possibly also had *M. tuberculosis*, which might have been outcompeted in MGIT culture (Dinnes et al., 2007; Ssengooba et al., 2014). The usefulness of GeneXpert in culture negative samples, especially in HIV seropositive patients, warrants urgent investigation in order to address the increasingly observed MGIT culture limitations in this group. Future studies with more robust identification methods such as 16S rDNA sequence based identification (Patel et al., 2000) on negative cultures are also recommended to eliminate the possibility of culture false-negatives.

Table 5
Performance outcomes of SSM, GeneXpert, and NWU-TB stratified by HIV status, against MGIT culture as gold standard in the cohort.

Parameter	SSM % (95% CI)	GeneXpert % (95% CI)	NWU-TB % (95% CI)
Sensitivity			
Cohort (n = 306)	62.1 (55.3–68.4)	85.3 (79.9–89.6)	80.8 (75.0–85.7)
HIV positive (n = 167)	62.3 (53.1–70.9)	86.1 (78.6–91.7)	78.7 (70.4–85.6)
HIV negative (n = 54)	63.2 (46.0–78.2)	89.5 (75.2–97.1)	86.8 (71.9–95.6)
Specificity			
Cohort (n = 306)	56.1 (44.7–67.0)	73.2 (62.2–82.4)	75.6 (64.9–84.4)
HIV positive (n = 167)	51.1 (35.8–66.3)	71.1 (55.7–83.6)	73.3 (58.1–85.4)
HIV negative (n = 54)	50.0 (24.7–75.3)	75.0 (47.6–92.7)	81.3 (54.4–96.0)
PPV			
Cohort (n = 306)	79.4 (72.7–85.2)	89.7 (84.8–93.4)	90.0 (85.1–93.8)
HIV positive (n = 167)	77.6 (68.0–85.4)	89.0 (81.9–94.0)	88.9 (81.4–94.1)
HIV negative (n = 54)	75.0 (56.6–88.5)	89.5 (75.2–97.1)	91.7 (77.5–98.2)
NPV			
Cohort (n = 306)	35.1 (27.0–43.9)	64.5 (53.9–74.2)	59.0 (49.0–68.5)
HIV positive (n = 167)	33.3 (22.4–45.7)	65.3 (50.4–78.3)	55.9 (42.4–68.8)
HIV negative (n = 54)	36.4 (17.2–59.3)	75.0 (47.6–92.7)	72.2 (46.5–90.3)

All values are in percentages (95% CI).

Table 6
Frequencies of non-tuberculosis mycobacteria found in the cohort.

NTM	Frequency	Growth rate
M. abscessus	1	Rapid grower
M. avium	24	Slow grower
M. chelonei	3	Rapid grower
M. fortuitum complex	4	Rapid grower
M. intracellulare	1	Slow grower
M. kansasii	25	Slow grower
M. Simiae	1	Slow grower

The smear positive-culture positive group is associated with high mycobacterial burden and most TB detection systems show high sensitivity (94–100%) in this group. Sensitivities for GeneXpert and NWU-TB in both subgroup and cohort analyses were within the reported ranges of other molecular based systems in literature (Boehme et al., 2011; Castan et al., 2014; Marlowe et al., 2011). However, in the problematic, smear negative-culture positive group, NWU-TB showed a marginally better sensitivity compared to GeneXpert in the subgroup and cohort analyses. GeneXpert had a similar performance to that reported in another South Africa study with a comparable HIV prevalence (Table 7) (Scott et al., 2011). NWU-TB performed better (Table 7) than other molecular based MTB diagnosis in literature in the smear negative-culture positive group.

Tuberculosis is a disease more commonly seen in developing countries and is typically associated with poverty. Although the GeneXpert system is simple with good performance even in high HIV prevalence settings, it's high costs ultimately leads to limited use (Helb et al., 2010). In this study, the NWU-TB system shows comparable performance to GeneXpert and due to the simplicity of the technology we estimate the cost per test to be less than US$4 compared to US$18 for GeneXpert (Lawn and Nicol, 2011). One of the main advantages of the NWU-TB system is the small sputum sample volume (250 μL) required to obtain an accurate result compared to GeneXpert, which requires a minimum volume of 0.7 mL (Boehme et al., 2010). This is beneficial for some patient populations such as the elderly, children, and HIV infected individuals who have difficulties in expectorating sputum (A. Harries et al., 1998; A.D. Harries et al., 1998; Zar et al., 2005). The sputum sample matrix is also known for its complex PCR inhibitors, and our ability to directly amplify (without need of a nucleic acid extraction step) lysed sputum samples with sensitivity comparable to that of GeneXpert is significant.

The total assay time (TAT) for NWU-TB system is about 1 h 30 min consisting of a 7 minute cell lysis step, 25 min PCR cycling, 30 min agarose gel processing and 30 min for manipulation procedures in between the steps. The semi-automation of the NWU-TB system is one of the next steps in its development and this will further reduce TAT to about 1 h and should lower cost even further. This short TAT as in

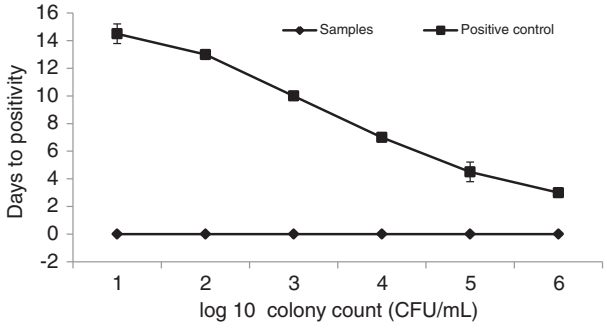

Fig. 4. Time to positivity after MGIT culture of NWU-TB system lysed 10-fold serial dilutions of *M. tuberculosis* over a period of 42 days.

Table 7
Reported diagnostic performance of different NAATs in smear-negative sputum TB presumptive individuals in different countries.

Location	Test	HIV prevalence	Sensitivity % (95% CI)	Reference
Brazil	Gen-Probe A-MTD	100%	70.8 (48.0–87.3)	Barreto et al. (2014)
Zambia	Gen-Probe A-MTD	75%	60.0 (43.0–87.3)	Kambashi et al. (2001)
Zambia	IS6110 PCR	75%	40.0 (25.0–57.0)	Kambashi et al. (2001)
South Africa	GeneXpert	70%	61	Scott et al. (2011)
South Africa	GeneXpert	75.3%	61. 5	This study
South Africa	NWU-TB	75.3%	76.1	This study

GeneXpert (2 h) will reduce patient dropout rates ensure timely initiation of effective treatment for infected patients and timely quarantine of infected individuals, which has a significant role in controlling spread of tuberculosis.

Potential dangers during mycobacterial culture are well known and widely reported in literature (Bemer-Melchior and Drugeon, 1999; Cruciani et al., 2004). For SSM, there is limited epidemiologic data linking smear preparation with tuberculosis risk; however, most national guidelines recommend sputum handling and AFB smear preparation inside a biological safety cabinet (BSC) (Chedore et al., 2002; Kent, 1985). An ideal POC-TB test should carry less risk than SSM and should aim to eliminate all infectious risk. Safety during lysis and handling of lysates for health care workers is of primary concern and must be thoroughly investigated before deployment of any system. There is contrasting evidence as to the efficiency of heat of inactivation of M. tuberculosis. For example, Zwadik et al. reported the occurrence of living microorganisms after 30 min at 95 °C (Zwadyk et al., 1994). In NWU-TB, rapid cell lysis is achieved through a combination of thermal, chemical and mechanical means (Grobler et al., 2012). The 7 minute heat lysis step of the NWU-TB system may point toward a biohazard risk due to incomplete inactivation of the mycobacteria. However, the inclusion of a chemical lysis buffer which weakens the bacterial cell wall enable use of such a short lysis time as demonstrated by zero mycobacterial growth after 42 days of MGIT culture in this preliminary study. The LMR used for lysis is fitted with a filter (Fig. 5) to trap bioaerosols formed during heating at the high temperatures. Although smoke-tests were performed, in-depth studies using a Biosampler to trap aerosols exiting the LMR filter during lysis step are needed before reaching final conclusion on the biosafety. From the preliminary assessment it can be concluded that there is complete mycobacterial inactivation after the 7 minute lysis step. Bacillary load has been reported to be one of the main variables in causing incomplete mycobacteria inactivation but in this study there was complete inactivation independent of the bacillary load (Castro et al., 2009).

In conclusion, the NWU-TB system has shown good sensitivity and specificity results comparable to GeneXpert, which is the best in class molecular based TB detection test. The system is also safe, and based on a simple and efficient sputum sample preparation method. Further development of the NWU-TB system is ongoing.

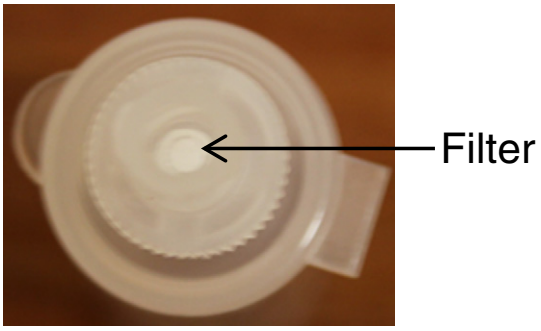

Fig. 5. Aerial view of LMR showing a fitted filter to trap bioaerosols during heat lysis.

5. Limitations of the study

The patient cohort does not represent the normal population and the high accuracy reported in this study could be due to the high prevalence of MTB strains with multiple copies of the insertion sequence IS6110 in this cohort (Alonso et al., 2013). Fresh sputum samples were used for GeneXpert, SSM and MGIT culture, but frozen samples stored for up to a year were used for NWU-TB analysis. Association between the use of frozen sputum samples and reduced the sensitivity of another (GeneXpert) molecular test has been previously reported (Steingart et al., 2013). The use of frozen samples may have altered the performance of NWU-TB and future evaluation studies must include fresh samples. Like all other current molecular based systems, our system does not distinguish between live and dead mycobacteria, which could partly explain the lower specificity especially in such a high TB prevalence setting.

Funding

This study was supported by the Technology Innovation Agency of South Africa, MRC — Strategic Health Innovation Partnerships, and the South African TB Research & Innovation Initiative (SATRII).

Conflict of interest

The authors declare that they have no conflicts of interest. All rights in patents were assigned to North-West University.

Acknowledgments

The authors would like to thank the staff at the AngloGold Ashanti Orkney-Westvaal Hospital for performing smear microscopy, GeneXpert and mycobacterial culture for this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mimet.2015.06.007.

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