Field evaluation of a novel preservation medium to transport sputum specimens for molecular detection of *Mycobacterium tuberculosis* in a rural African setting

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Abstract

OBJECTIVES To assess the performance of an innovative method of transporting sputum to centralised facilities for molecular detection of *Mycobacterium tuberculosis*: using a swab to inoculate sputum in a transport medium, PrimeStore® Molecular Transport Medium (PS-MTM).
METHODS Two sputum specimens were obtained from suspected patients with tuberculosis (TB) at rural healthcare facilities in South Africa. A swab was taken from each specimen and placed into PS-MTM, prior to it being processed by either liquid culture or Xpert MTB/RIF assay (Xpert).
RESULTS A total of 141 patients (including 47 with laboratory-confirmed TB) were included in this analysis. *M. tuberculosis* was detected at 29% by culture and 29% by Xpert, whereas 31% tested positive by *IS6110* real-time PCR of PS-MTM from the culture and 36% from the Xpert-paired specimen. Concordance between the method under evaluation with culture was 82% (McNemar, $P = 0.55$) and 84% (McNemar, $P = 0.05$) for Xpert. Stratified by culture result, the detection rate by *IS6110* real-time PCR of PS-MTM was similar to Xpert for patients with positive culture ($P = 0.32$), but significantly higher if culture was negative ($P = 0.008$).
CONCLUSIONS These results suggest that swab collection of sputum into PS-MTM for transport is a promising method for diagnosis of TB in rural healthcare settings, thereby potentially improving the options available for molecular diagnosis of TB in countries incapable of applying decentralised high-tech molecular testing.

keywords specimen transport medium, sputum, molecular detection, *Mycobacterium tuberculosis*, preservation

Introduction

Tuberculosis (TB) remains one of the greatest eradication challenges this century, with most of the disease burden occurring in resource-constrained settings [1]. Important factors that hamper the eradication of TB include the ineffective vaccine protection [2–4], delayed turnaround time and suboptimal sensitivity of current diagnostic tests, poor uptake of new diagnostic tools, operational and logistic delays, undiagnosed cases and treatment default. The countries with the highest burden remain reliant on smear microscopy with minimal access to the diagnostic gold standard culture [5–7]. The use of molecular methods for detecting *Mycobacterium tuberculosis* provides a rapid alternative approach for the diagnosis of TB. Several studies highlight the benefits of molecular testing for the detection of *M. tuberculosis* [8, 9]. The endorsement by WHO of the Xpert MTB/RIF assay (Xpert) [10] for screening of TB suspects has revolutionised testing for TB globally. In South Africa, Xpert has been implemented as the baseline diagnostic test for pulmonary tuberculosis at previous smear microscopy laboratories [11]. A review by Steingart *et al.* [12] has shown that using pooled sensitivity estimates, Xpert could diagnose 88% of cases *vs.* 65% by smear microscopy.

Despite the successful implementation of molecular diagnostics in South Africa, including the Xpert, several challenges exist. Xpert requires samples to be processed within 3 days if kept at ambient temperature, or samples can be stored at 2–8 °C for a maximum of 7 days if delays are expected, both of which may not be realistic

in many poorly resourced countries [13]. On average, a specimen may take up to 3–5 days before testing in a laboratory mainly due to delays in transport from outlying areas with poor infrastructure [14] and laboratory capacity [15]. Furthermore, Xpert generally uses the complete sputum volume, necessitating collection of an additional specimen should further tests be required; this may be complex in rural settings. Other challenges include the requirement of refrigeration in countries with temperatures exceeding the 28 °C reagent threshold, increased cost associated with reagents, staff and maintenance, and the availability of a stable uninterrupted power supply [7, 13]. These conditions require financial, operational and logistical support [16] which may exist in urban and periurban locations [17], but are difficult to achieve in rural settings in poor countries.

An alternative approach to Xpert testing may be provided by sputum specimen collection and transport to centralised facilities for molecular processing. PrimeStore® – Molecular Transport Medium (PS-MTM, Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) was developed to collect and transport respiratory specimens under extreme environmental conditions by stabilising nucleic acids over time until processing. The transport medium is able to inactivate purified cultured *M. tuberculosis* isolates within 5 s and *M. tuberculosis*-spiked sputum samples within 30 min; DNA remains stable over 28 days at ambient temperature in the medium and is compatible with a range of nucleic acid extraction systems [18]. This medium effectively inactivates pathogens, including *M. tuberculosis,* within 30 min, inhibits nuclease activity and preserves nucleotides at ambient temperature for at least 4 weeks [19, 20]. PS-MTM has been successfully evaluated for detection of respiratory viruses, but may also provide a useful method for molecular detection of *M. tuberculosis*, particularly in low-resource settings where operational and logistic challenges are frequent, the climate is hot, and considerable delays between specimen production and testing may occur. In this study, we evaluate a system of centralised molecular testing of swabbed sputum specimens inoculated in PS-MTM, as an alternative to Xpert or mycobacterial culture, for the detection of *M. tuberculosis* in a rural African setting.

Methods

Study design and sputum specimens

Participants were recruited from a larger cohort study in which individuals with a cough lasting more than two weeks were recruited at primary healthcare facilities in rural Mopani District, South Africa (Peters *et al.,* submitted). Fifty-three (37.6%) specimens were from males and 88 (62.4%) from females. The median age of patients was 40 years with 29 (21%) having been previously treated for tuberculosis. HIV status was known for 84% of patients, of whom 59% were positive. Two sputum specimens, produced at least 1.5 h apart, were obtained from each patient: one for Xpert testing (at point of care or in the NHLS laboratory) and one for mycobacterial culture at the University of Pretoria. The order of specimens from each patient was randomised for the two tests. For this evaluation, paired PS-MTM specimens were selected from 141 patients: those that had a positive result for Xpert and/or MGIT ($n = 47$), and combined with randomly selected patients with negative specimen result ($n = 94$) at a 1:2 ratio.

At the study site, shortly after production by the patient, a swabbed sample was collected from each sputum specimen by rotating the flocculated cotton swab (Copan Diagnostics Inc., Brescia, Italy) a minimum of five times within each specimen container and placing it into PS-MTM collection tubes. The PS-MTM specimens were batched and sent for molecular processing biweekly at ambient temperature across approximately 500 km from the study sites to the National TB Reference Laboratory, Johannesburg, South Africa. The time lag between sample collection and molecular processing was sufficient to ensure total inactivation by the transport medium of mycobacteria that may have been present in the sample.

Routine microbiological tests

IS6110 real-time PCR results of sputum collected in PS-MTM were compared to those of routine Xpert, smear microscopy and liquid culture. Of the two collected sputum specimens, one was tested using the Xpert MTB/RIF assay (version G4 cartridges) (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions and the other decontaminated and concentrated using the NaLC-NaOH method [21]; sediments were used to prepare smears for light microscopy by Ziehl–Neelsen staining [22], and 0.5 ml was inoculated for culture using the Mycobacterial Growth Indicator Tubes (MGIT) 960 system (BD, Sparks, MD, USA) as described by the MGIT procedure manual [23]. Smears were graded according to WHO recommendations [22], and a culture was considered negative by the MGIT 960 system after 42 days of incubations.

Detection of *M. tuberculosis* in specimens collected in PS-MTM

The sputum specimen in PS-MTM was vortexed using the Vortex Genie (Scientific Industries Inc., USA) at maximum speed for 1 min, and a 200 µl aliquot was processed using the generic protocol on the NucliSENS easyMAG (bioMerieux, Marcy l'Etoile, France) to extract DNA. A real-time PCR targeting the *M. tuberculosis* complex-specific insertion sequence element (IS) 6110 for detection of DNA was performed on the StepOne Plus (ThermoFisher Scientific, Waltham, MA USA) following manufacturers' instructions [24]. The PCR comprised 40 cycles, and threshold of 0.1 was applied for analysis. Specimens were considered either positive, indeterminate or negative based on the following pre-defined cycle threshold (CT) values: ≤38 positive, 38.01–39.99 indeterminate and ≥40 (no amplification signal) as negative. In general, the real-time PCR cycle threshold is indicative of the level of target template DNA; reactions with a CT below 37 have moderate-to-high levels of template, whereas 38–40 may indicate a low level of template or an environmental contamination (and usually requires another clinical sample for confirmation).

Statistical analysis

Descriptive statistics are provided including frequency measurements. Results of *IS6110* real-time PCR of sputum collected in PS-MTM were compared to Xpert and MGIT culture as standards through cross-table comparison (using concordance rate and McNemar test instead of sensitivity/specificity due to the selection of specimens). A composite reference standard was used with the definition for TB positivity as specimen positive in either MGIT or Xpert. For the purposes of comparison, an indeterminate result of PCR on PS-MTM was excluded from analysis, as an additional patient sample could not be requested to confirm the presence or absence of *M. tuberculosis*. Statistical analyses were performed using Statistix v.7.0 (Analytical Software, Tallahassee, FL, USA).

Ethics approval

Ethics approval for this study was obtained from the Human Research Ethics Committee at the University of Witwatersrand, Johannesburg, South Africa (Ref: M120226).

Results

Detection of *M. tuberculosis* in sputum specimens

A total of 282 paired sputum specimens from 141 patients were evaluated. At most, 2.8% of sputum specimens were not available for testing within each test set. A diagnosis of TB was made for 25 (18%) cases by microscopy, 41 (29%) by culture and 40 (29%) by Xpert (Figure 1). MGIT culture and Xpert were both positive for *M. tuberculosis* for 34 samples with an additional seven for culture only and six for Xpert only. Further, *M. tuberculosis* DNA was detected in specimens collected in PS-MTM for 43 patients (31%) paired to culture specimens and 49 patients (36%) paired to Xpert specimens. There was no difference in detection of *M. tuberculosis* DNA between first (32%) or second (36%) sputum specimen stored in PS-MTM (McNemar, $P = 0.21$).

Concordance between *IS6110* real-time PCR of sputum in PS-MTM and liquid culture

Mycobacterium tuberculosis DNA was detected from sputum in PS-MTM in 21/25 specimens that were smear microscopy positive and 29/41 specimens that were positive by culture (71%) (Table 1). An indeterminate result was observed for one culture-positive specimen and for two culture-negative specimens. Agreement, excluding indeterminate results, was 73% for culture-positive and 85% for culture-negative samples resulting in an overall agreement of 82% (McNemar, $P = 0.55$). *IS6110* real-time PCR of DNA from PS-MTM samples showed discordance for 11 positive and 14 negative specimens by culture (Table 2). When comparing this discordance to the same patient's Xpert specimen result, the Xpert result concurred with six of the 11 culture positives and 11 of the 13 negative cultures (1 Xpert not done) (Table 2).

Concordance between *IS6110* real-time PCR from sputum in PS-MTM and Xpert

Of the Xpert-positive samples, *IS6110* real-time PCR of DNA from sputum in PS-MTM was positive for 32/39 (82%) (Table 1). One Xpert-positive and two Xpert-negative sputum specimens had indeterminate results of PCR from sputum in PS-MTM. Agreement, excluding indeterminate results, between *IS6110* real-time PCR from PS-MTM and Xpert, was 84% for both positive and negative Xpert results, giving an overall agreement of 84% (McNemar, $P = 0.05$). The relatively low agreement for negative specimens is the result of a higher detection rate by *IS6110* real-time PCR of PS-MTM: 15 Xpert-negative specimens had *M. tuberculosis* DNA detected from samples in PS-MTM. When

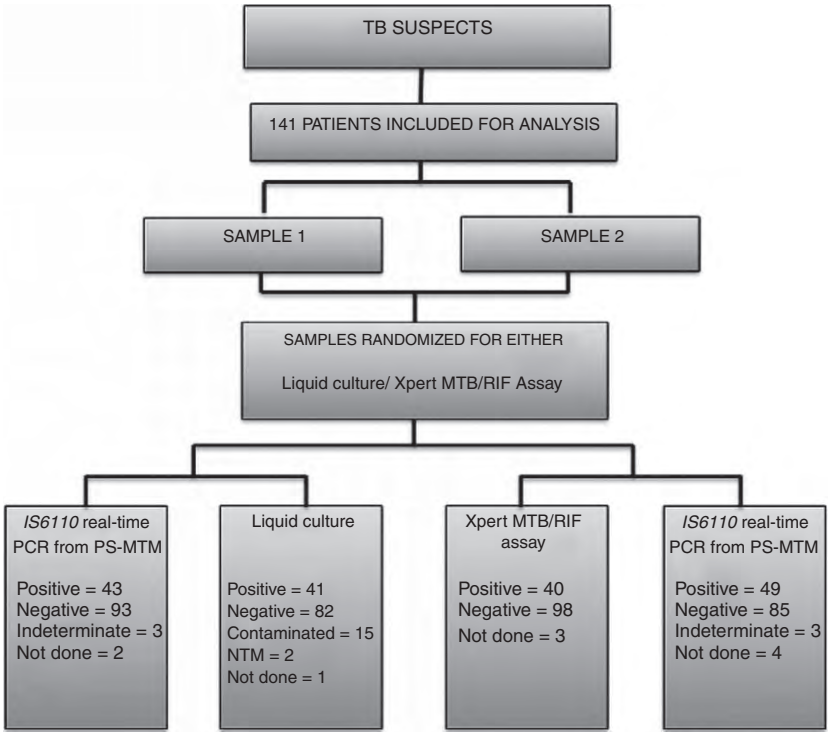

Figure 1 Study design and results of testing sputum specimens for the presence of *Mycobacterium tuberculosis* by liquid culture (MGIT 960 system), Xpert MTB/RIF assay and *IS6110* real-time PCR of sputum in PrimeStore® – Molecular Transport Medium (PS-MTM).

Table 1 Concordance of *IS6110* real-time PCR detection of *Mycobacterium tuberculosis* DNA from sputum in PS-MTM compared to liquid culture and Xpert

	Liquid culture		Xpert	
IS6110 real-time PCR from PS-MTM	Positive (n = 41)	Negative (n = 98)	Positive (n = 39)	Negative (n = 95)
No. (%*) PCR positive	29 (71)	14 (14)	32 (82)	15 (16)
No. (%*) PCR negative	11 (27)	82 (84)	6 (15)	78 (82)
No. (%) PCR indeterminate	1 (2)	2 (2)	1 (3)	2 (2)
Concordance* (McNemar)	82% (P = 0.55)		84% (P = 0.05)	

*Excluding specimens with indeterminate PCR result from the denominator.

Table 2 Detection of *Mycobacterium tuberculosis* DNA by *IS6110* real-time PCR from PS-MTM samples compared to Xpert result stratified by the MGIT culture result

	Culture positive		Culture negative	
IS6110 real-time PCR from PS-MTM*	Xpert positive (n = 32)	Xpert negative (n = 5)	Xpert positive (n = 6)	Xpert negative (n = 87)
No. (%) PCR positive	29 (90.6)	1 (20.0)	3 (50.0)	14 (16.1)
No. (%) PCR negative	3 (9.4)	4 (80.0)	3 (50.0)	73 (83.9)
Concordance* (McNemar)	89% (P = 0.32)		82% (P = 0.008)	

*Excluding specimens with indeterminate PCR result.

stratifying Xpert results by the result of liquid culture, there was good concordance between *IS6110* real-time PCR of DNA in PS-MTM and Xpert (Table 2).

A significant difference was observed for the culture-negative specimens (McNemar, P = 0.008), in which *M. tuberculosis* was detected exclusively in 14 PS-MTM samples *vs.* three by the Xpert (Table 2).

Sputum *M. tuberculosis* DNA load by result of routine test

In cases of positive *IS6110* real-time PCR result of sputum in PS-MTM, the median CT-value was significantly higher for specimens with a positive result paired to culture than a negative (28.47 *vs.* 34.47; $P < 0.001$). This significant difference was also observed when comparing CT-value between specimens with positive and negative paired Xpert result (28.34 *vs.* 34.81; $P < 0.001$).

Discussion

This field evaluation in a rural setting with poor infrastructure shows that PS-MTM provides a promising tool that could support centralised molecular testing for *M. tuberculosis* as an alternative to the Xpert assay and mycobacterial culture. We have previously shown that this is a safe way of transporting sputum specimens as PS-MTM inactivates the bacilli, thereby making it safe from an infection control perspective, and that stability of DNA is achieved over time at ambient temperature [18]. These findings concurred with previous studies evaluating these technical properties [19, 20]. In our laboratory evaluation, we further demonstrated good sensitivity and specificity of *IS6110* real-time PCR detection of *M. tuberculosis* from clinical samples inoculated into PS-MTM [18]. This study confirms those results in the field setting of rural healthcare facilities.

In this study, *IS6110* real-time PCR detection of *M. tuberculosis* from PS-MTM specimens showed a slightly higher positivity rate than culture and Xpert. A few samples had an indeterminate result of *IS6110* real-time PCR from PS-MTM as low concentration of DNA was detected (CT-value > 38 cycles). In these cases, the origin of *M. tuberculosis* DNA is not clear and, as background contamination cannot be ruled out, a repeat specimen would normally be requested in clinical practice for further interpretation. It is of note that one of the specimens with indeterminate result was culture positive and another Xpert positive.

Concordance of *IS6110* real-time PCR from PS-MTM with culture and Xpert was good for both positive and negative results, except for the concordance with Xpert-negative specimens: a considerable number of samples were *IS6110* real-time PCR from PS-MTM positive and Xpert negative ($n = 15$). When stratified by culture result, the detection rate of *IS6110* real-time PCR from PS-MTM was equal to that of Xpert among culture-positive patients, but significantly higher than Xpert in specimens from patients with negative paired culture result. A potential explanation for the latter observation is that the genetic target of the real-time PCR used for the PS-MTM (*IS6110*) occurs multiple times throughout the *M. tuberculosis* genome, whereas the Xpert's has a single-copy target; this would in theory allow for more sensitive detection by the PS-MTM approach used in this study [25].

Mycobacterium tuberculosis DNA load (reflected by higher CT-value) of sputum collected in PS-MTM was significantly lower in negative paired culture or Xpert results. This observation could possibly be due to the low organism load in a specimen that may be killed during the harsh decontamination procedure of sputum prior to culture [26], which resulted in lack of growth in culture, the presence of non-viable organism (up to 6 months) in patients that may have previously been treated for *M. tuberculosis* infection or latently infected patients [27–30].

Conclusion

As culture is the reference standard for the diagnosis of pulmonary TB [31] and Xpert has a known high sensitivity for the detection of *M. tuberculosis* [25, 32, 33], the good level of agreement between these diagnostic methods and *IS6110* real-time PCR from PS-MTM makes the latter a promising approach to enhance availability of molecular diagnostic systems in resource-poor settings with often challenging logistics and climate. An additional advantage is that only an aliquot of the sputum specimen in PS-MTM is tested by *IS6110* real-time PCR, allowing for further molecular or phenotypic characterisation and repeat testing of the remaining sputum volume (which is not possible in Xpert where the entire sputum volume is consumed). Improving control of TB is reliant on improving diagnosis and treatment outcomes [34]. In an effort to contain the disease, South Africa is the only high-burden country to have implemented two molecular methods in routine diagnostic approach: the Xpert for first-line diagnosis and the line probe assay for detection of drug resistance. Many countries are unable to afford the costs associated with this implementation to appreciate true benefit, in particular, the infrastructural and operational costs [7, 35].

High-quality sputum specimens that are stabilised and transported at ambient temperature to a central diagnostic facility may provide a safe alternative approach for molecular detection of *M. tuberculosis* in resource-constrained settings and offer a unique opportunity for detection of additional positives in low-load specimens.

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