

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

Molecular tests are revolutionizing diagnosis of tuberculosis (TB). Disease burden is concentrated in resource-poor countries with inadequate infrastructure and capacity resulting in delays for specimens to reach the laboratory. We assessed the performance of an innovative method using a swab to inoculate sputum in a transport medium, PrimeStore® - Molecular Transport Medium (PS-MTM) for subsequent molecular detection of *Mycobacterium tuberculosis* at a centralized facility. A sputum specimen was obtained from suspected TB patients at rural healthcare facilities in South Africa and a swab taken and placed into PS-MTM from this specimen, prior to it being processed by either liquid culture or Xpert MTB/Rif assay (Xpert). A subset from a larger cohort study of 141 patients was included for analysis, which included 47 laboratory-confirmed TB patients. *M. tuberculosis* was detected at 29% by culture, 29% by Xpert and 31% and 36% by real-time PCR of PS-MTM for the culture and Xpert specimen respectively. 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, detection rate by 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 ($p=0.008$). These results suggest that swab collection of sputum into PS-MTM provides a promising application for diagnosis of TB in rural healthcare settings thereby potentially improving the options available for the diagnosis of TB in countries incapable of applying decentralized high-tech molecular testing.

INTRODUCTION

Tuberculosis (TB) remains one of the greatest eradication challenges world-wide in 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 protection from the vaccine (2-4), long turnaround time and suboptimal sensitivity of current diagnostic tests, poor uptake of new diagnostic tools, operational and logistic delays, undiagnosed cases and treatment default (1). Mycobacterial culture is the gold standard for diagnosis, despite its known delay to report a negative result (5-7). In countries with the highest burdens of TB, microscopy remains the cornerstone for microbiological diagnosis, especially in settings where culture methods are not readily accessible such as at rural health care facilities. The use of molecular methods for detection of *M. tuberculosis* provides an alternative approach for rapid diagnosis of TB. Several studies highlight the benefits of molecular testing for the detection of *M. tuberculosis* (8, 9). The endorsement by the World Health Organization (WHO) of the Xpert MTB/Rif assay (Xpert) (10) for screening of TB suspects has revolutionized testing for TB globally. In South Africa, Xpert has been implemented as 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 compared to 65% by smear microscopy.

Despite the success of Xpert introducing molecular diagnostics to South Africa, there are several implementation challenges. Xpert requires samples to be processed within 3 days if kept at ambient temperature or stored at 2-8° C for a maximum of 7 days if delays are expected, both of which may not be realistic in many poor-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 of specimens

from outlying areas with poor infrastructure (14) and laboratory capacity (15). Furthermore, Xpert generally uses the complete sputum volume necessitating new specimen collection in case additional testing is 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 stable uninterrupted power supply (7, 13). These conditions require financial, operational and logistical support (16) which may exist in urban and peri-urban locations (17), but are difficult to achieve in rural settings in the same countries.

An alternative approach to Xpert testing may be provided by sputum specimen collection and transport to centralized facilities for molecular processing. PrimeStore® - Molecular Transport Medium (PS-MTM) was developed to collect respiratory specimens and to allow for transport of these under extreme environmental conditions by stabilizing nucleic acids over time until processing. The medium effectively inactivates pathogens and nucleases and preserves nucleotides at ambient temperature for at least 4 weeks (18, 19). PS-MTM has been successfully evaluated for detection of respiratory viruses, but may also provide a useful method for molecular detection of *Mycobacterium 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 (Omar SV *et al.*, manuscript submitted for publication). In this study, we evaluate a system of centralized molecular testing of sputum specimens inoculated in PS-MTM, using a swab and directly after production by the patient, for detection of *M. tuberculosis* in a rural African setting.

MATERIALS AND METHODS

Study design and sputum specimens. Individuals were recruited for a larger cohort study when they presented with a cough for more than two weeks at primary healthcare facilities in rural Mopani District, South Africa (Peters *et al.* submitted). Two sputum specimens, produced at least 1.5 hours 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 randomized 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 (Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) collection tubes. The PS-MTM specimens were batched and for molecular processing sent bi-weekly at ambient temperature across ~500 km from the study sites to the Centre for Tuberculosis, National TB Reference Laboratory, National Institute for Communicable Diseases, South Africa.

Routine microbiological tests. PCR results of sputum collected in PS-MTM were compared to those of routine Xpert, smear-microscopy and liquid culture. Sputum specimens were tested using the Xpert MTB/Rif assay (version G4 cartridges) (Cepheid, Sunnyvale, CA, USA) according to the manufacturer's instructions. The other sputum specimen of each patient was decontaminated and concentrated using the NaLC-NaOH method (20); **sediments were used to**

prepare smears for light microscopy by Ziehl-Neelsen staining (21) and 0.5 ml was inoculated for culture using the Mycobacterial Growth Indicator Tubes (MGIT) 960 system (BD, Sparks, Maryland, USA) as described by the MGIT procedure manual (22). Smears were graded according to WHO recommendations (21) 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 one minute and a 200 μ l aliquot was processed on the NucliSENS EasyMAG (Biomerieux, Marcy l'Etoile, France) using the generic protocol to extract DNA. Real-time PCR was carried out on the StepOne Plus (ThermoFisher Scientific, Waltham, MA USA) using a hydrolysis probe targeting the insertion sequence element (IS) 6110 for detection of *M. tuberculosis* DNA as per manufacturers' instructions (23). Specimens were either considered positive, indeterminate or negative based on the following predefined Cycle-Threshold (CT) values; ≤ 38 positive, 38.01 – 39.99 indeterminate and ≥ 40 negative. In general, 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 to 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 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). Furthermore, a composite reference standard was used with the following definition for TB positivity: specimen positive in either MGIT or Xpert. For purposes of comparison an indeterminate result of PCR on PS-MTM

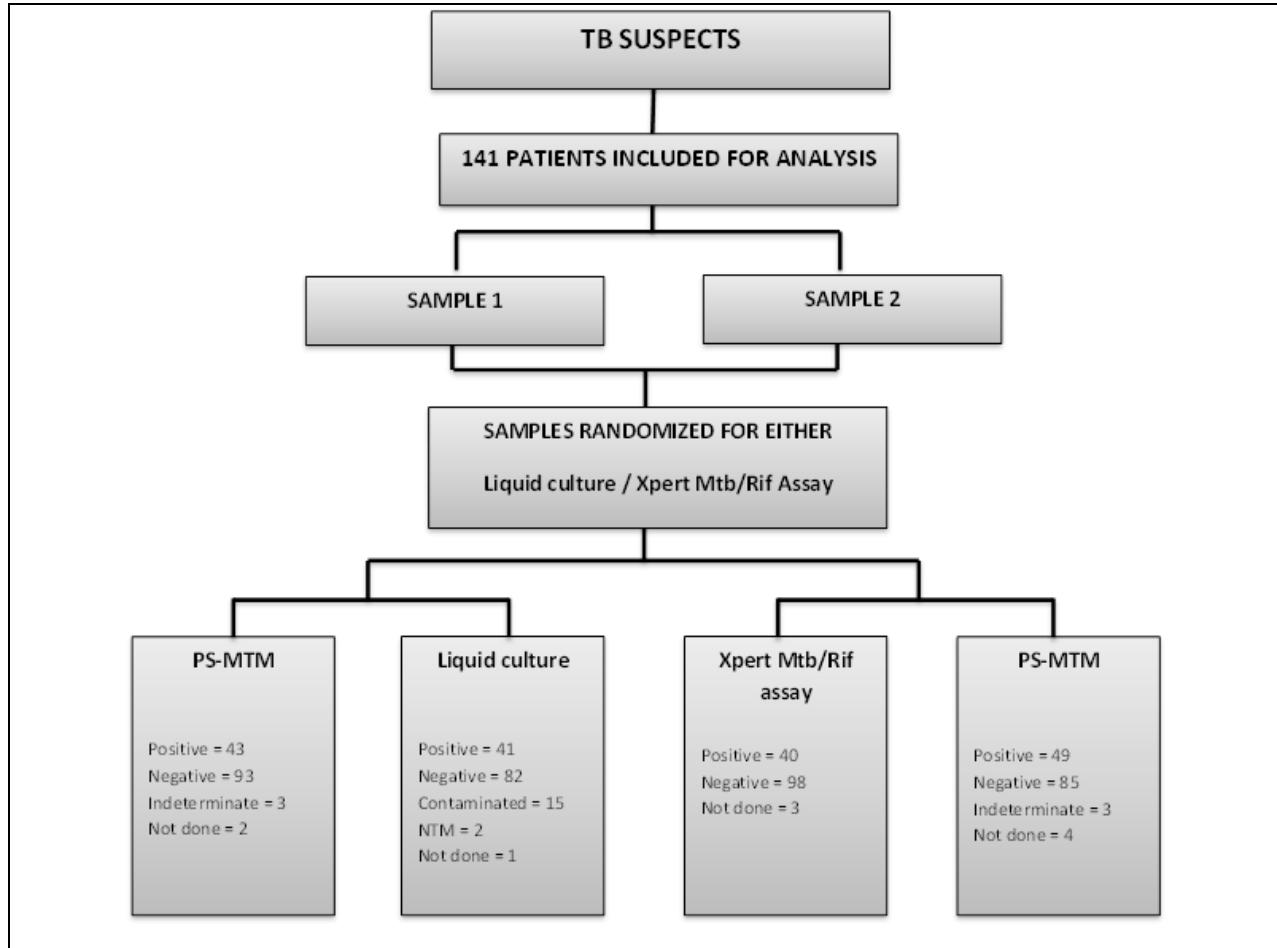
was excluded from analysis as an additional patient sample could not be requested to confirm the presence or absence of *M. tuberculosis*. Statistical analyses were performed using Statistics (v.7.0).

RESULTS

Detection of *M. tuberculosis* in sputum specimens. A total of 141 patients (282 paired-sputum specimens) were evaluated. At most 2.8% of sputum specimens were not available for testing within each test set. Overall, a diagnosis of TB was made for 25 (18%) cases by microscopy, 41 (29%) by culture and 40 (29%) by Xpert (Fig. 1). MGIT culture and Xpert were both positive for *M. tuberculosis* for 34 samples with an additional 6 for culture only and 6 for Xpert only. As such, the detection of *M. tuberculosis* was similar for both specimens. 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 PCR of sputum in PS-MTM and liquid culture. *M. tuberculosis* DNA was detected from sputum in PS-MTM in 21/25 sputum specimens that were smear-microscopy positive and 29/41 specimens that were positive by culture (71%) (Tab. 1). An indeterminate result was observed for one culture-positive specimen and for two culture-negative specimens. Agreement, excluding indeterminate results, was 73% for positive and 85% for culture-negative samples resulting in an overall agreement of 82% (McNemar, p=0.55). Real-time PCR of DNA from PS-MTM samples showed discordance for 11 positive culture specimens and 14 negative by culture (Tab. 2). When comparing this discordance to the same patient's Xpert specimen

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



result; the Xpert result concurred with 6 of the 11 culture positives and 11 of the 13 negative cultures (1 Xpert not done) (Tab. 2).

Concordance between sputum in PS-MTM and Xpert. Of the Xpert-positive samples, real-time PCR of DNA from sputum in PS-MTM was positive for 32/39 (82%) (Tab. 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 real-time PCR and Xpert was 84% for both negative Xpert results resulting in an overall agreement of 84%

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

Real-time PCR in PS-MTM	Liquid culture		Xpert	
	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.

(McNemar, p=0.05). The relatively low agreement for negative specimens is the result of higher detection rate by real-time PCR of PS-MTM: 15 Xpert-negative specimens had detected *M. tuberculosis* DNA detected from samples collected in PS-MTM. When stratifying Xpert results by the result of liquid culture, there was good concordance between PCR of DNA in PS-MTM and Xpert (Tab. 2).

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

Real-time PCR in PS-MTM*	Culture Positive		Culture Negative	
	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.

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 in the negative group compared to three by the Xpert (Tab. 2).

Sputum *M. tuberculosis* DNA load by result of routine test. In cases of positive real-time PCR result of sputum in PS-MTM, the median CT value was significantly higher for specimens with positive result of paired culture than negative concurrent culture (28.47 vs. 34.47; p<0.001) (Tab. 3). This was also observed when comparing CT-value between specimens with positive and negative paired Xpert result (28.34 vs. 34.81; p<0.001).

Table 3: Distribution of Cycle-Threshold (CT) values of *M. tuberculosis* detected by real-time PCR in PS-MTM amongst Culture and Xpert positive and negative specimens.

	Number of samples	Median CT value	Range of CT-values
Positive (CT score <38.00)			
Culture positive*	29	28.47	20.58 – 35.48
Culture negative	14	34.47	26.06 – 37.66
Xpert positive*	32	28.34	20.34 – 37.01
Xpert negative	15	34.81	22.09 – 37.61
Indeterminate (CT score 38.00 - 39.99)			
Culture positive	1	38.04	-
Culture negative	2	38.13; 38.24	-
Xpert positive	1	38.46	-
Xpert negative	2	38.43; 38.83	-

*P<0.001 for comparison of median CT-value between positive and negative routine tests.

DISCUSSION

This field evaluation shows that PS-MTM provides a promising tool that could support centralized molecular testing for *M. tuberculosis* in rural settings with poor infrastructure. Sputum specimens were transported ~500km from facilities in rural South Africa to the National TB Reference Laboratory in Johannesburg. Previous research has 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 (19). In a laboratory evaluation, we recently demonstrated could sensitivity and specificity of real-time PCR detection of *M. tuberculosis* from clinical samples inoculated into PS-MTM (Omar SV *et al.*, submitted for publication). This study confirms those results in the field setting of rural healthcare facilities whereby sputum specimens were, using a swab, inoculated into PS-MTM by the research nurse shortly after they were produced by the patient.

In this field evaluation, 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 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, since 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 real-time PCR of PS-MTM with culture and Xpert was good for both positive and negative results, except for the concordance with Xpert negative outcome: a considerable number of samples were real-time PCR positive and Xpert negative (n=15). When stratified by

culture result, detection rate of 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 compared to the Xpert's single copy target; this would in theory allow for more sensitive detection by the PS-MTM approach used in this study.

M. 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 (24) and this resulted in lack of growth in culture or the presence of non-viable organism (up to six months) in patients that may have previously been treated for *M. tuberculosis* or latently infected patients (25-28). Further investigation is required to determine the true interpretation of these isolated positive PCR results if these could be due to non-cultivable/non-viable mycobacteria, early infection or detection of the pathogen in latently infected persons.

Since culture is the reference standard for diagnosis of pulmonary TB (29) and Xpert has a known high sensitivity for the detection of *M. tuberculosis* (30-32), the good level of agreement between these diagnostic methods and 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 real-time PCR allowing for further molecular or phenotypic characterization 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 (33). 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, 34).

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

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