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Molecular detection of *Mycobacterium tuberculosis* from sputum transported in PrimeStore® from rural settings

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SUMMARY

SETTING: Mopani District, South Africa.

OBJECTIVE: To explore remote, molecular detection of *Mycobacterium tuberculosis* from sputum transported using PrimeStore® Molecular Transport Medium (PS-MTM) compared to settings where microscopy or Xpert® MTB/RIF is used as the baseline test.

DESIGN: Two sputum specimens were collected from patients with cough of ⩾2 weeks at clinics in rural South Africa. Shortly after expectoration and before processing using Xpert, microscopy and liquid culture, a flocked swab was swirled in each of these specimens and placed in PS-MTM. Swabs were stored and transported to the United States at ambient temperature for real-time PrimeMix® polymerase chain reaction (PM-PCR).

RESULTS: Of 132 patients, 23 (17%) were positive on microscopy, 39 (30%) on Xpert and 44 (33%) by PS-MTM/PM-PCR. Concordance of PS-MTM/PM-PCR with positive microscopy and Xpert was respectively 96% and 85%. Of 107 microscopy-negative samples, 22 (21%) were positive using PS-MTM/PM-PCR, while 11/91 (12%) Xpert-negative samples were PS-MTM/PM-PCR-positive. PS-MTM/PM-PCR positivity was significantly higher than smear microscopy positivity ($P < 0.001$), but similar to Xpert ($P = 0.33$).

CONCLUSION: PCR testing of specimens transported in PS-MTM would enhance TB diagnosis in settings where smear microscopy is the baseline diagnostic test, and could provide an alternative in settings where Xpert testing is not available.

KEY WORDS: specimen transport; PrimeMix; real-time PCR; specimen detection

THE MAJORITY OF NEW TUBERCULOSIS (TB) cases in sub-Saharan Africa occur in low-resource areas, with human immunodeficiency virus (HIV) infection a strong driver of the epidemic. The public health impact of the rising TB burden is further complicated by the continued spread of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of tuberculosis (TB). An increasing number of MDR- and XDR-TB cases have heightened the urgency to detect *Mycobacterium tuberculosis* rapidly and to identify resistant strains in order to provide appropriate treatment, reduce morbidity and mortality, and prevent transmission.[1,2]

Sputum smear microscopy is an inexpensive, widely used baseline detection test for diagnosing pulmonary *M. tuberculosis* in resource-poor settings. However, despite providing a quick diagnosis in some patients, microscopy has lower sensitivity than culture and Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA), provides no information on drug resistance and has reduced yield in patients with HIV co-infection.[3,4] To improve the detection of *M. tuberculosis*, South Africa has recently replaced smear microscopy by Xpert testing as the baseline diagnostic test for pulmonary TB. This has improved the rapid diagnosis of patients with TB and detection of rifampin resistance.[5,6] However, the financial resources to implement Xpert may not be widely available across other regions of sub-Saharan Africa, and smear microscopy has retained its role as the baseline diagnostic test in many of these settings. A drawback of Xpert is that the entire volume of sputum is usually processed, necessitating the collection of a new sputum specimen in case of sample error or if further analysis is required. Culture systems (both solid and liquid) still serve as the reference standard for *M. tuberculosis* detection. However, a dedicated laboratory infrastructure is required, the turnaround time is sometimes considerable, and cultures are prone to contamination.[7] Furthermore,

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longer specimen transport times contribute to loss in viability and reduced culture sensitivity.[8]

PrimeStore® Molecular Transport Medium (PS-MTM; Longhorn Vaccines & Diagnostics, San Antonio, TX, USA) has been demonstrated to inactivate *M. tuberculosis* and to rapidly stabilize and preserve the DNA from sputum specimens at ambient temperature and above for real-time polymerase chain reaction (PCR).[9,10] This system allows ambient temperature collection, storage, and transport of sputum specimens for molecular testing by real-time PCR at a central laboratory. It also provides the option to perform secondary molecular analysis of the same sample, as only a small part of the sample volume is required and the integrity of the primary sample is not compromised.

In the present study, we evaluated the collection of sputum samples in PS-MTM for transport to a centralized facility for the detection of *M. tuberculosis* using real-time PrimeMix™ (Longhorn Vaccines & Diagnostics) PCR (PM-PCR). The potential value of this system was assessed for settings where smear microscopy or Xpert would be used as the baseline diagnostic test for TB.

METHODS

Study design

This evaluation was embedded in a prospective cohort study addressing the potential impact of clinic-based Xpert compared to central, laboratory-based Xpert for the diagnosis of TB at primary health care (PHC) facilities in rural Mopani District, South Africa. The study was conducted from June 2012 to May 2013; 508 adult patients with cough of ⩾2 weeks were recruited. After providing informed consent, patients were instructed to produce two sputum samples (±5 ml), at least 90 min but not more than 4 h apart, while still at the PHC facility. Both sputum samples were randomly allocated for testing either by Xpert (clinic- or laboratory-based) or by liquid culture at the laboratory of the Medical Research Council in Pretoria, South Africa. Before randomization, approximately 100 µl of sputum was transferred into 1.5 ml PS-MTM using a sterile flocked swab (Copan Diagnostics, Brescia, Italy) that was swirled a minimum of five times. For each patient, two PS-MTM samples were therefore available, with each of the corresponding sputum samples submitted for either Xpert testing or liquid culture. PS-MTM samples were stored and transported at ambient temperature within 48 h to the TB laboratories of the Department of Medical Microbiology, University of Pretoria, Pretoria, South Africa. Volumes of ±200 µl were prepared from the PS-MTM specimens, assigned unique recoding for blinded testing, and shipped commercially at ambient temperature to Longhorn Vaccines & Diagnostics in the United States for molecular analysis.

Routine laboratory testing

Sputum samples were tested the same day by clinic-based Xpert or transported to the local National Health Laboratory Services branch of the Mopani District Hospital in Tzaneen within 24 h for testing by Xpert. Ziehl-Neelsen (ZN) stained slides were prepared for microscopic detection of acid-fast bacilli (AFB) directly from the sputum samples. Liquid culture using the BACTEC™ MGIT™ 960 TB system (BD, Cockeysville, MD, USA) was performed according to routine practice.

Collection, selection, and transport of sputum samples

For molecular testing all specimens were collected, stored, and transported at ambient temperature in PS-MTM. After Xpert and liquid culture results were obtained, PS-MTM samples from selected patients (n = 141) were identified for inclusion in the analysis: samples from patients with *M. tuberculosis* detected using Xpert or liquid culture ($n = 47$) were combined with samples from randomly selected patients (n = 94) who had tested negative on Xpert and liquid culture at a ratio of 1:2. Of the 141 patients selected, 70 (49.6%) were HIV-positive, 48 (34.0%) were HIV-negative and 23 (16.3%) had unknown HIV status; 29/141 (20.6%) had previously received anti-tuberculosis treatment.

Molecular evaluation of PS-MTM samples

Total genomic DNA from sputum in PS-MTM was purified using PrimeXtract™ (Longhorn Vaccines & Diagnostics) according to the manufacturer's instructions. The H37Rv *M. tuberculosis* reference strain and water were included throughout as extraction and real-time amplification positive and negative controls, respectively.

Real-time PCR amplification for *M. tuberculosis* detection was performed using PrimeMix® MTB Complex (PM-PCR; Longhorn Vaccines & Diagnostics), a mixture of primers, buffers, salts, and enzyme that targets a conserved region of the TB insertion sequence (IS) *6110* region. Real-time amplification was carried out in a final volume of 20 µl containing 15 µl PrimeMix and 5 µl extracted DNA using an ABI 7500 thermocycler (ThermoFischer, Waltham, MA, USA). Thermocycling parameters were 95°C for 5 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 32 sec. For instrument analysis, the 0.1 baseline threshold was used. Samples were classified as positive if the cycle threshold (CT) value was below 38, indeterminate if CT was 38–40, and negative if no amplification signal was observed. For quantitative analysis, each sample was tested in duplicate to obtain an average CT.

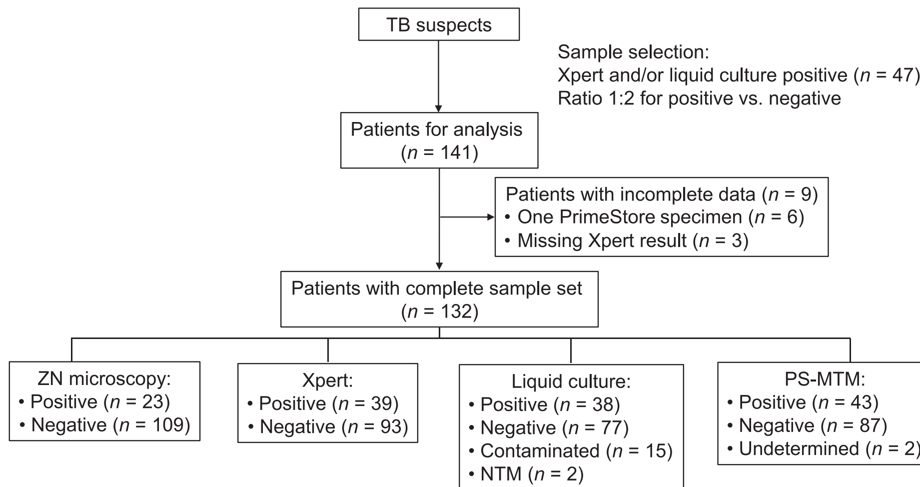

Figure Flow chart of patients and samples included in this evaluation. TB = tuberculosis; Xpert = Xpert® MTB/RIF assay; PS-MTM = PrimeStore® Molecular Transport Medium; ZN = Ziehl-Neelsen; NTM = non-tuberculous mycobacteria.

Statistical analysis

In this analysis, we used the result of PS-MTM testing of first sputum specimens to determine its diagnostic performance in comparison to ZN smear microscopy, Xpert, and liquid culture in 2x2 tables. When there was a positive PS-MTM/PM-PCR result, in particular in the context of negative Xpert and/or liquid culture, the results of the second PS-MTM sample with first sample results were used to facilitate interpretation. Two programmatic scenarios were analyzed in describing the role of PS-MTM based real-time PCR in those settings, taking into account demographic, clinical, and other microbiological data: 1) ZN smear microscopy used as baseline test and MGIT as a secondary test; and 2) Xpert used as baseline diagnostic test, followed by MGIT as a secondary test. The McNemar test was used to determine statistical significance between detection methods.

RESULTS

Characteristics of sample collection

A total of 282 specimens (141 pairs) in PS-MTM were evaluated using PM-PCR. These include 47 TB patients who were detected using Xpert ($n = 40$) and/or MGIT ($n = 41$). Paired samples from 132 patients were available for analysis; samples from nine patients were excluded from further analysis due to incomplete data for PS, Xpert and/or culture (Figure). Of the 132 patients included in the evaluation, 44 (33%) had a diagnosis of TB by ZN microscopy ($n = 23$), Xpert ($n = 39$) and/or liquid culture ($n = 38$). The 23 positive ZN microscopy samples included three samples with a scanty AFB result (1–9 AFB observed); two of these were also positive on Xpert, MGIT and PS-MTM/PM-PCR, whereas a third was negative on all three tests. Forty-four patients (33%) had a positive test for PS-MTM/PM-PCR; another two (1.5%) had an undetermined result (i.e., CT value between 38 and 40).

The majority of the samples (38/44, 86%) with a positive PS-MTM/PM-PCR result in the first sample also had a positive PS-MTM/PM-PCR result for the second sample. The six samples with a negative PS-MTM/PM-PCR result for the second sample had significantly lower median CT values than the samples with concordant positive PS-MTM/PM-PCR tests (34.8, range 31.7–36.8 vs. 31.2, range 22.4–37.1; $P = 0.030$).

Comparison of centralized PS-MTM with sputum smear microscopy

Of the 132 patient samples available, two samples had an undetermined PS-MTM/PM-PCR result and were excluded from the analysis (Table 1). Of 23 ZN-positive samples, 22 (96%) were also positive in PS-MTM; in the PS-MTM negative sample, 1–9 AFB were observed, but Xpert, liquid culture, and the second PS-MTM sample were negative. Among the 107 smear-negative samples, 22 (21%) were found to be positive on PS-MTM/PM-PCR. Of these 22 PS-MTM/PM-PCR-positive samples, 11 (50%) could be confirmed by culture ($n = 9$) or Xpert ($n = 11$). The interpretation of the positive PS-MTM/PM-PCR results of the 11 patients not confirmed by other methods (microscopy, Xpert, or culture) is presented below. The median CT value of PS-MTM was higher in patients with a positive than in those with a negative result for ZN microscopy (29.1, range 22.4–36.3 vs. 34.6, range 29.4–37.1; $P < 0.001$).

Comparison of centralized PS-MTM with Xpert

Excluding the two samples with indeterminate results, Xpert and PS-MTM/PM-PCR were analyzed

Table 1 Comparison of sputum collected in PS-MTM and tested using real-time PCR (PM-PCR) with smear microscopy, Xpert® MTB/RIF, and liquid culture for detection of *M. tuberculosis*

	Smear microscopy		Xpert		Liquid culture	
First PS-MTM specimen	Positive (n = 23) n (%)	Negative (n = 109) n (%)	Positive (n = 39) n (%)	Negative (n = 91) n (%)	Positive (n = 38) n (%)	Negative (n = 94) n (%)
Positive	22 (96)	22 (20)	33 (85)	11 (12)	31 (82)	13 (14)
Negative	1 (4.3)	85 (78)	6 (15)	80 (88)	7 (18)	79 (84)
Undetermined	0	2 (1.8)	0	2 (2.2)	0	2 (2.1)

PS-MTM = PrimeStore® Molecular Transport Medium; PCR = polymerase chain reaction.

for 130 samples (Table 1). Of the 39 Xpert-positive samples, 33 (85%) were concordantly positive. Of these, 31/33 (94%) Xpert- and PS-MTM/PM-PCR-positive samples were also MGIT-positive. Of the 91 Xpert-negative samples, 11 (12%) had a positive PS-MTM/PM-PCR result and another 5 (5.5%) were MGIT-positive.

Six patients had a positive result in Xpert and a negative first PS-MTM/PM-PCR test (Appendix Table A.1).* In four cases, there was additional microbiological support for a diagnosis of TB: in two cases liquid culture was positive and in another two the second PS-MTM/PM-PCR test was positive. Two cases were positive on Xpert only (i.e., MGIT and both PS samples negative), without clinical evidence of HIV and/or severe immunosuppression. In these two patients, treatment was initiated on the basis of the Xpert result, and both patients improved on anti-tuberculosis treatment.

Eleven patients had a PS-MTM/PM-PCR positive first sample PS-MTM result, but a negative Xpert test; liquid culture was also negative in all these patients. Based on clinical and microbiological characteristics, 2/11 patients likely had TB. One of these patients had a history of previous TB, and a second PM-PCR test was also positive. The second case also had a positive second PM-PCR test, and presented with cough, dyspnea and night sweats, and had household contact with TB. There were another 4/11 patients with possible TB; one had HIV infection (CD4 = 99 cells/mm^3) with cough and weight loss. The other three cases were PM-PCR-positive on a second sample. Interpretation of the single PS-MTM/PM-PCR result was unclear in another five patients and required follow-up. CT scores ranged from 31.1 to 37.1 (Appendix Table A.2), and there was no clear visible pattern of any particular CT value with specific clinical variables. One patient who was only positive on PS-MTM/PM-PCR was started on anti-tuberculosis treatment, as clinical symptoms and chest X-rays were consistent with TB. All patients were followed for 2 months, and most reported doing well without initiating anti-tuberculosis treatment.

Clinical and microbiological characteristics were compared between patients with a positive Xpert result, those with only a positive first PS-MTM/PM-PCR result, and those with a negative result for these three tests (Table 2). HIV status was not significantly different between the groups. The 11 patients with an isolated first sample PS-MTM/PM-PCR positive result had a lower sputum DNA load (higher median CT value) than those with TB confirmed by Xpert and/or MGIT (32.7, range 22.4–40 vs. 35.3, range 31.1–37.1; $P < 0.001$). Persistent cough was less frequently reported at 2 months follow-up in the PM-PCR-positive group than in patients without a positive TB culture or PCR test.

DISCUSSION

Xpert assay is increasingly used as the baseline test for the detection of TB in individuals presumed to have TB, following the recommendations of the World Health Organization.[11] Xpert has shown considerably higher sensitivity than smear microscopy (pooled sensitivity in meta-analysis 88% vs. 65%).[12] In comparison with smear microscopy, Xpert increased TB detection among culture-confirmed cases by 23% (95% confidence interval [CI] 15–32).[12] Concomitant HIV infection often results in a lower probability of AFB detection on sputum smear microscopy, but may not affect detection by molecular-based methods such as Xpert.[6,13]

In many low-resource areas of the world, however, smear microscopy remains the primary method for detecting TB,[3,4] with smear-positive patients assigned to treatment and smear-negative patients often started on treatment empirically while awaiting the results of culture or other diagnostic investigations.[14] PS-MTM-transported specimens tested at a distant laboratory using the PM-PCR assay showed high concordance with positive liquid culture and Xpert results, and may detect additional people with TB over microscopy. Safe and efficient transport of sputum specimens to central or regional laboratories for molecular testing may allow the detection of TB patients who are missed on smear microscopy alone.

* The appendix is available in the online version of this article, at http://www.ingentaconnect.com/content/iuatld/ijtld/2015/00000019/00000005/art00011

Table 2 Association of demographic, clinical and microbiological characteristics with first sample

	Confirmed TB* (n = 44) n (%)	Only PrimeStore-positive (n = 11) n (%)	No TB (n = 75) n (%)	P value
Age, years, median (range)	37 (19–82)	49 (20–72)	42 (18–76)	NS
Household contact with TB	12 (27)	2 (18)	11 (15)	NS
History of TB	7 (16)	2 (18)	19 (25)	NS
Smoker	10 (30)	1 (13)	11 (19)	NS
Duration of cough, days, median (range)	30 (14–2190)	21 (14–90)	21 (14–730)	NS
HIV status				
Positive	21 (49)	3 (27)	42 (55)	NS
Unknown	4 (9.3)	4 (36)	11 (15)	
Negative	18 (42)	4 (36)	22 (29)	
On ART	5 (24)	1 (33)	15 (36)	NS
Median CT value in first PrimeStore, median (range)	32.7 (22.4–40)	35.3 (31.1–37.1)	—	<0.001
Persistent cough at 2 months follow-up	10 (28)	1 (10)	21 (32)	$P < 0.001$ for PrimeStore-positive vs. no TB

* TB diagnosed by Xpert and/or liquid culture.
TB = tuberculosis; NS = non-significant; HIV = human immunodeficiency virus; ART = antiretroviral therapy; CT = cycle threshold.

In our study, six cases were Xpert-positive and PS-MTM/PM-PCR-negative (Appendix Table A.1). PS-MTM/PM-PCR and liquid culture of the second specimen was positive in 2/6 cases not detected on the first specimen. Real-time CT values for repeat positive specimens were 33.2 and 30.6, suggesting that the sputum *M. tuberculosis* burden was low and variable. Both patients for whom these observations were made had symptoms suggestive of TB, and one had a low CD4 count.

These observations might be of importance, as many HIV-infected patients may control *M. tuberculosis* infection, but could have an episodic release of active *M. tuberculosis* into the respiratory tract and thus transmit TB despite testing smear-negative.[3,15,16] Eleven PS-MTM/PM-PCR-positive cases were negative on Xpert and liquid culture (Appendix Table A.2). Of these, five also tested positive in a second PS-MTM specimen. In these samples, CT values were generally above 33, suggesting a low bacillary burden. In these specimens, i.e., specimens with <1000 genomic copies in the sputum, detection of TB would generally be difficult without more sensitive assays such as liquid culture or a multi-copy target PCR assay.

It may be important to adopt additional strategies for *M. tuberculosis* detection in low-resource rural settings, in particular those with high HIV prevalence.[4,17,18] As HIV patients may have few symptoms and low sputum bacillary burden, microscopy and clinical symptoms may miss TB, and the turnaround time for culture may be too long. In addition, the rise of MDR-/XDR-TB has made it important not only to detect patients with TB, but also to identify resistant strains to be able to treat the patient optimally and prevent the spread of MDR-/XDR-TB. One approach would be to collect a PS-MTM specimen at the microscopy center and have it available to send to a central laboratory for next-generation sequencing of drug resistance genes. Smear-positive patients could be started immediately on treatment while the results of drug susceptibility testing are pending from the central laboratory. It is operationally feasible to process PS-MTM specimens with PCR in <7 days from expectoration, even if referred to remotely located laboratories.[19] Samples collected in PS-MTM are compatible with most extraction systems, and PM-PCR works well with several real-time platforms (S V Omar, unpublished data). In other settings, the collection of sputum for PS-MTM/PM-PCR detection could be followed by molecular analysis for drug resistance to further guide treatment.

In conclusion, there is a need to expand and improve TB detection, especially in high prevalence HIV-TB areas with few resources. This study demonstrates the value of sputum collection and shipment in PS-MTM, followed by PM-PCR detection at central laboratory sites, even when the facility is very distant from the clinic area. Such an operational system would increase the diagnosis of TB over smear microscopy and could provide an alternative in settings where Xpert testing is not available. Central or regional laboratory testing expands capabilities for high quality, accurate *M. tuberculosis* detection with the added potential for high throughput volume and molecular drug resistance analysis.

Conflicts of interest: none declared.

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