Evaluation of the Xpert MTB/RIF Assay for the Diagnosis of Pulmonary Tuberculosis in a High HIV Prevalence Setting

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***Rationale*: Xpert MTB/RIF is a novel automated molecular diagnostic recently endorsed by the World Health Organization. However, performance-related data from high HIV prevalence settings are limited.**

***Objectives*: The impact of sample-related factors on performance and the significance of Xpert MTB/RIF-positive culture-negative discordance remain unclear.**

***Methods*: Xpert MTB/RIF was evaluated using single archived spot-sputum samples from 496 South African patients with suspected TB. *Mycobacterium tuberculosis* culture positivity and phenotypic resistance to rifampicin served as reference standards.**

***Measurements and Main Results*: Overall, Xpert MTB/RIF detected 95% (95% confidence interval [CI], 88–98%; 89 of 94) of smear-positive culture-positive cases and the specificity was 94% (91–96%; 320 of 339). The sensitivity in smear-negative cases was 55% (35–73%; 12 of 22) when the analysis was restricted to 1 ml of unprocessed sputum and culture time-to-positivity of less than or equal to 28 days. Compared with smear microscopy (n = 94), Xpert MTB/RIF detected an additional 17 cases (n = 111) representing an 18% (11–27%; 111 vs. 94) relative increase in the rapid TB case detection rate. Moreover, compared with smear microscopy, the inclusion of Xpert MTB/RIF-positive culture-negative TB cases (ruled-in by an alternative diagnostic method) resulted in the detection of a further 16 cases (n = 127), thus significantly increasing the rapid TB case detection rate to 35% (95% CI, 26–45%; 94 to 111 vs. 94 to 127; $P < 0.01$), the overall specificity to 99.1% (97–100%; 320 of 323; $P < 0.001$), and sensitivity in smear-negative TB to 60% ($P = 0.12$). Performance strongly correlated with smear status and culture time-to-positivity. In patients infected with HIV compared with patients uninfected with HIV Xpert MTB/RIF showed a trend to reduced sensitivity ($P = 0.09$) and significantly reduced negative predictive value ($P = 0.01$). The negative predictive value for rifampicin resistance was 99.4%.**

***Conclusions*: XpertMTB/RIF outperformed smear microscopy, established a diagnosis in a significant proportion of patients with smear-negative TB, detected many highly likely TB cases missed by culture, and accurately ruled out rifampicin-resistant TB. Sample-specific factors had limited impact on performance. Performance in patients infected with HIV, especially those with advanced immunosuppression, warrants further study.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Xpert MTB/RIF is an accurate rapid diagnostic tool for tuberculosis (TB) and rifampicin resistance. Although it has been recently endorsed by the World Health Organization, there are limited data about the impact of HIV coinfection and sample-related factors on test performance. The significance of Xpert MTB/RIF-positive culture-negative samples remains unclear.

What This Study Adds to the Field

HIV coinfection, but likely not sputum volume and processing methods, may impact on assay performance. Almost all Xpert MTB/RIF-positive culture-negative samples are likely true positives and this approximately doubles the number of detected TB cases over and above that of smear microscopy.

Keywords: smear-negative tuberculosis; tuberculosis; diagnostics; HIV; PCR

Tuberculosis (TB) is a major global health priority and kills approximately 1.7 million people annually (1). The incidence of multidrug resistant (MDR) TB is increasing with almost 0.5 million estimated new cases in 2008 (2). Although smear microscopy is widely used for the rapid diagnosis of TB, it does not detect drug resistance and sensitivity in individuals coinfected with HIV varies between 20% and 50% (3). Results of mycobacterial culture often only become available after 2–8 weeks (4). This creates a diagnostic delay that hampers disease control, enhances transmission, and increases healthcare costs (5).

Xpert MTB/RIF (Cepheid, Sunnyvale, CA) is an automated user-friendly real-time polymerase chain reaction (PCR) assay designed for the rapid and simultaneous detection of *Mycobacterium tuberculosis* and rifampicin resistance (6–8). The assay amplifies a *M. tuberculosis* complex-specific region of the *rpoB* gene, which is probed with molecular beacons to detect the

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presence of rifampicin resistance-determining mutations (9). In December 2010, the World Health Organization (WHO) endorsed the scale-up of Xpert MTB/RIF and recommended its use as the initial test in patients coinfected with HIV and TB and patients with suspected MDR TB (10, 11). The performance of the test with the first-generation software using both NALC-NaOH decontaminated and unprocessed sputum from 1,730 patients with suspected TB was recently assessed as part of a large multicentre study (7). Using a single assay on a single unprocessed sputum sample *M. tuberculosis* complex-specific DNA was detected in 98% of smear-positive cases and in 72% of smear-negative cases using culture positivity as a reference standard. However, only 67 (39%) of all smear-negative cases were from a high HIV prevalence setting. Moreover, there is no information regarding Xpert MTB/RIF performance stratified by CD4 count. Thus, data about performance in patients infected with HIV, particularly those with smear-negative TB, are limited.

There are several other gaps in the knowledge. (*1*) There are limited data about the effects of an alteration in sample volume (using the recommended 1 ml vs. <1 ml) or processing methods (raw vs. liquefied sputum) on assay performance. Additionally, the relationship between bacterial load (measured using smear grade and culture time-to-positivity [TTP]) and assay performance is unclear. These factors have important implications for data interpretation in sputum-scarce patients, the integration of the assay into existing laboratory work flows, and the design of future clinical trials. (*2*) What additional yield Xpert MTB/RIF can offer, if any, over culture is unknown. Thus, the significance of Xpert MTB/RIF-positive, culture-negative samples remains unclear. (*3*) The impact, given resource constraints, of combining smear microscopy and Xpert MTB/RIF requires clarification. (*4*) Finally, we evaluated, hitherto untested, the specificity of a recently released second-generation software algorithm for the simultaneous detection of *M. tuberculosis* and rifampicin resistance.

The goal was to validate test performance further using a single cartridge in the context of high HIV prevalence and to assess the impact of the previously mentioned factors on assay performance. These issues gain importance as countries prepare to roll-out and scale-up the Xpert MTB/RIF assay (10).

METHODS

Study Sites and Population

Sputa were collected from 496 consecutively recruited ambulant patients with suspected TB (≥ 18 yr of age) between February 2007 and April 2010 at two primary care clinics in Cape Town, South Africa. Informed consent was obtained from all participants and the study was approved by the University of Cape Town, Faculty of Health Sciences Research Ethics Committee. Detailed patient and laboratory-specific information were recorded on a standardized case record form and captured using double data entry. An HIV test was performed after appropriate counseling. All chest radiographs were independently scored by two trained readers using the Chest Radiographic Reading and Reporting System (12, 13). Chest radiographs were scored as compatible or unlikely to be compatible with active TB. Discrepant results were adjudicated by a third senior reader.

TB Case Definitions

Each patient was allocated to one of three diagnostic categories: (*1*) definite TB: a clinical presentation compatible with TB with at least one spot sputum sample culture-positive for *M. tuberculosis*; (*2*) probable TB: a clinical–radiologic picture highly suggestive of TB or anti-TB treatment was initiated by an attending clinician based on clinical suspicion but the patient did not meet the criteria for definite TB (no culture-based evidence of *M. tuberculosis*); or (*3*) non-TB: no evidence of TB based on smear microscopy and culture, no anti-TB treatment initiated with response to alternative treatment where appropriate, and when available no radiologic evidence to support the diagnosis of TB.

Microbiology

At the first visit two paired spot sputa were concurrently collected from each patient. One arbitrarily selected sample was decontaminated in NALC-NaOH, submitted for routine concentrated fluorescence smear microscopy, and cultured for *M. tuberculosis* using the BACTEC MGIT 960 system (BD Diagnostics, Franklin Lakes, NJ) (14). The second sputum sample was stored (liquefied immediately or as raw sputum) at −20°C for later analysis using the Xpert MTB/RIF assay. Patients who returned for postenrolment follow-up provided additional sputum samples at each visit. Smear grading according to the WHO/International Union Against Tuberculosis and Lung Disease method was performed (15). Culture-positive isolates underwent phenotypic drug susceptibility testing for rifampicin and isoniazid using the MGIT 960 SIRE kit (BD Diagnostics) (16). Cultures with a TTP of more than 28 days and only one out of four positive follow-up cultures (when available) were considered to be possible cross-contaminants (also analyzed separately) (7). Unless otherwise stated, all Xpert MTB/RIF and culture results were generated from paired samples taken at the same visit, and patients accordingly classified.

Sample Processing and Storage for Later Analysis

The second sputa from the first 101 patients were liquefied using a 2:1 vol of 0.1% dithiothreitol (17) before storage at −20°C. The remaining 395 samples were unprocessed and stored at −20°C on collection.

Sample Preparation and XpertMTB/RIF Procedure

Sputum sample preparation was performed as described previously (6, 18, 19) by a trained operator masked to clinical information. Briefly, the sample reagent (Cepheid) was mixed at a 2:1 ratio with 1 ml of sputum (either liquefied or unprocessed) and homogenized. If the sample volume was less than 1 ml, sterile phosphate buffer (Merck, Darmstadt, Germany) was added to bring the final volume to 1 ml. Two milliliters of homogenized mixture was transferred into an Xpert MTB/RIF assay cartridge and inserted into the GeneXpert instrument (6).

Resolution of Discordant Results

For all Xpert MTB/RIF and culture discordant results, the cartridge-generated amplicon was extracted, amplified, and sequenced as previously described (6). In addition, a GenoTypeMTBDR*plus* test (HainLifescience, Nehren, Germany) or a PCR (using primers and conditions described previously) (6) followed by sequencing of the reaction products was performed on the stored sputum sediment or stored sample (18). Postenrolment sputum cultures were also analyzed and chest radiographs scored for likelihood of TB. Thus, performance was evaluated based on culture alone (Tables 1–4) or a combination of culture and these additional diagnostic investigations (Table 5). For the resolution of discordance in rifampicin resistance (phenotypic MGIT culture vs. Xpert MTB/RIF) a GenoTypeMTBDR*plus* test was performed on the culture isolate. When appropriate, the *rpoB* gene from the sample sediment or the culture isolate was amplified and sequenced (6).

Test Performance Assessment and Statistical Analysis

For the analysis of assay sensitivity, culture positivity and phenotypic susceptibility to rifampicin using simultaneously obtained paired samples (Xpert MTB/RIF vs. culture) were the reference standards. Specificity calculations were based on paired culture-negative samples from both culture-negative groups (probable and non-TB). Comparative specificity using the non-TB group only was also obtained. Test performance assessment and chi-square analyses were performed using OpenEpi (version 2.3.1; www.openepi.com) (20). Graphpad Prism (version 5.0; GraphPad Software, San Diego, CA) was used for the analysis of linear regression.

TABLE 1. DEMOGRAPHIC INFORMATION AND CLINICAL CHARACTERISTICS STRATIFIED BY SMEAR STATUS

Demographic or Clinical Characteristic	Study Cohort (%)	Smear-positive, Culture-positive (%)	Smear-negative, Culture-positive (%)	*P* Value
No. of TB suspects	480	94 (20)	47 (10)	
Median age (range)	36 (18–83)	35 (19–64)	37 (19–71)	0.56
Male	325 (68)	70 (22)	29 (9)	0.13
Female	155 (32)	24 (15)	18 (12)	0.13
Race				
Black	340 (71)	73 (21)	37 (12)	0.89
Mixed ancestry (colored)	132 (27)	19 (14)	10 (8)	0.88
White	9 (2)	2 (2)	0 (0)	0.44
Smoker (past or current)*	330 (73)	65 (20)	36 (11)	0.37
HIV positive†	130 (31)	23 (27)	23 (51)	<0.01
Median CD4 count (cells/ml) if HIV positive (range)‡	182 (0–935)	213 (0–439)	162 (10–465)	0.65
Previous TB§	158 (34)	24 (15)	16 (10)	0.30

Definition of abbreviation: TB = tuberculosis.
* Excludes 26 patients with no smoking-related data.
† Excludes 59 patients who refused testing and 5 patients who had no data.
‡ Excludes seven patients who were HIV positive with no CD4 count data.
§ Excludes 19 patients with no data about previous TB history.

RESULTS

Patient Population

After the exclusion of samples from 16 patients (Figure 1), 480 patients with suspected TB were eligible for inclusion into the analysis. Figure 1 depicts how samples were processed and archived, and patients categorized by diagnostic subgroup. Patient demographic and clinical characteristics are shown in Table 1.

A total of 141 (29%) of 480 patients had definite TB. Of these, 94 (67%) of 141 patients had smear-positive, culture-positive TB, whereas 47 (33%) of 141 had smear-negative, culture-positive TB. A total of 182 (38%) patients were classified as probable TB, whereas 157 (33%) patients were classified as non-TB. Of the 141 patients with positive cultures from their first sputum, 2 (1%) were found by phenotypic drug susceptibility testing to have MDR isolates (resistance to rifampicin and isoniazid), and 21 (15%) were found to have isoniazid monoresistant isolates.

Overall Xpert MTB/RIF Performance

The performance of Xpert MTB/RIF versus liquid culture performed on a simultaneously obtained paired spot sputum sample supplied at enrolment is shown in Table 2. The overall sensitivity of the assay was 78.7% (95% confidence interval [CI], 71.3–84.7;

TABLE 2. PERFORMANCE OUTCOMES OF XPERT MTB/RIF FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPARED TO SMEAR MICROSCOPY, AND STRATIFIED BY HIV STATUS

	All Patients (*n* = 480)		Patients Uninfected with HIV (*n* = 286)*		Patients Infected with HIV (*n* = 130)*	
	Sens. (95% CI)†	Spec. (95% CI)‡	Sens. (95% CI)	Spec. (95% CI)‡	Sens. (95% CI)§	Spec. (95% CI)‡
Sputum smear	66.7 (58.5–73.9) 94 of 141	99.7 (98.4–100) 338 of 339	73.2 (62.7–81.6) 60 of 82	100 (98.2–100) 204 of 204	50 (36.1–63.9) 23 of 46 (*P* = 0.01)	98.8 (94.6–99.8) 83 of 84
Xpert MTB/RIF	78.7 (71.3–84.7) 111 of 141 (*P* = 0.02)	94.4 (91.4–96.4) 320 of 339	82.9 (73.4–89.6) 68 of 82	95.6 (91.8–97.7) 195 of 204	69.6 (55.2–80.1) 32 of 46 (*P* = 0.09)	91.7 (83.8–95.9) 77 of 84
Sputum smear or Xpert MTB/RIF	82.3 (75.1–87.7) 116 of 141 (*P* < 0.01)	94.1 (91.1–96.2) 319 of 339	85.4 (76.1–91.4) 70 of 82	95.6 (91.8–97.7) 195 of 204	73.9 (59.7–84.4) 34 of 46	87.5 (75.3–94.1) 76 of 84
Xpert MTB/RIF in smear-negative, culture-positive cases	46.8 (33.3–60.8) 22 of 47	N/A	45.5 (26.9–65.3) 10 of 22	N/A	47.3 (29.2–67) 11 of 23	N/A
	PPV (95% CI)	NPV (95%CI)†	PPV (95% CI)	NPV (95% CI)	PPV (95% CI)	NPV (95% CI)§
Sputum smear	99 (94.3–99.8) 94 of 95	87.8 (84.1–90.7) 338 of 385	100 (93.4–100) 60 of 60	90.3 (85.7–93.5) 204 of 226	95.8 (79.8–99.3) 23 of 24	78.3 (69.5–85.1) 83 of 106 (*P* < 0.01)
Xpert MTB/RIF	85.4 (78.3–90.4) 111 of 130	91.4 (88–93.9) 320 of 350	88.3 (79.3–93.4) 68 of 77	93.3 (89.1–96) 195 of 209	82.1 (67.3–91) 32 of 39	84.6 (75.8–90.6) 77 of 91 (*P* = 0.02)
Sputum smear or Xpert MTB/RIF	85.3 (78.4–90.3) 116 of 136	92.7 (89.5–95) 319 of 344 (*P* = 0.03)	88.6 (79.8–93.9) 70 of 79	94.2 (90.1–96.7) 195 of 207	85 (70.1–93) 34 of 42	77.8 (65.1–86) 76 of 88 (*P* < 0.001)
Xpert MTB/RIF in smear-negative, culture-positive cases	53.4 (38.8–68) 22 of 41	92.7 (89.5–95) 319 of 344	52.6 (31.7–72.3) 10 of 19	94.2 (90.1–96.7) 195 of 207	61.1 (38.6–79.7) 11 of 18	86.4 (77.7–92) 76 of 88 (*P* = 0.03)

Definition of abbreviations: CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value.
* Excludes 59 patients who refused testing and 5 patients who had no data.
† *P* values < 0.10 in the "all patients" category are shown for comparisons between assays (microscopy vs. Xpert MTB-RIF, or microscopy vs. a combination of both).
‡ Specificity calculations were based on culture-negative samples obtained from both culture-negative groups (probable and non-TB)
§ Assay-specific (microscopy or Xpert MTB-RIF or a combination of both) *P* values < 0.10 comparing patients infected versus patients uninfected with HIV.

TABLE 3. PERFORMANCE OUTCOMES OF XPERT MTB/RIF FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* COMPARED WITH SMEAR MICROSCOPY IN PERSONS INFECTED WITH HIV, AND STRATIFIED BY CD4 COUNT

	Patients Infected with HIV ($n = 130$)*		Patients Infected with HIV with CD4 count ⩾ 200 cells/ml† ($n = 57$)		Patients Infected with HIV with CD4 count <200 cells/ml ($n = 66$)	
	Sens. (*95% CI*)	Spec. (*95% CI*)‡	Sens. (*95% CI*)	Spec. (*95% CI*)‡	Sens. (*95% CI*)§	Spec. (*95% CI*)
Sputum smear	50 (36.1–63.9) 23 of 46	98.8 (94.6–99.8) 83 of 84	61.9 (40.1–79.3) 13 of 21	97.2 (85.8–99.5) 35 of 36	39.1 (22.2–59.2) 9 of 23 ($P < 0.01$)	100 (91.8–100) 43 of 43
Xpert MTB/RIF	69.6 (55.2–80.1) 32 of 46	91.7 (83.8–95.9) 77 of 84	76.2 (54.9–89.4) 16 of 21	97.2 (85.8–99.5) 35 of 36	65.2 (44.9–81.2) 15 of 23	93 (81.4–97.6) 40 of 43
Sputum smear or Xpert MTB/RIF	73.9 (59.7–84.4) 34 of 46	87.5 (75.3–94.1) 76 of 84	81 (60–92.3) 17 of 21	4.3 (81.4–98.4) 33 of 35	69.6 (49.3–84.4) 16 of 23	93 (81.4–97.6) 40 of 43
Xpert MTB/RIF in smear-negative, culture-positive cases	47.3 (29.2–67) 11 of 23	N/A	50 (21.5–78.5) 4 of 8	N/A	50 (26.8–73.2) 7 of 14	N/A
	PPV (*95% CI*)	NPV (*95% CI*)	PPV (*95% CI*)	NPV (*95% CI*)	PPV (*95% CI*)	NPV (*95% CI*)§
Sputum smear	95.8 (79.8–99.3) 23 of 24	78.3 (69.5–85.1) 83 of 106	92.9 (68.5–98.7) 13 of 14	81.4 (67.4–90.3) 35 of 43	100 (70–100) 9 of 9	75.4 (62.9–84.8) 43 of 57 ($P < 0.01$)
Xpert MTB/RIF	82.1 (67.3–91) 32 of 39	84.6 (75.8–90.6) 77 of 91	94.1 (73–99) 16 of 17	87.5 (73.9–94.5) 35 of 40	83.3 (60.8–94.2) 15 of 18	83.3 (70.4–91.3) 40 of 48 ($P = 0.04$)
Sputum smear or Xpert MTB/RIF	85 (70.1–93) 34 of 42	77.8 (65.1–86) 76 of 88	89.5 (68.8–97.1) 17 of 19	89.2 (75.3–95.7) 33 of 37	84.2 (71–96) 16 of 19	85.1 (72.3–92.6) 40 of 47 ($P > 0.05$)
Xpert MTB/RIF in smear-negative, culture-positive cases	61.1 (38.6–79.7) 11 of 18	86.4 (77.7–92) 76 of 88	80 (37.6–96.4) 4 of 5	89.5 (75.9–95.8) 34 of 38	70 (39.7–89.2) 7 of 10	85.1 (72.3–92.6) 40 of 47 ($P > 0.05$)

Definition of abbreviations: CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value

* Excludes seven HIV-positive patients with no CD4 count data.

† Assay-specific performance in the patients infected with HIV with a CD4 count greater than 200 cells/ml group did not differ significantly compared with any other group.

‡ Specificity calculations were based on culture-negative samples obtained from both culture-negative groups (probable and non-TB).

§ Assay-specific *P* values (microscopy or Xpert or a combination of both) less than 0.10 comparing patients infected with HIV with a CD4 count less than 200 cells/ml versus patients uninfected with HIV.

111 of 141). In smear-positive culture-positive cases the sensitivity was 94.7% (88.2–97.7; 89 of 94), whereas in smear-negative culture-positive cases it was 46.8% (33.3–60.8; 22 of 47).

The overall specificity for the diagnosis of TB using both culture-negative groups (probable and non-TB) was 94% (91.4–96.4; 320 of 339). Using only the non-TB group, Xpert MTB/RIF specificity was 95% (91.7–98.4; 132 of 137; $P = 0.39$). The assay was negative in all eight cases that were culture-positive for non-TB mycobacteria (Figure 1), and negative in 14 of 15 cases with sputum isolates that were contaminated by bacterial overgrowth. Only 1 (0.2%) of 496 evaluated samples yielded an indeterminate Xpert MTB/RIF result.

A single Xpert MTB/RIF assay outperformed smear microscopy and showed an 18% relative increase in the rapid (potentially within 24 h) TB case-detection rate (17 additional cases) compared with 94 smear-positive cases and thus detected significantly more patients than smear microscopy (111 [78.7%] of 141 vs. 94 [66.7%] of 141; $P = 0.02$).

When a positive smear microscopy or Xpert MTB/RIF result were combined, the sensitivity improved further to 82.2% (75.1–87.7; 116 of 141) compared with smear microscopy alone (66.7% [68.5–73.4; 94 of 141]; $P < 0.01$) (Table 2).

Performance of Xpert MTB/RIF in Patients Infected with HIV

Smear microscopy was significantly less sensitive in subjects infected with HIV versus subjects uninfected with HIV (23 [50%] of 46 vs. 60 [73.2%] of 82; $P = 0.01$) (Table 2). Although the sensitivity of Xpert MTB/RIF was lower in the HIV-infected group, this did not reach significance (32 [69.6%] of 46 vs. 68 [82.9%] of 82; $P = 0.09$). The same pattern was seen in those with a CD4 count above or equal to versus below 200 cells/ml. Sensitivity of Xpert MTB/RIF in the smear-negative group was unaffected by HIV status or CD4 count (Tables 2 and 3). By contrast, the negative predictive value (NPV) of Xpert MTB/RIF decreased significantly in patients infected with HIV versus patients uninfected with HIV (195 [93.3%] of 209 vs. 77 [84.6%] of 91; $P = 0.02$), and was lower in those with a CD4 count less than 200 cells/ml versus those with a CD4 count greater than or equal to 200 cells/ml (40 [83.3%] of 48 vs. 34 [87.5%] of 40; $P = 0.60$). The same pattern was seen for smear microscopy.

When the assays were directly compared within patient subgroups, the sensitivity of Xpert MTB/RIF was higher than smear microscopy in persons infected and uninfected with HIV, and in those with a CD4 count less than 200 cells/ml, but this difference was not significant for all three groups (Tables 2 and 3). The NPV for Xpert MTB/RIF did not differ significantly from that of smear microscopy in any of these groups.

The combination of smear microscopy and Xpert MTB/RIF had a significantly better sensitivity than smear microscopy alone in patients infected with HIV (34 [73.9%] of 46 vs. 23 [50%] of 46; $P = 0.02$) and in those with a CD4 count less than 200 cells/ml (16 [69.6%] of 23 vs. 9 [39.1] of 23; $P < 0.05$). Likelihood ratios stratified by smear status, HIV status, and CD4 count are included in the online supplement.

Sample Processing and Volume, and Impact of Bacterial Burden

Sensitivity (77.9% vs. 81.1%; $P = 0.70$) and specificity (94.3% vs. 94.7%; $P > 0.99$) was similar in 386 unprocessed compared with 94 liquefied samples (Table 4). Similarly, sputum sample volume had limited impact on sensitivity (71.2% in samples less than the recommended 1 ml [median 575 μl; interquartile range {IQR} 300–700 μl] vs. 83.2% in samples of 1 ml [$P = 0.10$]; 40.9% in smear-negative culture-positive samples <1 ml [median 500 μl; IQR 300–600 μl] vs. 52% in samples of 1 ml [$P = 0.47$]). Restricting the analysis to include only unprocessed

TABLE 4. IMPACT OF SAMPLE PROCESSING, SAMPLE VOLUME, AND CULTURE TIME TO POSITIVITY CUT-OFF ON XPERT MTB/RIF PERFORMANCE

		Sensitivity (*95% CI*)* Number of Patients			
Sample Type		Culture-positive	Culture-positive, Smear-positive	Culture-positive, Smear-negative	Specificity (*95% CI*) Number of Patients
All	All samples ($n = 480$)	78.7 (71.3–84.7) 111 of 141	94.7 (88.2–97.7) 89 of 94	46.8 (33.3–60.8) 22 of 47	94.4 (91.4–96.4) 320 of 339
Sample processing	Unprocessed ($n = 386$)	77.9 (69–84.8) 81 of 104	93 (84.6–97) 66 of 71	45.5 (29.9–96) 15 of 33	94.3 (91–96.5) 266 of 282
	Liquefied ($n = 94$)	81.1 (65.8–90.5) 30 of 37	100 (85.7–100) 23 of 23	50 (26.8–73.2) 7 of 14	94.7 (85.6–98.2) 54 of 57
	P value†	0.70	0.24	0.78	0.95
	1 ml ($n = 294$)	83.2 (74–89.5) 74 of 89	95.3 (87.1–98.4) 61 of 64	52 (33.5–70) 13 of 25	95.1 (91.2–97.3) 195 of 205
Sample volume	<1 ml ($n = 186$) Median volume (IQR) = 575 μl (300–700)	71.2 (57.7–81.7) 37 of 52	93.3 (78.7–98.2) 28 of 30	40.9 (23.3–61.3) 9 of 22	93.3 (87.7–96.4) 125 of 134
	P value†	0.10	0.70	0.47	0.48
Culture time to positivity	Samples with a TTP >28 d excluded ($n = 1$)‡	79.3 (71.3–85.2) 111 of 140	94.7 (88.2–97.7) 89 of 94	47.8 (34.1–61.9) 22 of 46	94.4 (91.4–96.4) 320 of 339
All of the above filters	Unprocessed samples only with 1 ml of sputum available for the assay, and a culture TTP not >28 d with all subsequent sputum samples culture-negative ($n = 241$)	81.7 (71.2–90) 58 of 71	93.9 (83.5–97.9) 46 of 49	54.6 (34.7–73.1) 12 of 22	94.7 (71.2–90) 162 of 171
	P value†	0.62	0.83	0.56	0.89

Definition of abbreviations: CI = confidence interval; IQR = interquartile range; TTP = time-to-positivity.

* Culture positivity for *M. tuberculosis* using a sputum sample obtained at enrolment was used as the reference standard.

† *P* values (two-tailed Mid-P chi-square test) comparing sputum processing method, sample volume, and samples with a TTP greater than 28 days excluded versus samples with a volume of 1 ml and a culture TTP not greater than 28 days (provided no subsequent cultures were positive).

‡ Three patients had a first spot sputum sample culture TTP greater than 28 days. One patient gave a later sample that was culture-positive. One patient was lost to follow-up. For the remaining patients all three subsequent samples were culture-negative, indicating probable cross-contamination.

samples with 1 ml of available sputum and those with a culture TTP less than or equal to 28 days (provided no postenrolment samples gave a positive culture) (21) did not significantly improve assay sensitivity (46.8%–54.6%; $P = 0.56$) for smear-negative TB.

Higher bacterial loads, as determined by both smear grade and MGIT TTP, were associated with earlier detection by assay and more frequent positive results (Figure 2). The average cycle threshold value was significantly lower in smear-positive compared with smear-negative cases (22 ± 0.5 vs. 32 ± 0.9; $P < 0.0001$). A similar relationship was seen using smear grade and culture TTP as markers of bacterial load (Figure 2). Using spiked sputum samples the limit of detection of the MTB/RIF assay was found to be 100 cfu/ml and freeze thaw

TABLE 5. XPERT MTB/RIF PERFORMANCE OUTCOMES IN PATIENTS WITH DEFINITE TB (ALL CULTURE-POSITIVE CASES) VERSUS A GROUP CONTAINING CULTURE-POSITIVE AND HIGHLY LIKELY TB CASES*

		Sens. (*95% CI*)	Spec. (*95% CI*)	PPV (*95% CI*)	NPV (*95% CI*)	Relative Increase in no. of Xpert MTB/RIF Diagnosed Cases Versus Smear Microscopy
All TB cases	Definite TB (culture-positive)	78.7 (71.3–84.7) 111 of 141	94.4 (91.4–96.4) 320 of 339	85.4 (78.3–90.4) 111 of 130	91.4 (88–93.9) 320 of 350	94–111† (18%)
	Definite TB + highly likely group‡	80.9 (74–86.3) 127 of 157	99.1 (97.3–99.7) 320 of 323	97.7 (93.4–99.2) 127 of 130	91.4 (88–93.9) 320 of 350	94–126 (35%)
	P value	0.64	<0.001	<0.001	>0.99	<0.01
Smear-negative TB	Definite TB (culture-positive)	46.8 (33.3–60.8) 22 of 47†	94.4 (91.4–96.4) 319 of 338	53.4 (38.8–68) 22 of 41	92.7 (89.5–95) 319 of 344	N/A
	Definite TB + highly likely group*	60.3 (48–71.5) 38 of 63	99.1 (97.3–99.7) 319 of 322	92.7 (80.6–97.5) 38 of 41	92.7 (89.5–95) 319 of 344	N/A
	P value	0.12	<0.001	<0.001	>0.99	N/A

Definition of abbreviations: CI = confidence interval; NPV = negative predictive value; PPV = positive predictive value; TB = tuberculosis.

* *P* values indicate a comparison between groups. The same analysis for patients with smear-negative TB is also shown.

† Xpert MTB/RIF did not detect five smear-positive, culture-positive samples.

‡ Sixteen culture-negative, Xpert MTB/RIF-positive patients are included here (five patients were found to be culture-positive by a second sputum obtained within 2 weeks from enrolment, five had *Mycobacterium tuberculosis* DNA in their sputum by sequencing, and six patients had typical radiologic evidence of active TB). All Xpert MTB/RIF amplicons were confirmed to contain *M. tuberculosis* DNA.

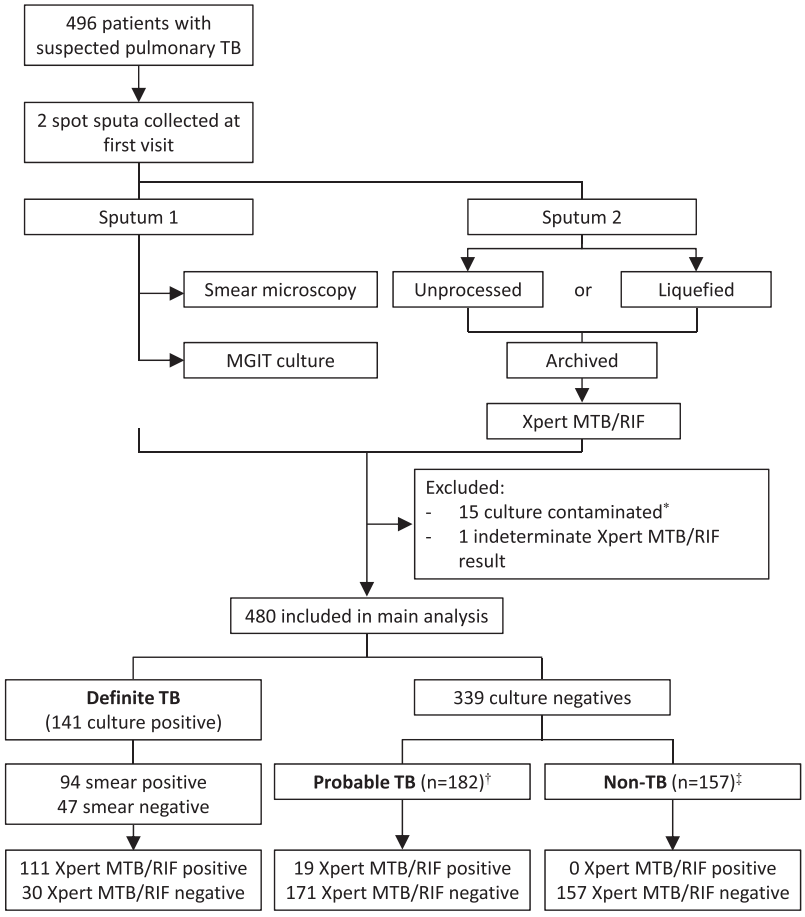

Figure 1. Flow diagram outlining patient enrolment, sample processing, and outcomes stratified by diagnostic category. TB = tuberculosis; MGIT= mycobacterial growth indicator tube.

*1 patient who had a contaminated spot sputum culture was Xpert® MTB/RIF-positive. No follow up culture results or radiological evidence was available for this patient.
†Includes 5 Xpert® MTB/RIF-negative individuals culture-positive for non-tuberculosis mycobacteria
‡ Includes 3 individuals culture-positive for non-tuberculosis mycobacteria.

experiments showed no decrease in assay sensitivity (data not shown).

Performance of Xpert MTB/RIF for the Detection of Rifampicin Resistance Using the Second-generation Software

Xpert MTB/RIF identified six samples as rifampicin resistant. By contrast, MGIT DST identified five of these isolates as sensitive to rifampicin. Five out of the six samples where confirmed to be genotypically resistant by sequencing of DNA extracted from the isolate or a GenoTypeMTBDR*plus* test. We were unable reliably to compute sensitivity given the small number of drug-resistant cases but Xpert MTB/RIF correctly determined susceptibility to rifampicin in 151 of 152 cases, and hence the specificity was 99.4% and the NPV was 98.7%.

Discordance Between Xpert MTB/RIF and Culture (discrepant analysis)

Xpert MTB/RIF-positive results in the probable group. As shown in Figure 1, there were 19 Xpert MTB/RIF-positive patients

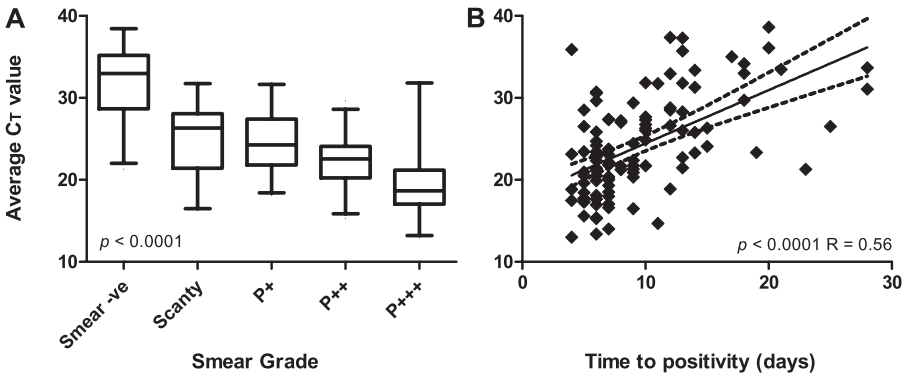

Figure 2. Correlation of the average cycle threshold (C_T) value with smear grade (*A*) and liquid culture time to positivity (*B*).

who were culture-negative based on their simultaneously obtained paired sputum sample. Of these, five (26%) were found to be culture-positive on a second sputum obtained within 2 weeks of enrolment. A further 10 (53%) had *M. tuberculosis* DNA detected in their archived sputum using sequencing or a GenoTypeMTBDR*plus* test. In one (1%) additional patient the chest radiograph was compatible with and suggestive of active TB. Furthermore, in all 16 cases diagnosed with TB using either culture, sequencing of DNA from sputum or sample sediment, a GenoTypeMTBDR*plus* test, or chest radiography, the sequencing of the Xpert MTB/RIF cartridge amplicon confirmed *M. tuberculosis*. Thus, 16 of the 19 Xpert MTB/RIF-positive culture-negative patients were deemed likely to be true positives and designated as "highly likely TB." In the remaining three patients a chest radiograph was unavailable precluding meaningful classification, although they were placed on treatment by the attending clinician based on clinical suspicion.

When the 16 of 19 patients were combined with the definite TB group and the data were reanalyzed (Table 5), there was no significant change in sensitivity (111 [78.7%] of 141 to 127 [80.9%] of 157; $P = 0.64$) (Table 5). However, specificity (320 [94.4%] of 339 increased to 320 [99.1%] of 323; $P < 0.001$) and the positive predictive value improved significantly (111 [85.4%] of 130 to 127 [97.7%] of 130; $P < 0.001$), and the relative increase in the proportion of patients diagnosed compared with smear microscopy improved significantly (94–111 [18%] vs. 94–127 [35%]; $P < 0.01$). The positive predictive value in individuals with smear-negative TB improved significantly (22 [53.4%] of 41 to 38 [92.7%] of 41; $P < 0.001$) and the sensitivity increased to 60.3%. Patients in the culture-negative Xpert MTB/RIF-positive patient group had a higher mean average cycle threshold value compared with those who were culture-positive, Xpert MTB/RIF-positive (29.3 [23.9–34] vs. 23.6 [19.6–27.3]; $P < 0.001$).

Xpert MTB/RIF-negative culture-positive results. Thirty patients were culture-positive but Xpert MTB/RIF-negative. There was a higher proportion of smear-negative individuals in this group compared with culture-positive, Xpert MTB/RIF-positive individuals (83% vs. 20%; $P < 0.001$). The median TTP (IQR) was significantly longer in this group (18 [13–25] vs. 7 [6–12] d; $P < 0.001$). There was no significant difference in median sample volume across these groups (0.9 vs. 1 ml; $P = 0.13$).

DISCUSSION

The WHO recently endorsed Xpert MTB/RIF (11); however, there are limited data about performance outcomes in high HIV prevalence settings where smear-negative TB is a formidable diagnostic challenge. The key findings of this preliminary study using archived samples were: (*1*) Xpert MTB/RIF outperformed smear microscopy because it diagnosed a significant proportion of smear-negative TB cases, and increased the relative proportion of potentially rapidly diagnosed cases by 18%; (*2*) HIV coinfection was associated with a significantly reduced assay NPV and there was a trend to reduced sensitivity; (*3*) taking into account Xpert MTB/RIF-positive culture-negative samples obtained from highly likely patients with TB the proportion of potentially rapidly diagnosed cases relative to smear microscopy significantly improved from 18–35%; (*4*) sputum volume and processing methods had a nonsignificant impact on assay performance, but by contrast bacterial load correlated strongly with performance; and (*5*) the specificity and NPV of the second-generation software for rifampicin resistance was almost 100%. Thus, Xpert MTB/RIF outperformed smear microscopy and simultaneously ruled out rifampicin resistance with great accuracy.

There are limited data about Xpert MTB/RIF performance in persons infected with HIV and none stratified by CD4 count. Our preliminary data indicate that the NPV is significantly reduced in this group ($\sim$ 15% of those with negative results have TB). The effects are most marked in those with advanced immunosuppression. Thus, in persons infected with HIV Xpert MTB/RIF is a good rule-in test but may have limited rule-out value compared with persons uninfected with HIV. This may be caused by the lower concentration of mycobacteria in the sputum of persons infected with HIV and possibly reduced specificity caused by occult or subclinical disease. Our data add to the limited existing knowledge base about the impact of HIV on Xpert MTB/RIF performance. However, there are several important limitations regarding our data (discussed in detail later) and thus further studies are required to clarify these findings. The added rule-out value of a second test in persons infected with HIV who are smear- and Xpert MTB/RIF-negative remains to be determined.

The key advantage of Xpert MTB/RIF is that it diagnosed 47% (95% CI, 33–61%) of smear-negative TB cases in a high HIV prevalence setting and 55% (35–73%) when a restricted analysis was performed. A recent multicenter study showed a sensitivity in the smear-negative group when using a single cartridge of 73% (65–79%) (7). The differing sensitivities likely reflect differences in study design or represent a chance finding given that the CI overlap in both the studies ($P = 0.10$ vs. the restricted samples). Our results may reflect the effect of using frozen samples; however, preliminary experiments showed no significant effect of repeated freeze–thaw cycles on assay performance and similar observations have been reported by Helb and colleagues (6). That early morning sputum samples (known to have a higher diagnostic yield relative to spot sputum samples) (22, 23) were included in the study by Boehme and colleagues (7) and classification using at least one of four culture results per individual (which would decrease the number of culture-negative, Xpert MTB/RIF-positive cases) are other possible reasons for this discrepancy. Nevertheless, even with our detected sensitivity in the smear-negative group, there was an almost 20% increase in TB case detection compared with smear microscopy alone. This increase is dependent on local HIV prevalence rates.

Previous studies have not evaluated the significance of culture-negative Xpert MTB/RIF-positive samples. Are these true or false-positive results? When the significance of these results was clarified using short-term follow-up cultures, sequencing, and a suggestive radiologic picture using a standardized scoring system there was a 35% relative increase in the number of detected cases. Given the well-known limitations of *post hoc* discrepancy analyses (24), larger studies are now required in different settings to evaluate better discordant cases with long-term follow-up.

Our data suggest that sensitivity in smear-negative TB was limited by bacterial load. Studies correlating Xpert MTB/RIF Ct values with bacterial load are important because they inform contact tracing policies, treatment monitoring, and definition of a benchmarking threshold against which competitor assays can be measured. The limit of detection in our hands using spiked sputum samples was 100 CFU/ml. This is in keeping with the findings of Helb and colleagues (6), who report a limit of detection of 132 cfu/ml. Nevertheless, published studies confirm that those with smear-negative TB often have bacterial loads substantially below 100 cfu/ml (25). Future studies are required to examine if sample concentration can improve sensitivity.

A key advantage of Xpert MTB/RIF over smear microscopy is the simultaneous assessment for rifampicin resistance. We are unable to comment on sensitivity for rifampicin resistance given

the limited number of cases in this category, but we can confirm, similar to the findings of Boehme and colleagues (7) using the first-generation software, that the specificity and NPV are high (7). Thus, the assay using the second-generation software can reliably rule out rifampicin resistance in a high HIV prevalence setting. This is crucial given the increasing burden of MDR-TB and XDR-TB in Africa (26–28).

A major hurdle to widespread implementation of the Xpert MTB/RIF assay in resource-poor settings is cost (29). Thus, a possible interim strategy to enhance uptake might be to perform the assay only in smear-negative rather than smear-positive patients suspected of having TB (8, 28). Our data lend credence to this strategy because a combination of the two diagnostic methods showed the best sensitivity and specificity. However, the downside is that information about drug susceptibility is unavailable in smear-positive patients.

There are no existing published data on the effect of sputum volume on Xpert MTB/RIF performance. This is an important consideration in Africa, where HIV coinfection may result in a higher proportion of sputum-scarce patients who produce suboptimal sputum volumes. Our data indicate that volumes below the recommended limit of 1 ml (median volume, 575 μl) do have some impact on performance, although not significant, and thus could still be used for Xpert MTB/RIF in sputum-scarce patients. However, further studies are required to confirm these findings and to accurately determine the minimum volume of sputum that can be reliably used in sputum-scarce or paucibacillary individuals. It is possible that using volumes greater than 1 ml improves detection in smear-negative TB. That an approximately twofold reduction in volume has minimal impact on performance is not surprising given that PCR amplifies its DNA target by over a billionfold (30). Similarly, liquefied sputum had a minimal effect on performance. These data are important because in cases where the Xpert MTB/RIF assay is indeterminate or if an additional test is required then the decontaminated stored sample can be used to clarify the status of the donor.

There are several limitations of our study findings. First, our results may reflect the bias of sample storage and freeze–thaw, which may impact on DNA integrity or sputum viscosity. However, this is not supported by our preliminary freeze–thaw data or the data of Helb and colleagues (6), which suggest that prolonged storage and freeze–thaw have limited impact on sensitivity. Nevertheless, even under less rigorous and well-controlled study conditions compared with published data (7) we show that Xpert MTB/RIF comprehensively outperforms smear microscopy. We only performed one paired sputum-Xpert MTB/RIF culture per patient and used only a single liquid culture as a reference standard. Thus, we may have erroneously estimated outcomes given that a MGIT culture on a second specimen, compared with a MGIT culture on a single specimen from persons infected with HIV, may detect an additional 17% of cases (31). Our study findings may have limited relevance to low HIV prevalence settings where TB prevalence rates are lower (in patients infected with HIV and patients uninfected with HIV). The conclusiveness of our findings is especially limited by the small number of patients, particularly in the smear-negative and HIV-infected subgroups, which is a consequence of the study design. Prospective studies in these patient subgroups are now urgently needed. However, we establish a firm rationale and provide a foundation for the design of larger and more comprehensive studies to evaluate Xpert MTB/RIF in populations infected with HIV. Finally, our data do not inform on how Xpert MTB/RIF tests perform at the point-of-treatment, where the assay can have the greatest impact on patient care. Thus, controlled studies evaluating outcomes at point-of-treatment in high HIV prevalent settings are urgently required.

In summary, the Xpert MTB/RIF assay is an accurate rapid rule-in test for pulmonary TB. It outperformed smear microscopy given that it established a diagnosis in a significant proportion of patients who are smear-negative. It may also detect additional culture-negative patients and has excellent rule-out value for MDR TB. However, because sample size, volume, and use of frozen samples were important limitations of this study, further studies are required to clarify test rule-out value in persons infected with HIV and the role of this assay in current TB treatment algorithms in high HIV prevalence settings.

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