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# Evaluation of the Xpert® MTB/Rif test, microscopic observation drug susceptibility test and nitrate reductase assay, for rapid and accurate diagnosis of smear-negative tuberculosis in HIV patients

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ABSTRACT

Diagnosis of smear-negative tuberculosis (TB), which is frequently seen in HIV-infected patients, is a challenge without conventional culture methods. Since 2007, the WHO (World Health Organization) has endorsed new or improved tests for increased and rapid diagnosis of TB. This study was undertaken in an effort to evaluate the accuracy of two rapid culture methods: the Microscopic Observation Drug Susceptibility assay (MODS) and Nitrate Reductase Assay (NRA), and the molecular based test Xpert® MTB/Rif (Xpert), for diagnosis of smear-negative TB in HIV patients using the mycobacteria growth indicator tube (MGIT) in the BACTEC™ MGIT™ 960 system as the reference test. 430 smear-negative patients with presumptive TB were enrolled in a cross-sectional study at a tertiary care facility in Uganda. Their sputum was tested on MODS, NRA, Xpert and MGIT. Of the 430 patients, 373 had complete results to compute test accuracy. *Mycobacterium tuberculosis* (MTB) was detected in 43 patients by MGIT. The sensitivity and specificity were 24.4% and 98.1% for MODS, 41.5% and 92% for NRA, 48.8% and 95.1% for Xpert, respectively. The low sensitivity of the tests implies that additional diagnostics such as chest X-ray and conventional liquid culture methods might still be needed to detect TB in smear-negative HIV patients. The high specificity of the tests is useful to confirm TB in HIV patients with symptoms suggestive of TB.



## Introduction

The World Health Organization (WHO) estimates that 8.7 million people develop tuberculosis (TB) each year worldwide [1]. Of these, 13% are co-infected with HIV, while of the 1.4 million deaths that occur, 30% are HIV-related. The African region, especially sub-Sahara Africa, accounts for 80% of the HIV-associated TB cases worldwide. Unfortunately, diagnosis

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of TB in this region relies mainly on sputum microscopy, which due to its relatively low sensitivity, performs poorly in HIV-infected patients. This is owing to the paucibacillary nature of TB in HIV-infected patients; therefore, active TB disease is often not detected by smear examination [2,3].

Culture is a more sensitive way to detect paucibacillary forms of TB, which are common in HIV patients and children [4]. However, in several resource-limited settings, such as in sub-Sahara Africa, mycobacterial culture is inaccessible to the majority of the population. Even when available, the commonly used solid culture on Löwenstein–Jensen culture media (L–J) is time-consuming and growth of mycobacteria is typically detected after several weeks [5]. Commonly, therefore, diagnosis of TB in smear-negative HIV patients is made empirically. This is associated with delayed detection and initiation of TB treatment, and a higher mortality rate compared with patients who are smear-positive [6].

Since 2007, the pipeline for new TB diagnostics has expanded. In parallel, WHO has endorsed several new tests for increased and more rapid TB diagnoses. However, despite a major drive to incorporate these tests into the current diagnostic algorithms, data of their accuracy for diagnosis of smear-negative TB in HIV patients is still inadequate [7]. In this study, the accuracy of a molecular test and two rapid culture methods for diagnosis of smear-negative TB in HIV patients using the mycobacteria growth indicator tube (MGIT) in the BACTEC™ MGIT™ 960 system (Becton Dickinson Biosciences, Sparks, MD) as the reference test was evaluated [8].

### *Overview of tests*

The molecular test Xpert® MTB/Rif (Cepheid GeneXpert System, Sunnyvale, CA) (Xpert) is based on quantitative real-time PCR for detection of TB. This is the first molecular method for TB detection to be fully automated and to integrate all the steps required for PCR-based DNA testing. Thus, results are reported within three hours and with high accuracy [9,10]. Both processed and unprocessed sputum can be used in this test.

The two rapid culture methods evaluated were: the Microscopic Observation Drug Susceptibility assay (MODS), and the Nitrate Reductase Assay (NRA). The tests are described as being low-cost and easily implemented, and WHO has recommended that both can be used as direct or indirect tests for rapid diagnosis of multi-drug resistant TB [11].

The MODS relies on two well-known properties of *Mycobacterium tuberculosis* (MTB): first, the rate of growth in liquid medium is considerably higher than that on solid medium. Secondly, the morphology in liquid culture is characteristic and recognizable, consisting of so-called cord-like structures. By using an inverted light microscope to examine culture plates inoculated with sputum, MTB growth can be detected within 7–10 days, for both smear-positive and -negative samples, compared with conventional solid culture that takes 3–8 weeks [12,13].

The NRA assay relies on the ability of MTB to reduce nitrate to nitrite. The presence of nitrite, indicating metabolically active mycobacteria, can easily be detected by the addition of what is known as the Griess reagent into the culture tube, producing a visually observed pink to purple color change [14]. The NRA test was modified by using Middlebrook 7H9 broth (Difco) as the culture media, since the rate of growth in liquid medium is considerably quicker than that on solid medium. As reported previously, results can be available within 10–14 days when liquid culture media is used. These studies were, however, based on examination of microscopy positive sputum samples with a higher content of bacteria [15].

## Materials and methods

Ethical approval for the study was obtained from the research and ethics committee, of the College of Sciences of Makerere University (REC 2011-45).

### *Sample size*

The sample size was calculated based on methods described earlier [16]. It was hypothesized that the culture and molecular methods would have similar sensitivities and specificities of 90% and 98%, respectively. The 95% confidence interval was adopted, and the corresponding $Z$ value of 1.96 in the calculations, while the precision was set at ±10%. Based on data from the pilot study, it was expected that approximately 10% of HIV patients reported smear-negative would have a positive MTB culture when evaluated by fluorescent microscopy (FM). It was therefore required that at least 346 smear-negative patients be enrolled for the full study.

### *Patient recruitment*

Using an observational and cross-sectional study design, patients with symptoms of TB between March and November 2012 were enrolled in the study. The study patients were in attendance at an adult out-patient HIV clinic or were admitted to the medical department at Mulago National Referral Hospital, Kampala, Uganda. At the hospital, patients are screened for TB using a standard WHO/Ministry of Health intensified TB case finding form. According to the form, patients are presumed to have TB if they had a cough for $\geqslant$2 weeks, with or without fever, night sweats, loss of weight, or blood-stained sputum. Following consent of the enrolled patients, they provided a spot and early morning sputum sample. Patients were excluded who were already on TB treatment or on quinolone medication, or were unable to produce sputum. Detailed HIV care information was obtained from the patients' clinical charts and recorded on a standardized case record form.

### *Laboratory methods*

The collected sputum samples were maintained at 2–4 °C in an ice box at the clinic/ward, and were carried to a closely located bio-safety level 3, mycobacteriology research laboratory, twice daily. The laboratory is quality controlled by the National TB Reference Laboratory (NTRL).

Unprocessed sputum received in the laboratory was examined by fluorescent microscopy (FM) using standard auramine reagent at x40 objective (Olympus CX31 with LED attachment, Olympus Corporation, Tokyo, Japan). Samples found positive

had their results immediately issued to the clinic and department physicians to initiate TB treatment. Samples found negative were pooled and underwent further processing as follows (see Fig. 1): digestion and decontamination with NALC/NAOH 2% method for 15 min, followed by homogenization. The homogenized sample was then centrifuged at 3000*g* (Allegra® X-12 series) for 15 min to prepare a sediment [14]. The sediment was re-suspended with phosphate buffer (pH 6.8) to make 5 ml. Aliquots of the suspension (0.5 ml) were inoculated in MGIT and L–J tubes for mycobacteria culture. Residual aliquots were stored at 4–8 °C, batched and later tested on Xpert, software version 4.0 (1 ml), NRA (0.5 ml) and MODS (0.1 ml). All tests were performed by trained technicians blinded to results from the other tests.

For the Xpert MTB/RIF assay, a sample reagent was added to the processed sample in a 3:1 ratio. The mixture was incubated at room temperature for 15 minutes and was manually agitated. A total of 1 ml of sample was introduced into an Xpert MTB/RIF cartridge, which was then loaded into the GeneXpert instrument, where the subsequent steps of sample lysis, nucleic acid extraction, and amplification occurred automatically. When sufficient residual was available, repeat testing was carried out when an "Invalid" or "Error" result was obtained.

The MODS culture was performed in a 24-well tissue plate. Two wells were used for each processed and quality control sample. In brief, the MODS media was prepared with 4.7 g Middlebrook 7H9 broth (Difco, Sparks, MD) and 2 ml glycerol in 900 ml of distilled water. This media was autoclaved at 121 °C for 10 min, cooled to 45 °C and 100 ml of OADC added. This was stored at 4–8 °C. Each new batch of the media was quality checked for sterility by inoculating on blood agar before use. Next, 800 μl of the Middlebrook 7H9 broth, 100 μl of a cocktail of polymyxin B, Amphotericin B, Nalidixic acid, trimethoprim and azlocillin (PANTA: BD®), and 100 μl of the processed sample were transferred into each well giving a final volume of 1 ml/well. 100 μl of a suspension of H37Rv isolate 0.5 McFarland standard for positive

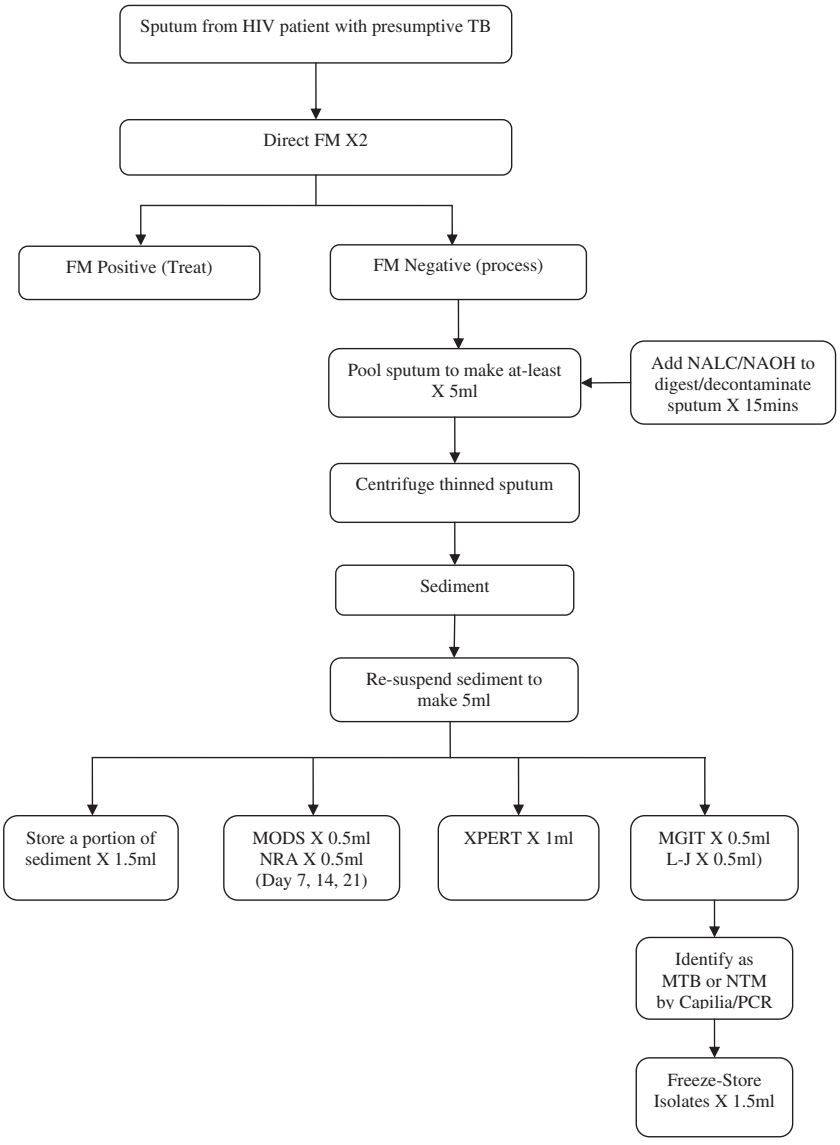


**Fig. 1 – Specimen flow diagram.**

control was used. For negative growth control, 800 μl of Middlebrook 7H9 broth, 100 μl PANTA without sample was used. The positive and negative growth control wells were included for each plate. Plates were sealed with tape and ziplock bags and incubated at 37 °C. The plates were examined under an inverted light microscope at magnifications of X10 and X40 for cord formation. Initially the examination was done on days 5, 7 and 14. However, following a preliminary analysis of the first 10% of the samples, which showed only a few wells with detecable growths by day 14, examination of the rest of the samples (90%) was extended to day 21. Wells that had rapid overgrowth or clouding were interpreted as contaminated.

The modified NRA test was performed using Middlebrook 7H9 broth, supplemented with 2 ml of glycerol, 10% OADC, PANTA (0.6 ml PANTA: 10 ml 7H9 media), and 1000 μg of potassium nitrate ($KNO_3$)/ml. The medium was apportioned into 20 ml screw-cap glass tubes in volumes of 7 ml. Next, 500 μl of the processed sample was inoculated into 3 tubes containing the above cocktail. Each batch included three corresponding positive growth control (H37Rv isolate) and the negative growth control (cocktail without sample). The tubes were incubated at 37 °C. On the day of examination, 200 μl of Griess reagent (a solution of hydrochloric acid 50% (vol/vol), sulfanilamide 0.2% wt/vol, and N-(1-naphtyl)ethylene-diamine dihydrochloride 0.1% (wt/vol) mixed in a ratio of 1:2:2 was added into each test tube and observed for change of color to pink-purple. When color change occurred, it was compared with the prescribed WHO color standard [14]. The NRA was initially examined on days 5, 7 and 14, also. However, for similar reasons for MODS above, examination was extended to day 21 as well. Tubes that had clouding were interpreted as contaminated. For the MGIT culture, 0.5 ml of the processed samples were inoculated following the protocol of Becton Dickinson, while for L–J, 0.5 ml of the processed sample was inoculated onto an L–J slant and incubated at 37 °C. MGIT culture tests were read up to day 42 before recording negative growth, while the L–J culture was read up to day 56. Positive cultures on MGIT were examined by ZN microscopy to confirm the presence of AFB. The Capilia tuberculosis (MPT64) assay [17] or PCR IS6110 method were used to determine if positive MGIT cultures were MTB or Non-Tuberculous Mycobacteria (NTM). All tests were interpreted after examination of the positive and negative controls. The GeneXpert system has built-in internal quality control for each sample, which is automatically performed on each run.

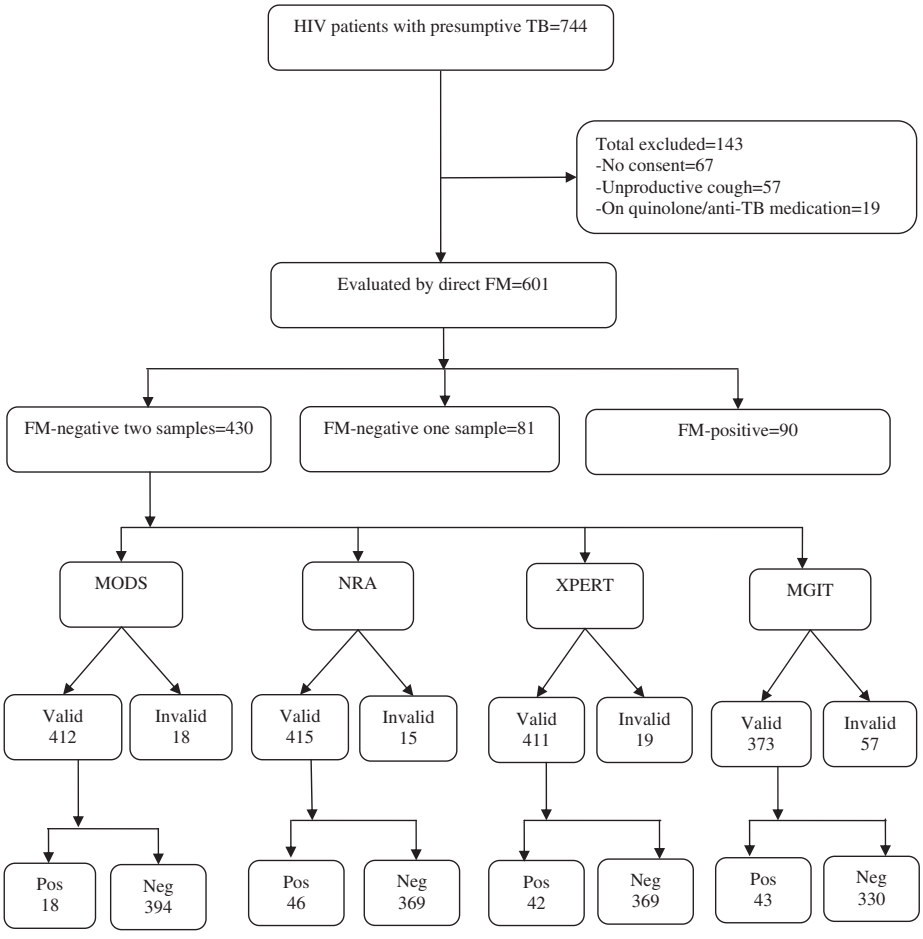


**Fig. 2 – Patient flow and test analysis diagram. Pos = Positive. Neg = Negative. MODS = Microscopic Observation Drug Susceptibility assay. NRA = Nitrate Reductase Assay. Xpert = Xpert® MTB/Rif test. Invalid = contaminated culture, non-tuberculous mycobacteria growth or "error" or "invalid" result in the case of the Xpert test.**

**Table 1 – Characteristics of smear-negative patients with presumptive TB.**

| | |
|---|---|
| Age: years, median (range), [IQR] | 34, (18–67), [29–40] |
| Female: *n*, (%) | 242, (56) |
| Duration, attendance in Clinic: Months, median (range), [IQR] | 14, (0–78), [1–46] |
| Percentage, patients on ART: n (%) | 263, (61%) |
| Duration on ART: Months, median (range), [IQR] | 12 (0–72) [1–38] |
| Most recent CD4 cell absolute count/mm$^3$: median (range), [IQR] | 280 (2–952) [119–439] |

IQR = Inter Quartile Range. ART = Anti-retroviral therapy.

### *Statistical methods (data management)*

Patients' clinical data captured on the standardized case record form was entered periodically into Epi Info™, version 3.5. Laboratory data was entered periodically into MS Access 2003. All data were exported to SPSS™, version 17.0 for analysis. Sensitivity, specificity and positive and negative predictive values were calculated using MGIT as the reference test. Analysis was performed on a per patient basis. NTM and contaminated cultures were excluded from the analysis. Samples that were reported contaminated on MGIT, and showed no growth on L–J, were considered as "no growth".

### *Patient and sample classifications*

An HIV patient was considered to have presumptive TB if they had a cough for two weeks or more with or without history of coughing blood-stained sputum. Patients with two sputum samples negative for AFB after microscopy examination and negative for MTB after culture were defined as smear and culture negative. Patients with at least one sputum sample positive for AFB after microscopy examination were defined as smear-positive pulmonary TB cases, and were not enrolled into the study. Patients who had an incomplete set of data for either the evaluated test or culture were defined as indeterminate and were excluded from analysis. A smear-positive sample was defined as one with scanty grade (1 to 10 AFB per 100 fields) or more bacilli. A culture-positive sample was defined as a sample with growth of MTB. A smear-negative pulmonary TB sample was one with at least two sputum samples negative for acid-fast bacilli (AFB) that was culture-positive for MTB.

## Results

### *Patient flow*

744 patients were identified with presumptive TB, and 143 of which were excluded owing to a lack of consent, unproductive cough or taking anti-TB or quinolone medication (see Fig. 2). The remaining 601 (81%) were evaluated with fluorescent microscopy and 90 were found smear-positive. Of the remaining smear-negative patients (511), 81 were excluded because only one sample was examined, leaving 430 patients with smear-negative results after examination of the second sample. Of the 430 smear- negative patients, 364 of the 412 had complete results to fill the $2 \times 2$ table for calculation of accuracy for MODS; 366 of the 415 for NRA; and 369 of the 411 for Xpert. In regard to MGIT, of the 430 patients, 373 patients had valid results used for calculation of the index test accuracy, of which 43 detected MTB growth. Index test results were considered invalid if they were contaminated or lacked a corresponding valid reference culture result. Reference culture results were considered invalid if they were contaminated on both MGIT and L–J, or if positive, had NTM growth. The demographic characteristics of the 430 smear-negative patients are summarized in Table 1.

### *Test accuracy*

The sensitivity and specificity were 24.4% and 98.1% for MODS, 41.5% and 92% for NRA, 48.8% and 95.1% for Xpert, respectively. The positive predictive values ranged from 39.5% for NRA to 62.5% for MODS. All three tests exhibited high negative predictive values of above 90%. The summary of the accuracy of the tests is shown in Table 2.

## Discussion

Poor diagnosis of TB is a major obstacle to TB treatment and control, especially in areas where smear microscopy generally is the only diagnostic tool. The situation is worse in HIV patients because the sensitivity of microscopy is even lower in these patients. Since 2007, new TB diagnostics have been developed, and WHO has endorsed some of them for rapid and increased TB diagnosis. The accuracy was evaluated of two non-commercial rapid culture tests (MODS and NRA) and one molecular test (Xpert), in detection of smear-negative TB in HIV patients. The study focused on smear-negative TB

**Table 2 – Sensitivity, specificity, positive and negative predictive value of the MODS, NRA and Xpert against MGIT culture as reference, including 95% confidence intervals.**

| | | MGIT | | | | | |
|---|---|---|---|---|---|---|---|
| Test | | Positive | Negative | Sensitivity (95% CI) | Specificity (95% CI) | PPV% | NPV% |
| MODS | Positive | 10 | 6 | 24.4 (12.4–40.3) | 98.1 (96–99.3) | 62.5 | 91.1 |
| | Negative | 31 | 317 | | | | |
| NRA | Positive | 17 | 26 | 41.5 (26.3–57.9) | 92 (88.5–94.7) | 39.5 | 92.6 |
| | Negative | 24 | 299 | | | | |
| Xpert | Positive | 21 | 16 | 48.8 (33.3–64.5) | 95.1 (92.2–97.2) | 56.8 | 96.3 |
| | Negative | 22 | 310 | | | | |

because of the pronounced difficulty in diagnosing this form of TB without conventional culture.

Currently the MODS and NRA are only endorsed for diagnosis of multi-drug resistant TB (MDR-TB) either as direct or indirect tests. The MODS and NRA were selected because the two tests were found to be highly accurate and the easiest to adopt in this setting in an earlier study that evaluated smear-positive patient population [18]. On the other hand, Xpert was selected because it is the only rapid molecular test which the WHO has endorsed for TB diagnosis in patient populations who are infected with HIV or are smear-negative on microscopy. Indeed, there are currently major efforts to roll out the Xpert in selected recipient countries, a number of them from high HIV-prevalent and resources-constrained settings [19].

A recent survey of published literature showed that information about the accuracy of these three tests in the diagnosis of smear-negative TB in HIV patients is currently still inadequate [20,21]. This study provides new information which would be useful for developing new diagnostic algorithms and define the role of MODS and NRA in TB/HIV management. This study also provides additional experience on the performance of the tests as a follow-up to microscopy in a high HIV-prevalent African population. In turn, this information could provide guidance for the further roll-out of the tests for improved diagnosis of HIV-associated TB, especially for the smear-negative form.

The sensitivity of MODS was low (24.4%). The sensitivity was better, but still low, for the NRA (41.5%) and Xpert (48.8%). All the tests exhibited relatively low and widely varied positive predictive values, from 39.5% for NRA to 62.5% for MODS. On the other hand, all the tests exhibited high specificity and negative predicative values of above 90%, (Table 2).

The Xpert has previously been reported to detect approximately 70% of smear-negative TB [10]. However, there is growing evidence from reports of studies conducted in high HIV African settings similar to this study setting, indicating that sensitivity could be lower, ranging from 40% to 50% [22–24]. The researchers of this study are unaware of studies that have evaluated MODS and NRA for diagnosis of smear-negative TB in HIV patients, although one recent study on MODS from India reported sensitivity of 38% for smear-negative TB. The report, however, did not clearly describe the patient population [25].

A possible explanation for the low sensitivity of the tests is the advanced immune deficiency of the study patients in these settings whose median CD4 count was 280/mm$^3$ in this study (Table 1). Patients with advanced HIV disease usually have few bacilli in their sputum, which could be below the detection limits of the tests [9,26]. No difference was seen in the CD4 count of the patients with positive and negative MGIT culture for each of the tests when compared. Therefore, the effect of immune suppression was uniform for all the three tests (data not shown).

It is also likely that the MODS and NRA had a low sensitivity because samples were examined after batching weekly following storage in a fridge (4–8 °C). Some loss of viability could therefore have occurred. On the other hand, it has been shown previously that there is no such effect on Xpert [27,28], which is not dependent on live bacteria for detection. This study did not determine the effect of this for the MODS and NRA. Further, ideally, aliquots of the stored samples should also have been inoculated into MGIT. However, since there were different technicians that performed the tests, it was not possible to perform all the tests at the same time. The feasible practice was to process the collected sputum, inoculate on culture and batch the rest of the samples for later testing weekly. This was the only possible workflow that could be implemented in the laboratory. In addition, the MODS and NRA were initially examined up to day14 and later up to day 21, whereas MGIT and L–J were examined up to 42 and 56 days, respectively. It is likely that more days were required before interpreting the two tests as negative. However, it was considered that a period beyond day 14 could limit the timeliness of these two tests for patient care. The timeliness would even be more limited if the reading time got beyond day 21. Lastly, because fluorescent microscopy was used, which is more sensitive than the commonly used Ziehl-Neelsen (ZN) method to determine smear-negative specimens [29], it is likely that in settings where ZN is the routine microscopy method for TB diagnosis, the tests could have been found to be more sensitive.

There were 68 patients with positive culture on MGIT, of which 38% (25/68) were identified as NTM. Although recovery of NTM is expected with liquid culture systems, the clinical implications of this have not been evaluated in this setting. This is important since microscopy, which is the main diagnostic tool for TB, cannot differentiate between MTB and NTM. Therefore, patients infected with NTM could mistakenly be labeled as "chronic" TB cases as they may not respond to standard TB treatment. Such a scenario has been recognized previously from Honduras [30].

### *Implications of study findings for practice*

Since the sensitivity and the positive predictive values of the tests were relatively low, this implies that additional diagnostic tools such as chest X-ray and conventional culture could still be critical for a sensitive diagnosis of smear-negative TB in this population and setting. It should be considered if, for example, "smear and Xpert negative" TB suspects would need further evaluation by conventional culture. However, this may be difficult in practice because of the resource constraints faced in many low-income settings. Thus, initiation of inappropriate TB medications and the associated side effects would be avoided. For HIV patients, there is additional benefit as this implies that they can receive other HIV services such as isoniazid preventive therapy for TB [31,32], and reduce the need to make several visits to the clinic for investigations for TB. This would only be necessary if symptoms persist.

### *Strength of the study*

The strength of this study is that it was performed under real clinical settings, where patients were enrolled prospectively and consecutively using pre-set inclusion and exclusion criteria. To further reduce bias from sample selection, sputum samples were also pooled during processing, although this may not be replicated as standard practice. Secondly, a strategy to back up MGIT with L–J culture was used. This reduced

loss of culture results due to MGIT contamination to 7% in this study compared with an earlier reported range between 8% and 15% [33].

## Conclusion

While the Xpert, MODS and NRA have been developed for use as frontline tests for rapid TB diagnosis, this study found their sensitivity for the detection of TB in smear-negative HIV patients to be low. Additional diagnostics such as chest X-ray and liquid culture could therefore still be required for TB diagnosis in this population. Even though the sensitivity was unfortunately low for all three tests, they showed a high specificity and were thus useful to correctly confirm TB in sputum smear-negative HIV patients with suggestive TB symptoms.

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