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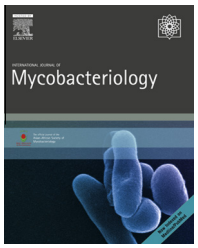

Full Length Article

Increased detection of smear-negative pulmonary tuberculosis by GeneXpert MTB/RIF® assay after bleach concentration

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ABSTRACT

Objective/background: The GeneXpert MTB/RIF assay (Xpert) was endorsed as the initial diagnostic tool in people suspected of human immunodeficiency virus-associated or drug-resistant tuberculosis (TB). However, information regarding the performance of Xpert for diagnosing smear-negative TB in high burden settings remains limited. We evaluated the diagnostic accuracy of Xpert and the impact of bleach concentration on the performance of Xpert using smear-negative sputum samples from human immunodeficiency virus-negative patients.

Methods: One spot and one morning smear-negative sputum samples per patient were examined using Xpert and culture at the Mycobacteriology Research Center of Jimma University, Ethiopia. The sputum culture on both Löwenstein–Jensen and/or Mycobacteria Growth Indicator Tube was the gold-standard.

Results: Of 185 smear-negative presumptive pulmonary TB cases, 19 (10.3%) had culture-proven TB. The sensitivity of Xpert on spot and morning sputum was similar (63.2%). Testing two specimens per patient insignificantly increased the sensitivity of Xpert. Bleach concentration and pelleting improved the sensitivity of Xpert over unprocessed sputum in paired samples (73.8% vs. 63.2%) without affecting the specificity (95%). Bleach concentration and pelleting allowed an additional seven cases of TB (missed on the first and second direct Xperts) to be detected, five of which were from culture-negative cases.

Conclusion: Testing of a single sputum sample by Xpert can reach reasonable sensitivity and results would be available on the same day, avoiding loss of patients and treatment delay. The sensitivity of Xpert was improved after bleach concentration and pelleting, although its added value needs further study on a larger scale.

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Introduction

Tuberculosis (TB) remains a major public health problem, accounting for 9 million incident cases and 1.5 million deaths worldwide [1]. Ethiopia ranks 10th among the 22 countries with a high TB burden [2,3]. Recently, the number of registered smear-negative TB cases transcended the smear-positive cases. This is a peculiar situation seen in Ethiopia for over a decade. In the national TB prevalence survey in 2010/11 smear-negative cases accounted for 57% of culture-positive cases [4]. Diagnosis of smear-negative pulmonary TB remains a challenge [3,5,6]. This is because of the lack of rapid and accurate diagnostic modalities that can be applied in resource-limited settings [3,7].

The GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA; hereafter referred to as Xpert) detects the presence of MTB complex (MTBC) and its resistance to RIF in a single reaction [8,9]. Xpert is an integrated fully automated specimen processing and nucleic acid-amplification test [8,10], strongly recommended by World Health Organization for the diagnosis of human immunodeficiency virus (HIV)-associated TB and multidrug-resistant TB [11]. In clinical evaluation studies, the sensitivity of Xpert in patients with smear-negative pulmonary TB was reported to be moderate, 55–86%, compared with 99–100% in patients with smear-positive TB [12–14]. This urges for a simple and efficient way to enhance the sensitivity of Xpert for detection of MTB in smear-negative patients.

House hold sodium hypochlorite (NaOCl) or bleach has been used for over a century to increase the yield of microscopic detection. Bleach at 5% concentration can digest the sputum products and inactivate the mycobacteria without altering their structures. This provides a greater safety for laboratory use. Further centrifugation or sedimentation concentrates the acid fast bacilli (AFB) in the mixture, increasing the rate of positivity [15]. In the current study, we primarily evaluated the diagnostic accuracy of direct Xpert test in smear-and HIV-negative patients. Secondly, we determined whether sputum processing using bleach combined with simple centrifugation can increase the sensitivity of Xpert in resource poor settings.

Materials and methods

Study participants

A cross-sectional prospective study was carried out at Jimma University Specialized Hospital, a public tertiary care hospital, in Southwest Ethiopia. Adult consecutive patients with presumptive pulmonary TB presenting at the health care facility were screened between February 2014 and August 2014. Inclusion criteria were based on the World Health Organization case definition for presumptive pulmonary TB [16] and included patients having a persistent cough for at least 2 weeks with or without one of the following: night sweats, unintentional weight loss, fever, chest pain, shortness of breath, loss of appetite, and contact with a TB patient.

Consenting patients were enrolled only if they had three sputum-negative results on smear microscopy, were HIV-negative, and could provide detailed clinical history along with adequate amount of sputum specimen. Demographic and clinical characteristics were collected through interview by using a pretested questionnaire. This study was approved by institutional review boards of Jimma University, Ethiopia (reference number: RPGC/510/2014). All participants gave written consent for use of routine clinical data for research purposes.

Sputum sample processing

A portion of the collected sputum specimen was used immediately for direct smear microscopy and the remaining portion was stored at 4 °C in a refrigerator for culture and Xpert. HIV test results were collected from the medical records after obtaining written consent from the clients. One spot and one morning sputum sample collected from each presumptive TB case were brought to room temperature and liquefied by N-acetyl L-cysteine (NALC) powder. Each sputum sample was then divided into two aliquots, which were carefully labeled and assayed independently in a blinded manner by a second person. The first aliquot of spot sputum was assayed for direct Xpert and the other aliquot for culture (Mycobacteria Growth Indicator Tube [MGIT 960] and Löwenstein–Jensen [LJ] medium). Likewise, the first aliquot of morning sputum was tested for direct Xpert and the second for bleach-concentrated Xpert.

Direct Xpert

Xpert was performed as previously described [8]. Sample reagent was added to the sputum specimens in a 2:1 ratio in a sterile falcon tube. The solution was vortexed and left to settle for 15 min, with vortexing halfway through. The supplied sterile transfer pipette was used to draw the liquefied sample up to the marked line (corresponding to 2 mL) and transferred to a cartridge. The Xpert cartridge was then loaded onto the Xpert machine (Cepheid, Dx System Version 4.0c). Results were reported as positive or negative for MTBC. RIF resistance results were reported as susceptible, resistant, or indeterminate.

Sputum culture and identification

Mycobacterial culture was done in MGIT 960 and on LJ medium. Sputa were decontaminated by the standard N-acetyl-L-cysteine and sodium hydroxide (NALC/NaOH) method with a final NaOH concentration of 1% [17]. An equal volume of standard NALC/NaOH solution was added to the specimen and incubated for 15 min at room temperature. After centrifugation for 15 min at 3000*g*, the sediment was resuspended in 1 mL of sterile phosphate buffered saline (PBS; pH 6.8). The resulting pellet was used to inoculate a MGIT 960 and LJ tube. The MTB $H_{37}Rv$ reference strain (*American Type Culture Collection* 27294) was processed with each run as a positive control. All positive cultures were confirmed for MTBC with SD Bioline MPT64TB Ag test (Standard Diagnostics, Yongin, South Korea).

Bleach-concentrated sputum for Xpert

Xpert from bleach-treated and concentrated sputum was carried out according to the optimized protocol in the validation experiment of this study (described hereunder). Briefly, the second part of the morning specimen was transferred to a conical centrifuge tube (15 mL; Sarstedt, Nümbrecht, Germany) and treated with an equal volume of 5% bleach (Chora Gas and Chemical Products Factory, Addis Ababa, Ethiopia). After mixing, the tube was left for 15 min at room temperature and shook for 30 s every five minutes. The PBS was added up to 15 mL and centrifuged at 3000*g* for 15 min using a simple centrifuge (a low-cost table-top centrifuge that is available in peripheral laboratories of Ethiopia). After centrifugation the supernatant was decanted carefully and the sediment was resuspended with 1 mL of sterile PBS (pH = 6.8). The sample reagent was added in a 3:1 ratio and shaken vigorously for 15 s. Subsequently, the procedure described for direct Xpert was followed [8].

Statistical analysis

Data were analyzed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Sensitivity, specificity, positive predictive value, and negative predictive value of Xpert and their respective 95% confidence intervals (CI) were calculated using a culture as reference standard. A positive culture result was defined as growth on LJ and/or in MGIT identified as MTB. We excluded data of patients with contaminated sputum culture results and indeterminate Xpert results from the diagnostic accuracy analysis. The study reporting conforms to the Standards for Reporting Diagnostic Accuracy guidelines for diagnostic accuracy reporting (www.stard-statement.org/).

Validation experiments

Effect of bleach-sputum processing on Xpert

The protocol was based on the pretreatment of the sputum sample with 5% bleach. It is known that house hold bleach (sodium hypochlorite) can digest and liquefy mucus and debris in sputum [15]. This releases the TB bacilli, which can be further concentrated by centrifugation. Two experiments were conducted to check whether the pretreatment of sputum with bleach could have an interfering effect on Xpert result.

In the first experiment, a total of 10 AFB-negative sputum samples ($n = 10$) spiked with the MTB $H_{37}Rv$ reference strain were included. Briefly, $H_{37}Rv$ was cultured on LJ medium. Using a sterile wire loop, three to five well-isolated colonies were emulsified in 3 mL of sterile physiological saline (0.9% weight/volume). The bacterial suspension's turbidity was matched with that of McFarland Number 0.5 (approximately 1×10^8 colony forming units/mL). This suspension was spiked into 2 mL of liquefied (kept overnight at room temperature) smear-negative sputum from a healthy volunteer. Each spiked sample was divided in two groups: one received the treatment with 5% bleach followed by centrifugation (3000*g* for 15 min) and the other did not receive any treatment with bleach, nor centrifugation. Bleach-treated and untreated samples were analyzed by Xpert (as per the manufacturer's instruction). Positive results were placed in one of four categories: very low, low, medium, or high based on the quantitative cycle threshold (C_t) value of probe A.

The experiment was expanded by testing known smear-positive sputum specimens ($n = 9$). The sputum samples were kept at room temperature for 24 h to allow liquefaction. This enabled us to split the sample into two equal parts (approximately 1 mL each). As in the case of spiked sputum, the first part was treated with 5% bleach and the other left untreated. Bleach-treated and bleach-untreated samples were tested with Xpert.

Validation results

A total of 19 paired (bleach-treated and bleach-untreated) samples were analyzed with Xpert. The test result showed that all bleach-untreated samples (10 $H_{37}Rv$ spiked smear-negative and nine smear-positive sputa) and 18 bleach-treated samples (nine $H_{37}Rv$ spiked smear-negative and nine smear-positive sputa) were Xpert positive. The result for one of the bleach-treated spiked sputum samples was uninterpretable (error). The mean C_t value was 13.77 for bleach-untreated and 14.90 for bleach-treated specimens. Bleach-treatment and centrifugation did not seem to affect *Ct* values (*t*-test values of 0.800 and 0.460 for spiked and naturally infected sputa; Table 1). We concluded that 5% bleach did not have an interfering effect on Xpert test results, although more studies are warranted to further validate the protocol.

Results

Characteristics of study participants

During the study period, a total 326 consecutive adult patients with presumptive pulmonary TB were screened. One hundred twenty-four patients were excluded from the study (56 were HIV-positive/unknown, 30 were smear positive, 19 provided a sample with inadequate volume, 13 did not provide three sputa, and six had missing AFB-smear results). Of the remaining 202 presumptive TB patients who had smear-negative sputum, 17 patients were excluded (13 culture contaminated and four Xpert MTB/RIF indeterminate results), leaving 185 patients for the final analysis. Overall study flow diagram is shown in Fig. 1. The majority, 61.6% (114/185), of study participants were men. The median age of patients was 38 years (inter-quartile range 23–55). The study participant characteristics are shown in Table 2.

Diagnostic accuracy of direct Xpert

Nineteen (10.3%) of 185 smear-negative presumptive cases had culture (LJ and/or MGIT) confirmed TB. Among the 185 screened, 7.6% tested positive on the first Xpert and an additional 1.6% on the second Xpert, if no pretest bleach treatment was performed. Using mycobacterial culture as the reference standard, the sensitivity of Xpert in spot and morning sputum was the same (63.2% [95% CI: 41.5–84.8]). However, when results of both spot and morning Xpert were considered

Table 1 – Pre-study Xpert validation results using paired artificially spiked smear-negative and naturally smear-positive sputum samples with or without pre-test bleach treatment.

Code	Sputum appearance	Bleach untreated		Bleach treated		Paired *t*-test
		Result	C_t value	Result	C_t value	*p*
H37Rv spiked sputum						
3830	Purulent	Positive	13.4	Positive	16.6	.080
3854	Bloody	Positive	14.8	Positive	14.7	
3830	Purulent	Positive	13.7	Error	–	
3864	Muco purulent	Positive	14.9	Positive	15.6	
3852	Purulent	Positive	12.9	Positive	12.5	
3830	Purulent	Positive	15.6	Positive	17.4	
3854	Purulent	Positive	15.6	Positive	16.3	
3839	Muco purulent	Positive	19.7	Positive	19.1	
3846	Muco purulent	Positive	9.7	Positive	10.9	
3846	Highly mucoid	Positive	10.4	Positive	16.5	
Smear positive sputum						
3558	Muco purulent	Positive	15.8	Positive	20.5	.460
3560	Purulent	Positive	16.2	Positive	14.3	
3577	Muco purulent	Positive	10.6	Positive	10.9	
3608	Muco purulent	Positive	13.5	Positive	12.7	
3612	Purulent	Positive	12.7	Positive	16.6	
3669	Purulent	Positive	10.0	Positive	9.0	
3778	Muco purulent	Positive	12.6	Positive	15.2	
3788	Muco purulent	Positive	13.9	Positive	18.4	
3836	Purulent	Positive	15.7	Positive	11.0	

Note: C_t = cycle threshold.

together, the sensitivity (68.4% [95% CI: 47.5–89.3]) slightly increased compared with either test alone, although the difference was not statistically significant (*p* value = .76). Among 166 culture-negative cases, two spot and four morning sputa were Xpert positive. The estimated specificity of Xpert was 98.8% in spot and 97.6% in morning sputum samples. A summary of the overall Xpert performance is depicted in Table 3.

Performance of Xpert on bleach-concentrated sputum

Twenty-two (12%) of the 185 patients with smear-negative sputum were positive using bleach-concentrated Xpert, with eight of them (36.4%) being culture negative. The sensitivity of a single direct Xpert for culture-confirmed TB was 63.2% (95% CI: 41.5–84.8) and rose to 73.8% (95% CI: 58.6–97) for bleach-treated samples, which is also higher than the sensitivity when testing two samples by direct Xpert (68.4% [95% CI: 47.5–89.3]). Bleach-concentration and pelleting allowed an additional seven cases of TB (missed on the first and second direct Xperts) to be detected, five of which were from culture-negative cases.

The specificity of Xpert was not significantly affected by bleach concentration and pelleting: 95.1% (95% CI: 92–98.4) for bleach-concentrated sputum versus 98.8% (95% CI: 97–100) and 97.6% (95% CI: 95–100) for direct Xpert in spot and morning sputa (*p* = .92). However, the positive predictive value was lower for bleach-processed sputum (63.6% [95% CI: 43.5–83.7]) compared with unprocessed spot (85.7% [95% CI: 63.4–100]) and morning (75% [95% CI: 53.8–96.2]) sputa (data not shown). There were five patients, whose cultures grew nontuberculous mycobacteria (NTM). One of these patients had a positive Xpert after bleach concentration and pelleting. There was no RIF resistance detected in any of the sputum samples tested by Xpert.

Indeterminate Xpert results

Higher numbers of invalid Xpert results were observed when using bleach-concentrated sputum samples compared with unprocessed samples (1.5% [3/202] vs. 0.3% [1/404]). Overall, Xpert was indeterminate in four of 606 tests performed (0.6%), a rate that was much lower than the overall culture-contamination rate of 5% (20 of 404 cultures, seven on both media, four only in MGIT, and two only on LJ). Three Xpert-indeterminate samples were culture negative and one was culture positive.

Discussion

Smear-negative pulmonary TB constitutes a major burden of undiagnosed TB in resource poor settings [7]. It is associated with poor treatment outcomes, including death due to delayed diagnosis or nondiagnosis [3,7]. Multiple studies have consistently shown that Xpert can identify a substantial proportion of smear-negative TB patients, particularly in HIV coinfected patients [9,12,18]. There is a paucity of data on the diagnostic accuracy of the Xpert in smear- and HIV-negative patients in areas of high TB prevalence such as Ethiopia.

Our study suggests that application of Xpert on a single smear-negative sputum specimen can substantially increase the yield of confirmed TB cases. This is consistent with those reported by others regarding the effectiveness of Xpert in accurately detecting the presence of MTB in smear-negative

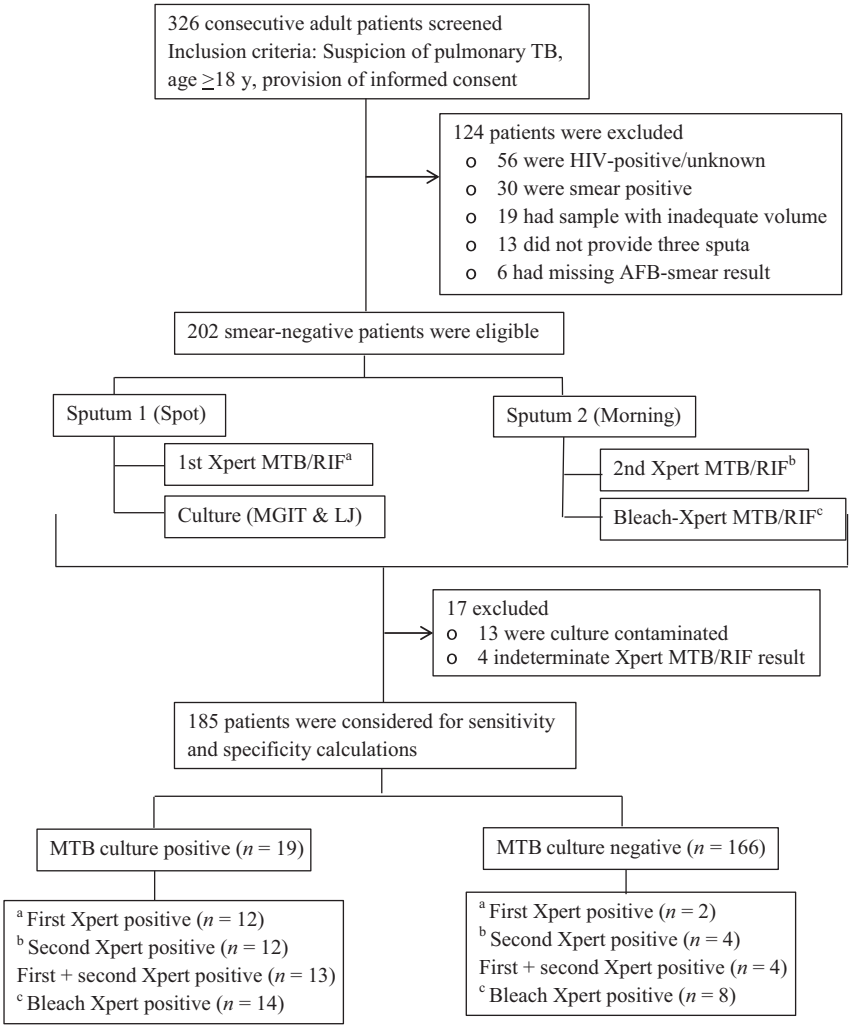

Fig. 1 – Overall study flow diagram explaining participants' recruitment, sample processing, and diagnostic test results. *Note*. AFB = acid fast bacilli; HIV = human immunodeficiency virus; LJ = Löwenstein–Jensen; MGIT = Mycobacteria Growth Indicator Tube; MTB = *Mycobacterium tuberculosis*; RIF = Rifampicin; TB = tuberculosis; y = year. [a]Xpert test on spot sputum. [b]Xpert test on morning sputum. [c]Xpert test on bleach-treated and concentrated sputum.

Table 2 – Demographic and clinical characteristics of the 185 study Participants.

Characteristics	Smear-negative presumptive pulmonary TB cases			*p*
	All patients (*n* = 185)	Culture positive (*n* = 19)	Culture negative (*n* = 166)	
Age, median (IQR)	38 (23–55)	32 (21–47)	40 (26–56)	.27
Male sex	114 (61.6%)	14 (73.7%)	100 (60.2%)	.25
Taking antibiotics in the last 2 wk	106 (57.3%)	11 (58%)	95 (57.2%)	.95
TB contact history in the last 2 y	38 (20.5%)	6 (31.6%)	32 (19.3%)	.21
Presenting TB symptom				
Cough > 4 wk	93 (50.3%)	13 (68.4%)	80 (48.2%)	.09
Chest pain	134 (72.4%)	16 (84.2%)	118 (71%)	.22
Night sweats	131 (70.8%)	14 (73.7%)	117 (70.5%)	.77
Fever	75 (40.5%)	9 (47.4%)	66 (39.8%)	.52
Weight loss	111 (60%)	15 (79%)	96 (58%)	.07
Shortness of breath	123 (66.5%)	12 (63.2%)	111 (67%)	.75
Loss of appetite	129 (69.7%)	15 (79%)	114 (68.7%)	.35

Note: IQR = interquartile range; TB = tuberculosis; wk = week; y = year.

Table 3 – Diagnostic Performance of Xpert MTB/RIF assay compared with culture as a reference standard, stratified to the type of sample or number of samples tested per patient.

Index test (Xpert MTB/RIF)	Reference standard		Sensitivity (95% CI)	Specificity (95% CI)
	Positive	Negative		
One spot sputum				
Positive	12	2	63.2% (41.5–84.8)	98.8% (97.1–100)
Negative	7	164		
One morning sputum				
Positive	12	4	63.2% (41.5–84.8)	97.6% (95.3–99.9)
Negative	7	162		
Bleach-concentrated pellet (morning)				
Positive	14	8	73.8% (53.8–93.5)	95% (91.9–98.4)
Negative	5	158		
Two sputa (spot + morning)				
Positive	13	4	68.4% (47.5–89.3)	97.6% (95.3–99.9)
Negative	6	162		
Three sputa (2 direct + 1 pellet)				
Positive	15	9	78.9% (60.6–97.3)	94.6% (91.1–98.0)
Negative	4	157		

Note: *Mycobacterium tuberculosis* culture positivity was used as reference standard, defined as identification of *M. tuberculosis* in at least one positive standard sputum culture (either on Löwenstein–Jensen slopes or Mycobacteria Growth Indicator Tube culture). CI = confidence interval.

specimens [18,19]. The observed sensitivity of a single Xpert (63.2%) for smear-negative TB is comparable with reported sensitivities ranged from 61% to 71.7% [13,18,20,21]. Repeating Xpert test using a different sputum specimen per patient insignificantly increased the sensitivity. In high TB prevalence settings such as Ethiopia, repeating Xpert test may increase laboratory workload and expenses, though further study is warranted to ascertain the incremental yield from the second Xpert test.

There is no published data to the best of our knowledge that evaluated the performance of Xpert using bleach-concentrated sputum samples. Previous studies demonstrated nonsignificant improvement in sensitivity of Xpert when sputum samples were centrifuged [22,23]. We documented an increased sensitivity by simple bleach concentration and pelleting of sputum samples, allowing the detection of seven cases who would have been missed by two direct Xperts. Similar to most previous studies performed the specificity was high for Xpert, ranging from 97% to 100% [13,23,24]. The difference in specificity of Xpert after bleach concentration and pelleting was not statistically significant from direct testing. Sodium hypochlorite (NaOCl) is cheap and available almost anywhere as household bleach. As a potent disinfectant, NaOCl also has the advantage of limiting the risk of laboratory infection. In addition, the relative centrifugal force needed for concentration of mycobacteria can easily be achieved by low-cost table-top centrifuge affordable under existing conditions of most TB laboratories in developing countries equipped with an Xpert device. However, bleach concentration and pelleting increased the rate of Xpert indeterminate test results. This might be due to the sputum being overconcentrated after centrifugation, generating viscous mixture debris and the NaOCl blocking the channels in the cartridge.

In our study, Xpert on bleach-concentrated sputum detected eight cases from smear-negative TB suspects which were not picked up by either of the culture methods. Similarly, in the study by Rachow and colleague [18], 9% of culture-negative but Xpert-positive patients were classified as clinical TB cases, with documented positive responses to anti-TB treatment. Unfortunately, due to logistic constraints, the eight cases in our study were not followed. As it is of paramount importance to ascertain that these cases detected only by Xpert in bleach-concentrated sputum are unambiguously true TB cases, a more thorough clinical evaluation study which specifically addresses such cases is warranted. Among culture-positive cases four were identified as NTM using the SD Bioline MPT64TB Ag test, one of which was positive on Xpert after bleach concentration. Previous studies by Moure et al. [13] and Marlowe et al. [25] reported 100% specificity of Xpert with 20 and 41 NTM samples, respectively. In the current study, due to limited resources, differentiation of NTM from MTBC was done by SD Bioline MPT64TB Ag test. It is possible that due to low MPT64 antigen expression by some lineage of MTBC, the SD Bioline MPT64TB Ag test can be false negative. If this is assumed true, the specificity of Xpert for NTM would be 100% in our study.

We documented a moderate sensitivity (63.2%) of single direct Xpert among smear-negative culture-positive cases. Xpert identified majority of the culture confirmed cases with high specificity. This gives the clinician sufficient confidence to initiate anti-TB treatment when Xpert is positive. A negative Xpert does not exclude a diagnosis of smear-negative TB given the fact that the test was unable to identify 36.8% of patients with culture confirmed TB. Patients with a high clinical probability of TB despite a negative Xpert should be started on anti-TB treatment. It is important to ensure that clinicians are aware of the Xpert limitations prior to its implementation. Despite this, Xpert has distinct advantages over culture; a faster turn-around time, providing same-day diagnosis which could potentially limit loss to follow-up during diagnostic evaluation of smear-negative TB patients.

This study has some limitations. Firstly, we did not document treatment outcomes of the patients. It is not clear how many of the patients testing positive for MTB by Xpert in this study would have been started on TB treatment based on the clinicians' decision if Xpert testing was not available. Among culture- and Xpert-negative cases there may be false-negative cases that started anti-TB treatment on clinical grounds and improved—cases that were most likely true TB. Secondly, all sputum samples received NALC-pretreatment which could have some effect on direct and bleach-concentrated Xpert results. Lastly, although the sensitivity of the Xpert in this study was improved on bleach-concentrated sputum sample compared with direct sputum, we had insufficient power to determine whether this difference was statistically significant.

In conclusion, our results suggest that direct Xpert can rapidly diagnose TB in smear-negative patients with modest sensitivity and excellent specificity. Smear-negative TB patients could benefit from Xpert particularly in those areas where no culture is available. Testing of single sputum by Xpert can reach reasonable sensitivity and results would be available on the same day, avoiding loss of patients and treatment delay. Moreover, bleach concentration and pelleting of sputum samples improves the sensitivity of Xpert, probably without affecting the specificity significantly. Further prospective studies including clinical outcome data (such as response to treatment) with a larger sample size are required to clarify these findings.

Conflicts of interest

We, the authors, declare that no conflict of interest exists.

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