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Rapid Detection of *Mycobacterium tuberculosis* and Rifampin Resistance by Use of On-Demand, Near-Patient Technology▽†‡

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Current nucleic acid amplification methods to detect *Mycobacterium tuberculosis* are complex, labor-intensive, and technically challenging. We developed and performed the first analysis of the Cepheid Gene Xpert System's MTB/RIF assay, an integrated hands-free sputum-processing and real-time PCR system with rapid on-demand, near-patient technology, to simultaneously detect *M. tuberculosis* and rifampin resistance. Analytic tests of *M. tuberculosis* DNA demonstrated a limit of detection (LOD) of 4.5 genomes per reaction. Studies using sputum spiked with known numbers of *M. tuberculosis* CFU predicted a clinical LOD of 131 CFU/ml. Killing studies showed that the assay's buffer decreased *M. tuberculosis* viability by at least 8 logs, substantially reducing biohazards. Tests of 23 different commonly occurring rifampin resistance mutations demonstrated that all 23 (100%) would be identified as rifampin resistant. An analysis of 20 nontuberculosis mycobacteria species confirmed high assay specificity. A small clinical validation study of 107 clinical sputum samples from suspected tuberculosis cases in Vietnam detected 29/29 (100%) smear-positive culture-positive cases and 33/39 (84.6%) or 38/53 (71.7%) smear-negative culture-positive cases, as determined by growth on solid medium or on both solid and liquid media, respectively. *M. tuberculosis* was not detected in 25/25 (100%) of the culture-negative samples. A study of 64 smear-positive culture-positive sputa from retreatment tuberculosis cases in Uganda detected 63/64 (98.4%) culture-positive cases and 9/9 (100%) cases of rifampin resistance. Rifampin resistance was excluded in 54/55 (98.2%) susceptible cases. Specificity rose to 100% after correcting for a conventional susceptibility test error. In conclusion, this highly sensitive and simple-to-use system can detect *M. tuberculosis* directly from sputum in less than 2 h.

An alarming increase in the global incidence of drug-resistant *Mycobacterium tuberculosis* infection has created a critical need for methods that can rapidly detect *M. tuberculosis* and identify drug-resistant cases (53). Failure to quickly and effectively recognize and treat patients with drug-resistant tuberculosis (TB), particularly multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis, leads to increased mortality, nosocomial outbreaks, and resistance to additional antituberculosis drugs (14, 37). However, MDR and XDR tuberculosis can be effectively treated if properly identified (35). A number of new diagnostic approaches have brought incremental improvements in detection and drug susceptibility testing (2, 9, 19, 24, 37, 41, 46); however, none can realistically provide actionable information within the time frame of a single office or clinic visit. Thus, despite technical advances, rapid diagnostics have not yet been able to have an impact on critical initial decisions regarding hospitalization, isolation, and the choice of treatment regimens for suspected tuberculosis patients.

Previously, we showed that direct molecular detection of *M. tuberculosis* and rifampin resistance could be accomplished simultaneously (27); more recently, our group developed a single-tube, molecular beacon-based real-time PCR assay for the detection of rifampin-resistant *M. tuberculosis* (42, 43). Mutations in the 81-bp rifampin resistance-determining region (RRDR) of the *rpoB* gene, which occur in 95 to 98% of all rifampin-resistant strains (and which are almost invariably ab-

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sent in rifampin-susceptible strains), were detected by five overlapping molecular beacons (34). The assay proved to be simple, rapid, specific, and highly sensitive in tests on isolates of *M. tuberculosis* from New York City, Madrid (42), India, and Mexico (51). As most rifampin-resistant isolates are also resistant to isoniazid, rifampin resistance can be used as a marker for MDR *M. tuberculosis* (36, 44, 49). However, like all nucleic acid amplification-based assays for *M. tuberculosis* detection (15), this assay was too complex and too prone to operator errors, sample cross-contamination, and biohazards for rapid near-patient use.

The Cepheid GeneXpert System (Sunnyvale, CA), a single-use sample-processing cartridge system with integrated multicolor real-time PCR capacity (45), has the potential to greatly simplify nucleic acid amplification tests. Here, we utilized this new technology to develop an on-demand, near-patient PCR assay that employs a novel six-color dye set to detect *M. tuberculosis* and identify rifampin resistance as a surrogate for MDR directly from a patient's sputum in less than 2 h. The many features of this system, including sample decontamination, hands-free operation, on-board sample processing, and ultrasensitive hemi-nested PCR, enabled us to create a low-complexity assay with a sensitivity that approached certain culture methods. This type of assay may prove to be useful in the initial management of suspected tuberculosis cases in both the United States and the world at large.

MATERIALS AND METHODS

Assay components. The GeneXpert Dx System (Cepheid, Sunnyvale, CA) is an integrated diagnostic device that performs sample processing and real-time PCR analysis in a single hands-free step. The Xpert MTB/RIF assay consists of two main components: (i) the Xpert MTB/RIF plastic cartridge, which contains liquid sample-processing and PCR buffers and lyophilized real-time PCR reagents; and (ii) the GeneXpert instrument, which controls intracartridge fluidics and performs real-time PCR analysis (45). The Xpert MTB/RIF assay was designed to amplify a sequence of the *rpoB* gene specific to members of the *M. tuberculosis* complex and to probe for mutations within the RRDR of the *rpoB* gene. Sequences of *M. tuberculosis rpoB* primers and *rpoB*-specific molecular beacons were modified from those described previously (11) to allow use of heminested PCR, to minimize cross-amplification of nontuberculosis mycobacterium (NTM) species, and to maximize mutation detection (see Table S1 in the supplemental material). A heminested molecular beacon assay to detect *Bacillus globigii* DNA was also included in the cartridge. This second assay tests for the presence of *B. globigii* spores, which are included in the Xpert MTB/RIF cartridge to serve as an internal control for sample processing and PCR (see Methods in the supplemental material). Fluorescent dyes and quenchers were developed to allow all six molecular beacons to be multiplexed within the same reaction.

Analytic studies. Analytic studies on DNA were performed by mixing known numbers of *M. tuberculosis* chromosomal DNA molecules with PCR reagents and adding the mixture to a chamber of the cartridge normally designated to receive eluted DNA from lysed *M. tuberculosis* cells in the full sputum-processing protocol.

Analytic studies of sputum were performed by spiking known numbers of bacterial CFU into discarded excess sputa, originally submitted for routine Gram staining and bacterial culture, from patients not suspected of tuberculosis at several clinical microbiology laboratories in the United States. Previously quantified frozen aliquots of *M. tuberculosis* strain H37Rv were used in all analytic studies unless otherwise indicated. In the limit of detection (LOD) studies, five different concentrations of *M. tuberculosis* cells were each spiked into 20 individual *M. tuberculosis*-negative sputum samples to final concentrations ranging from 10 to 300 CFU/ml. The sputum was then treated according to the normal sample-processing protocol as if it had come from a patient suspected of having tuberculosis, except that exactly 1 ml of the sputum (along with the added 2 ml of sample reagent) was added to each cartridge.

Clinical sputum samples. The experimenters performing the clinical validation studies using the Xpert MTB/RIF assay were blinded to the smear, culture, or rifampin susceptibility status of the samples until all experiments were completed. Unprocessed sputum samples were collected from 107 consecutively enrolled patients suspected of having tuberculosis at Pham Ngoc Thach Hospital, Ho Chi Minh City, Vietnam, between March 2007 and April 2007 and frozen for later analysis. The prespecified primary end points for this study were sensitivity and specificity of the assay in separate analyses of smear-positive and smear-negative tuberculosis patients. A posthoc analysis comparing assay sensitivity and specificity in smear-negative samples that were culture positive in solid medium only versus samples that were culture positive in either solid or liquid medium was also performed.

All clinical microbiology studies were performed on site at a microbiology laboratory experienced in clinical trials. Two sputum samples were collected from each patient. The first sputum sample was homogenized for 1 min with a vortexer and glass beads and split, with some aliquots frozen at −70°C for later analysis by the Xpert MTB/RIF assay and the remainder subjected to concentrated quantitative acid-fast bacillus (AFB) microscopy (17), quantitative culture on Lowenstein-Jensen medium (32), and Bactec MGIT 960 liquid culture (8). The second sputum was subjected to microscopy and culture but was not tested with the GeneXpert system. Positive cultures were confirmed to contain *M. tuberculosis* by MPT64 antigen detection using the Capilia TB test (20). Sputa were considered to be smear positive if both of the sputum samples had an AFB smear score of scanty or if either of the sputa had a score of 1+ or greater. The clinical characteristics and AFB smear definitions of the Vietnamese tuberculosis patients are shown in Table 1.

A second clinical validation study was designed to assess the sensitivity and specificity of the assay for detecting rifampin resistance in retreatment tuberculosis patients who are known to be at increased risk for rifampin-resistant tuberculosis (48). The study was performed using sputum samples from consecutively enrolled patients who were suspected of having tuberculosis and who also had a prior history of tuberculosis. Raw sputum was collected between February 2004 and March 2005 upon a patient's first visit to the Mulago Hospital tuberculosis clinic in Kampala, Uganda. Sputum samples were frozen at −70°C until tuberculosis was confirmed by culture; rifampin susceptibility using the Bactec 460 TB system on a contemporaneously obtained sputum sample was under way on-site at a microbiology laboratory experienced in clinical trials. The clinical characteristics of the Ugandan tuberculosis patients are shown in Table 1.

The prespecified primary end points of the second clinical validation study were sensitivity and specificity for detecting rifampin resistance as determined by conventional susceptibility tests. This study also had predefined secondary end points of sensitivity and specificity for detecting tuberculosis. A prespecified discrepancy analysis was also included, whereby the final rifampin resistance phenotype would be determined by sequencing the *rpoB* core region amplified from the sputum sample. Rifampin resistance would be considered to be present if mutations characteristically associated with rifampin resistance were detected in the amplicon.

Manual portion of the assay protocol. *M. tuberculosis*-negative *s*putum for the analytic studies was kept at 4°C for a maximum of 1 week before use. Sputa from the clinical validation studies were shipped frozen to the laboratory in the United States for analysis by the Xpert MTB/RIF assay and then thawed immediately prior to use. Two volumes of a proprietary NaOH- and isoproponyl-containing sample treatment reagent (SR) were added to each volume of sputum in all studies unless otherwise indicated. The sputum-SR mixture was vigorously shaken for 5 s, incubated at room temperature for 15 min, and shaken again. Two to three ml of digested sputum was then transferred to the Xpert MTB/RIF cartridge, the lid was closed, and the cartridge was loaded into the GeneXpert instrument, where all subsequent steps occurred automatically.

Automated portion of the assay protocol. The automated sample processing and real-time PCR procedure were initiated by placing the loaded assay cartridge into the GeneXpert instrument bay and then selecting the *M. tuberculosis* automated detection protocol from the included software. The detailed steps of the automated assay protocol are described in the Methods section in the supplemental material. At the end of the real-time PCR, the Xpert MTB/RIF assay's data analysis algorithm identified a sample as *M. tuberculosis* positive if at least two of the five *rpoB* probes were positive within two cycles of each other. Samples that were negative for *M. tuberculosis* but positive for a defined *B. globigii* signal were reported as *M. tuberculosis* negative; samples that were negative for both *M. tuberculosis* and *B. globigii* were identified as invalid. Rifampin resistance was detected by the failure of one or more of the *rpoB*-specific molecular beacons to hybridize properly to the *rpoB* amplicon. This was defined by the presence of greater than a 3.5-cycle difference in the cycle threshold (ΔC_T) values between

TABLE 1. Clinical characteristics of study patients

Characteristic	Value in the indicated group	
	Vietnamese patients (n = 107)[a]	Ugandan patients (n = 64)[a]
Patient characteristics		
Median age (yr [range])	34 (18–76)	34 (18–60)
No. of males (%)	74 (69)	38 (59)
No. of HIV-positive patients (%)	1 (0.9)[b]	20 (40)
No. of unavailable HIV result(s)	0	14
No. of previous TB episodes (no. of patients [%])		
1	2 (1.9)	54 (84)
2–3	0	7 (11)
4–5	0	3 (5)
Median duration of cough (days [range]	28 (14–336)	90 (21–365)
AFB smear grade (no. [%])[b]		0
Negative	53 (65)[c]	
Scanty (1–9/100 fields)[d]	8 (10)	
1+ (10–99/100 fields)[d]	8 (10)	
2+ (1–10/field)[d]	10 (12)	
3+ (>10/field)[d]	3 (4)	
1+ (1–9/100 fields)[e]		2 (3)
2+ (1–9/10 fields)[e]		8 (13)
3+ (1–9/field)[e]		4 (6)
4+ (>9/field)[e]		50 (78)
Days to positive BACTEC culture (no. [%])[f]		
<7	5 (6)	54 (84)
7–21	73 (89)	8 (13)
>21	4 (5)	2 (3)
Extent of disease on chest radiograph (no. [%])		
Normal	0	7 (12)
Minimal	21 (26)	2 (4)
Moderate	34 (41)	9 (16)
Severe	27 (33)	38 (68)
Presence of cavities (no. [%])	17 (21)	35 (63)
No radiograph available (no.)	0	8

[a] Except where indicated otherwise.
[b] Culture-positive subjects only.
[c] Eight subjects classified as AFB negative had one scanty sputum and one smear-negative sputum.
[d] Vietnam study smear definition.
[e] Uganda study smear definition.
[f] Days until growth was flagged by the instrument. BACTEC MGIT 960 was used in the Vietnam study; BACTEC 460 TB was used in the Uganda study.

the earliest and latest *rpoB* probe signals or if one to three *rpoB* probes did not produce any measurable signal.

Statistical analysis. The LOD for the assay was determined by converting the data to the percentage of positive responses for each concentration tested and fitting a binary logistic regression through the six concentrations. Lower and upper 95% confidence intervals (CI) were generated for the curve, and the 95% CI for the LOD was determined by points at which the 95% probability level crossed the upper and lower 95% CI. For sensitivity and specificity results, 95% CI were calculated according to the efficient-score method, corrected for continuity (39, 52).

Supplemental Methods. Additional details on the assay, the strains used in this study, their culture conditions, analysis of rifampin resistance, and tests of SR killing activity are detailed in Methods in the supplemental material.

Human subject protection. Identified samples were obtained under informed consent with approval by the appropriate institutional review boards.

RESULTS

Performance of the heminested PCR assay. The ability of the GeneXpert system to perform all assay fluidic and amplification steps directly within a completely self-contained cartridge in a hands-free manner made it possible for us to design a potentially very sensitive heminested PCR assay (12) that was free from amplicon cross-contamination. Analytical tests with *M. tuberculosis* DNA showed that the heminested PCR assay detected as few as 1 genome/reaction approximately 40% of the time and detected 7.5 genomes/reaction 100% of the time (Fig. 1A). None of the negative controls gave a positive signal. These results predicted an LOD for DNA analysis (where LOD is defined as the target number at which there is a 95% probability of a positive assay) of 4.5 genomes/reaction (95% CI, 3.3 to 9.7).

Limit of detection in sputum. We next tested the LOD with clinical sputum samples spiked with known numbers of *M. tuberculosis* cells. We found that the Xpert MTB/RIF assay has a calculated LOD of 131.0 CFU/ml of sputum (95% CI, 106.2

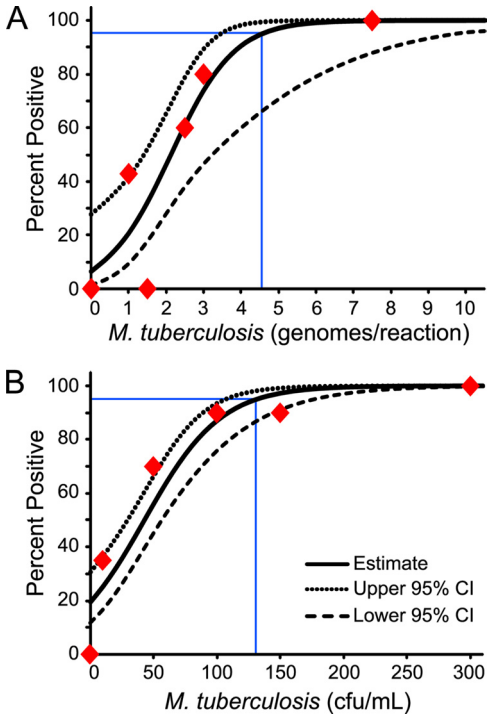

FIG. 1. Limit of detection for the in-cartridge heminested PCR. (A) DNA detection. *M. tuberculosis* DNA at final concentrations of 0, 1, 1.5, 2.5, 3, or 7.5 genomes per PCR was loaded into cartridges and processed according to the Xpert MTB/RIF protocol. For each genomic concentration tested (n = 5 to 7), the percentage of *M. tuberculosis*-positive cartridges was plotted. As determined by logistic regression, there is a 95% probability of detecting *M. tuberculosis* in samples containing at least 4.5 genomes per PCR (95% CI, 3.3 to 9.7). (B) Detection of *M. tuberculosis* cells in clinical sputum samples. *M. tuberculosis* cells were added to 1 ml of *M. tuberculosis*-negative sputum to final concentrations of 10, 50, 100, 150, or 300 CFU/ml (n = 20) and then processed according to the Xpert MTB/RIF protocol. The percentage of assays where *M. tuberculosis* was detected was then plotted for each concentration of cells. As determined by logistic regression, there was a 95% probability of detecting *M. tuberculosis* in samples containing at least 131 CFU/ml (95% CI, 106.2 to 176.4).

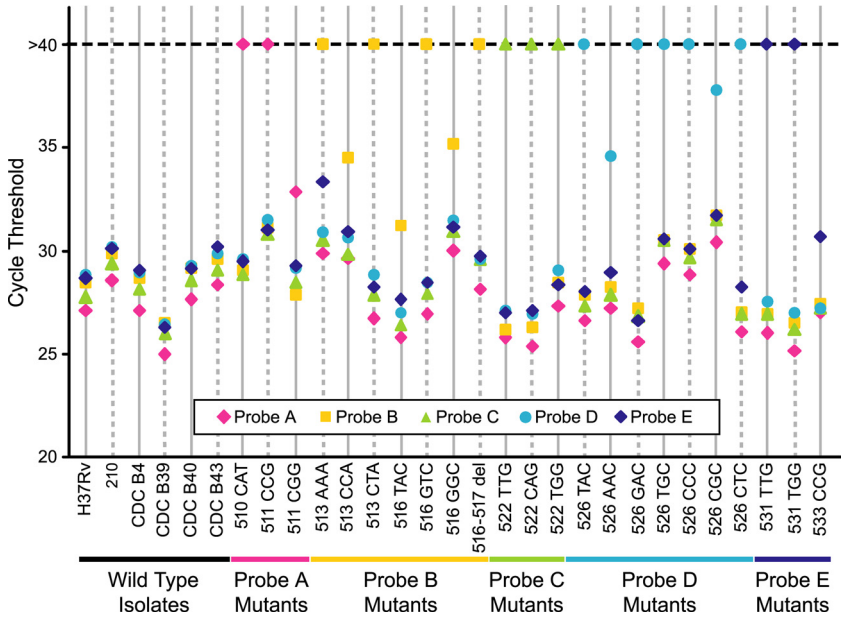

FIG. 2. Detection of RRDR mutations. Genomic *M. tuberculosis* DNA or artificial targets containing clinically relevant mutations were added to the wash buffer of cartridges that were then run with *M. tuberculosis*-negative sputum. Typical results from six rifampin-susceptible (wild-type RRDR) isolates and 23 RRDR mutants are shown. The results produced by each sample are indicated by a single vertical line on which the C_T of each of the five *rpoB*-specific molecular beacons (probes A to E) is plotted. ΔC_T values ≥3.5 cycles indicate rifampin resistance. All 23 RRDR mutants were correctly identified as rifampin resistant.

to 176.4) and was able to detect as few as 10 CFU/ml of sputum in 35% of samples (Fig. 1B). An additional LOD study was performed at a separate test site. In this study, known numbers of *Mycobacterium bovis* BCG CFU were spiked into sputum samples that had been frozen and then thawed. This second study resulted in a nearly identical LOD estimate (data not shown); thus, the freezing-thawing treatment of sputum samples does not appear to affect assay performance. None of the negative control samples were positive for *M. tuberculosis* at either test site. Thus, the limit of detection of the Xpert MTB/RIF assay in sputum appeared to be close to that of culture and at least 2 orders of magnitude more sensitive than the AFB smear (29).

Mutation detection. We tested the ability of the heminested assay to detect *M. tuberculosis rpoB* gene mutations associated with rifampin resistance. We obtained *M. tuberculosis* genomic DNA from drug-susceptible and rifampin-resistant clinical isolates, including isolates with 12 of the 19 most commonly described RRDR mutations (5, 7, 21, 22, 25, 26, 28, 31, 33, 46, 54). Full-length double-stranded oligonucleotides containing the remaining seven most common mutations were generated. Clinical DNA samples containing an additional four mutations were selected to ensure that all regions of each molecular beacon probe were tested. We found that the Xpert MTB/RIF assay detected all of the mutations tested using a predefined cutoff ΔC_T of 3.5 between the earliest and latest *rpoB* probe signals (Fig. 2). Sixteen of the 23 mutations caused at least one of the five *rpoB* molecular beacon probes in the assay to drop out completely (no detectible cycle threshold) (Fig. 2, indicated as C_T values >40), and the remaining mutations produced ΔC_T values greater than 3.5. All of the rifampin-susceptible DNA samples were identified as susceptible, with an average ΔC_T of 1.8 cycles (95% CI, 1.7 to 1.9).

Sample inactivation and sample stability in SR. The SR was developed to liquefy sputum samples so that they could be tested within the Xpert MTB/RIF cartridge and to decontaminate each sample to reduce possible biohazards. The goal was to achieve a minimum 6-log kill of the *M. tuberculosis* present in the sputum to comply with international decontamination standards (1, 6, 47). We tested the ability of the SR to kill high concentrations of *M. tuberculosis* in sputum after various periods of incubation. Live *M. tuberculosis* cultures added to fresh sputum samples and treated with SR at a 1:2 ratio showed an 8-log decrease in viability after a 15-min incubation in SR. After a 2-h SR incubation, the limits of the viability assay were reached, and viable bacteria could no longer be recovered. This corresponded to at least a 3.7×10^9 reduction in *M. tuberculosis* viability at the 2-h time point (Fig. 3A).

The effect of SR incubation time on assay sensitivity was also studied. The SR treatment time course experiment was repeated, this time adding only 150 CFU/ml of *M. tuberculosis* into the sputum sample before treatment with SR (Fig. 3B). At various time points, the sputum-SR mixture was placed into the Xpert MTB/RIF cartridge for analysis. This very small number of *M. tuberculosis* cells could be detected by the Xpert MTB/RIF assay even when samples were incubated in SR for 5 days before being processed within the cartridge. Furthermore, it appeared that the sample could be incubated in the SR for at least 24 h without causing a significant delay in the C_T value, suggesting that SR could be added to sputum samples in advance of sample processing without adversely affecting assay performance.

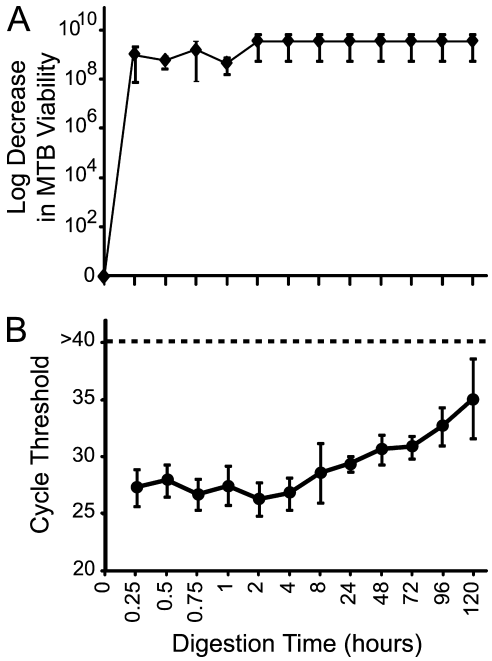

FIG. 3. Effect of sample reagent. (A) Biohazard reduction. Sputum samples spiked with high concentrations of *M. tuberculosis* cells were treated with 2 volumes of SR and incubated for 15 min to 5 days (n = 3). *M. tuberculosis* cells remaining viable after SR treatment were measured by quantitative culture. The log decrease in *M. tuberculosis* viability, compared to an untreated control, was plotted for each incubation time tested. (B) Assay performance. Sputum spiked with 150 CFU/ml of *M. tuberculosis* cells was treated with 2 volumes of SR and incubated for 15 min to 5 days and then processed in the Xpert MTB/RIF assay. Average C_T values were plotted for each incubation time (n = 3 to 4).

Cross-reactivity with other mycobacterial species. We studied assay specificity using unprocessed sputum containing 10^6 CFU/ml of 20 NTM species, including the 16 NTM commonly described as causing human disease (18). Several clinical isolates of the *Mycobacterium avium-intracellulare* complex were also included in our testing (see Table S2 in the supplemental material). The Xpert MTB/RIF assay's data analysis algorithm will not identify *M. tuberculosis* unless at least two of the *rpoB*-specific molecular beacon probes become positive within two cycles of each other. None of the NTM produced signals that fulfilled these criteria (Fig. 4A). However, 10^6 CFU of *Mycobacterium malmoense* produced two weakly positive *rpoB* molecular beacon signals with a ΔC_T of 5.2 (C_T of 31.5 for probe C and 36.7 for probe E). The *B. globigii* internal assay control was positive in all tests (data not shown).

We also tested whether high numbers of NTM mixed with low numbers of *M. tuberculosis* cells could affect detection of *M. tuberculosis* in a sputum sample. Two-hundred CFU of *M. tuberculosis* was added to fresh unprocessed sputum samples containing 10^6 CFU of *M. avium* (strains SmT and SmD), *M. intracellulare* (strains 35790 and 35776), *Mycobacterium kansasii*, or *M. malmoense* (Fig. 4B). *M. tuberculosis* was correctly detected in each case. However, the sample containing both 200 CFU of *M. tuberculosis* and 10^6 CFU of *M. malmoense* was identified by the Xpert MTB/RIF data analysis algorithm as containing rifampin-resistant *M. tuberculosis*. This error was due to an early C_T in probe C, caused by cross-hybridization with *M. malmoense*. We repeated these experiments, this time adding either 300 CFU of *M. tuberculosis* and 10^6 CFU of *M. malmoense* or 200 CFU of *M. tuberculosis* and 10^5 CFU of *M. malmoense* per ml of sputum. The Xpert MTB/RIF data analysis algorithm then correctly detected rifampin-susceptible *M. tuberculosis* in both of these cases (data not shown). Thus, a false-positive rifampin resistance result due to NTM coinfection is likely to be a very rare occurrence.

Clinical validation studies. We examined sputum aliquots from 107 consecutively enrolled patients suspected of having tuberculosis in Vietnam in a small study to assess the clinical sensitivity of the assay. The assay detected *M. tuberculosis* in 29 of 29 (100%; 95% CI, 85.4 to 100%) smear-positive samples and 38 of 53 (71.7%; 95% CI, 57.4 to 82.8%) smear-negative samples that were positive in either liquid and/or solid culture (Table 2). In a posthoc analysis where a positive culture on solid medium was used as the diagnostic standard, the Xpert MTB/RIF assay detected *M. tuberculosis* in 33 out of 39 smear-negative sputum samples (84.6%; 95% CI, 68.8 to 93.6%). Specificity was 100% (95% CI, 83.4 to 100%) in each case. Patients enrolled in this study also had a second sputum sample collected for acid-fast smear and culture. Eight patients were both smear and culture negative in the first sputum but smear negative, culture positive in the second sputum. Two of

TABLE 2. Performance of Xpert MTB/RIF assay

Sample origin	Smear sample type	Xpert MTB result	Detection in solid medium culture			Detection in liquid medium culture			Detection in liquid and solid medium culture			Specificity[a] (95% CI)
			No. of samples by culture type		Sensitivity (95% CI)	No. of samples by culture type		Sensitivity (95% CI)	No. of samples by culture type		Sensitivity (95% CI)	
			+	−		+	−		+	−		
Vietnam	Positive	+	29	0	100 (85.4–100)	29	0	100 (85.4–100)	29	0	100 (85.4–100)	
		−	0	0		0	0		0	0		
	Negative	+	33	0	84.6 (68.8–93.6)	37	0	71.1 (56.7–82.4)	38	0	71.7 (57.4–82.8)	100 (83.4–100)
		−	6	25		15	25		15	25		
Uganda	Positive	+	63	0	98.4 (90.5–99.9)	63	0	98.4 (90.5–99.9)	63	0	98.4 (90.5–99.9)	100 (80.0–100)
		−	1	20		1	20		1	20		

[a] Specificity values represent the proportions of culture-negative samples that were correctly identified as giving negative results by the Xpert MTB/RIF assay.

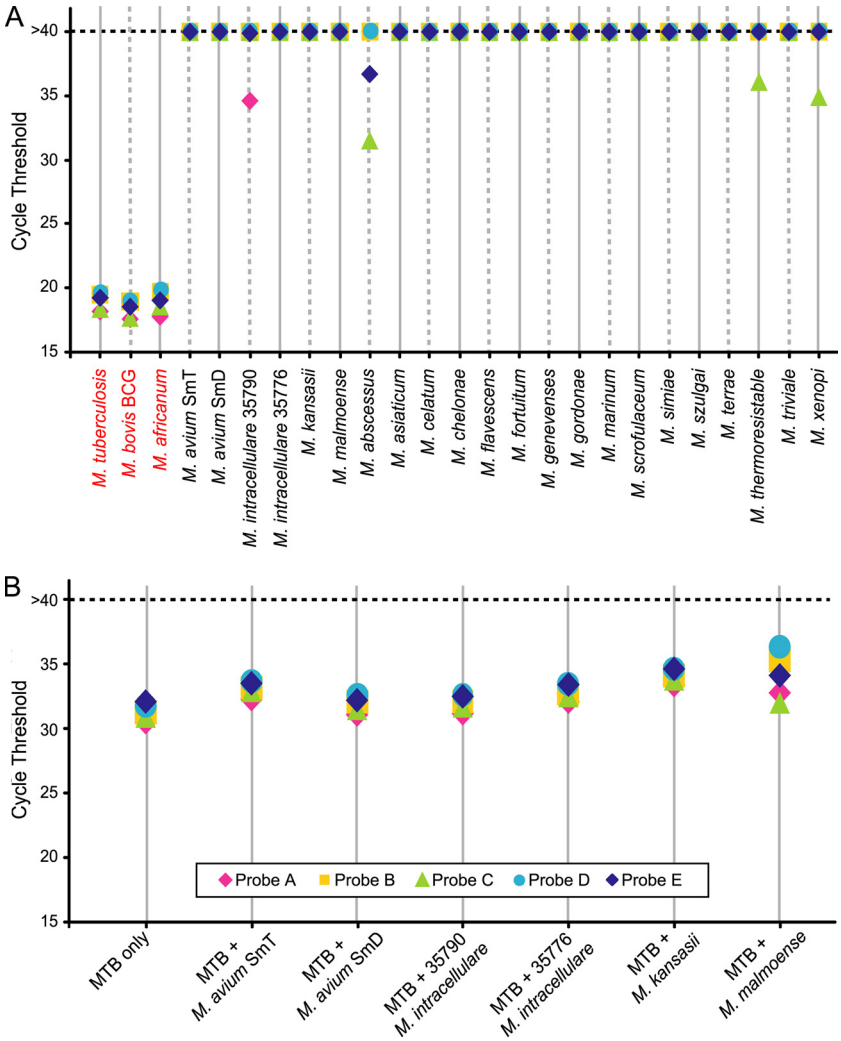

FIG. 4. Assay performance with nontuberculosis mycobacteria. (A) Lack of cross-reaction by NTM. A total of 10^6 CFU/ml of relevant NTM species was added into *M. tuberculosis*-negative sputum and processed according to the Xpert MTB/RIF protocol. The results produced by each species are indicated by a single vertical line on which the C_T of each of the five *rpoB*-specific molecular beacons (probes A to E) is plotted. For tuberculosis detection, the GeneXpert software requires a sample to have at least two positive molecular beacon probes with a ΔC_T of <2 cycles. In the example shown, the assay correctly identified members of the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis* BCG, and *Mycobacterium africanum*) as *M. tuberculosis* positive. However, none of the NTM samples produced ΔC_Ts that fulfilled the criteria for *M. tuberculosis* detection. (B) Lack of interference by NTM. Possible interactions between low concentrations of *M. tuberculosis* and high concentrations of NTM species were investigated. Sputum containing both 200 CFU/ml of *M. tuberculosis* and 10^6 CFU/ml of an NTM species was processed according to the Xpert MTB/RIF protocol, and cycle thresholds for all five *rpoB* probes were plotted. *M. tuberculosis* was accurately identified in all of these samples. However, the combination of high levels of *M. malmoense* with low concentrations of *M. tuberculosis* resulted in a false rifampin resistance result. Rifampin resistance was not seen when 200 CFU/ml of H37Rv was tested in combination with 10^5 CFU/ml of *M. malmoense* or when sputum contained 300 CFU/ml of H37Rv and 10^6 CFU/ml of *M. malmoense* (data not shown).

these eight patients were correctly identified as having *M. tuberculosis* in the first culture-negative sputum by the Xpert MTB/RIF assay. This suggests that assay sensitivity may be higher than culture in some circumstances.

We tested sputum samples from 64 sequential *M. tuberculosis* culture-positive patients in Uganda who had a prior history of tuberculosis in a small study to test the ability of the assay to detect clinical rifampin resistance. This patient group was studied because retreatment tuberculosis cases have higher rates of drug resistance than initial tuberculosis cases (48). The Xpert MTB/RIF assay detected rifampin resistance in all nine cases known to be rifampin resistant (sensitivity, 100%; 95% CI, 63.0 to 100%) and in 1/55 rifampin-susceptible cases (specificity, 98.2%; 95% CI, 89.0 to 99.9%). However, sequencing the *rpoB* gene of the single isolate that was discordant for rifampin resistance revealed a codon 511 CCG mutation that is one of the *rpoB* mutations commonly associated with rifampin resistance. An error in the conventional susceptibility test is the most likely explanation for this discordant result. Such errors have been reported in other studies (2) and is especially likely in this case since this isolate was known to be resistant to both isoniazid and ethambutol. Thus, the corrected specificity for

rifampin resistance was 100% (95% CI, 65.6 to 100%). The Xpert MTB/RIF assay also detected *M. tuberculosis* in 63 of the 64 sputum samples from culture-positive Ugandan patients (98.4%; 95% CI, 90.5 to 99.9%) (Table 2). Twenty laboratory control sputum samples from patients not suspected to have tuberculosis were negative.

Time to result. We measured the time required to analyze one or eight sputum samples, beginning at the moment that a potentially *M. tuberculosis*-containing sputum sample was placed into the possession of a laboratory technician. The time-to-result for one sputum sample processed alone was 1 h 55 min; the time-to-result for all eight samples processed together was 2 h.

DISCUSSION

This study demonstrates that the Xpert MTB/RIF assay system can rapidly detect the presence of *M. tuberculosis* and identify the mutations most frequently associated with rifampin resistance directly from smear-negative and smear-positive clinical sputum samples. The self-contained cartridge fluidics of the Xpert MTB/RIF assay made it possible to design a heminested PCR assay with a sensitivity that approached culture-based diagnostics. The assay appears to be relatively resistant to PCR inhibitors which may be present in sputum; however, PCR inhibitors may have been responsible for the one smear-positive sample from Uganda that was Xpert MTB/RIF negative. The retrospective nature of our clinical validation studies caused us to rely on tests of previously frozen sputum samples. Freezing may alter sputum viscosity, and it may improve nucleic acid recovery from mycobacteria. Thus, these results will need to be confirmed in larger prospective studies with fresh samples. However, we documented similar LODs regardless of whether the assay was performed with fresh or frozen sputum samples in our analytic studies using spiked sputum. Although the results presented here are most relevant to detection of pulmonary tuberculosis, preliminary studies with the Xpert MTB/RIF assay suggest that this system also has promise for detecting *M. tuberculosis* in other body fluids, such as cerebrospinal fluid; thus, the assay may have even broader utility.

Truly rapid results for drug susceptibility tests are particularly important in the management of drug-resistant tuberculosis (13). Currently available methods fall short of this promise (4, 9, 10, 19, 23, 30, 38, 40). Most rapid nucleic acid amplification methods to detect tuberculosis require skilled technicians and dedicated space for both setup and analysis in order to prevent amplicon cross-contamination. Assay setup can also present a significant biohazard, confining work to centers with specialized biocontainment equipment. These technical requirements cause most centers to batch samples and test for tuberculosis once a day at most. The Xpert MTB/RIF assay, however, is simple and robust enough to be performed by personnel with minimal training. Total hands-on time is less than 5 min, and results are typically available within 1 h 55 min. Each module within the GeneXpert instrument operates independently, which enables the user to test each sputum sample as it arrives in the laboratory instead of saving samples for batch processing. This important feature can potentially result in dramatically reduced turnaround times for tuberculosis detection, allowing decisions about respiratory isolation and treatment to be made in real time (3). False-positive results, often caused by carryover of amplified target, are mitigated by the use of closed cartridges that do not require any manual pipetting after the sample has been added to the cartridge. False-negative results, caused by operator errors, manufacturing defects, fluidics problems, or the presence of inhibitors in the sample, are controlled for by a multiplexed heminested PCR assay that detects a control target within *B. globigii* spores included within each cartridge.

Complete costing and cost effectiveness studies of the Xpert MTB/RIF test are planned and merit more thorough discussion in future publications. At present, instrumentation costs for the GeneXpert system are similar to those of an automated liquid culture system for tuberculosis, and per-assay running costs are also in the same range as culture, despite vastly superior performance in terms of speed, biosafety, and ease of use. Importantly, the assay makes use of a basic cartridge design and an instrument platform that are currently used in commercially available assays for rapid detection of methicillin-resistant *Staphylococcus aureus*, enteroviruses, *Bacillus anthracis* (50), and group B *Streptococcus* (16) and that is also likely to be used in the detection of a variety of infectious diseases, ranging from human immunodeficiency virus (HIV) and polio to sexually transmitted diseases and *Clostridium difficile*. Thus, the assay can take advantage of economies of scale that would not be possible with a tuberculosis-specific assay platform. For the developing world, where 95% of all TB occurs, the Xpert MTB/RIF assay will be available to the public sector at concessionary prices negotiated by the Foundation for Innovative New Diagnostics and will compete with the costs currently being paid locally for culture and conventional antibiotic susceptibility testing.

The point-of-care for tuberculosis for most people in the world is at a health post linked to a microscopy center. This is where tuberculosis is detected, registered, treated, and followed up. Diagnostic technology has been one of the major limitations at this level, with the insensitive and technologist-dependent performance of microscopy as the bottleneck to care in many situations and a major contributor to poor disease control. The Xpert MTB/RIF assay offers the first technical opportunity to bridge this gap, potentially bringing tests for both TB and drug resistance to levels of the health system where many seek care.

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D.H., E.S., C.B., P.B., R.B., N.T.N.L., E.C.J.-L., I.A., R.D.M., M.L., M.B., and M.D.P. declare that they have no conflicts of interest. E.W., K.H., J.K., M.R.O., R.R., B.M., E.W.-D., L.C., P.D., D.H.P., and M.J. are either employed by Cepheid or own stock or stock options in the company. D.A. is among a group of inventors who earn royalties on licensing fees for molecular beacon usage. D.A. also reports receiving income as a past participant in two Cepheid Scientific Advisory Board meetings unrelated to the current study.

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