Evaluation of the Analytical Performance of the Xpert MTB/RIF Assay[∇][†]

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We performed the first studies of analytic sensitivity, analytic specificity, and dynamic range for the new Xpert MTB/RIF assay, a nucleic acid amplification-based diagnostic system that detects *Mycobacterium tuberculosis* and rifampin (RIF) resistance in under 2 h. The sensitivity of the assay was tested with 79 phylogenetically and geographically diverse *M. tuberculosis* isolates, including 42 drug-susceptible isolates and 37 RIF-resistant isolates containing 13 different *rpoB* mutations or mutation combinations. The specificity of the assay was tested with 89 nontuberculosis bacteria, fungi, and viruses. The Xpert MTB/RIF assay correctly identified all 79 *M. tuberculosis* isolates and correctly excluded all 89 nontuberculosis isolates. RIF resistance was correctly identified in all 37 resistant isolates and in none of the 42 susceptible isolates. Dynamic range was assessed by adding 10^2 to 10^7 CFU of *M. tuberculosis* into *M. tuberculosis*-negative sputum samples. The assay showed a log-linear relationship between cycle threshold and input CFU over the entire concentration range. Resistance detection in the presence of different mixtures of RIF-resistant and RIF-susceptible DNA was assessed. Resistance detection was dependent on the particular mutation and required between 65% and 100% mutant DNA to be present in the sample for 95% certainty of resistance detection. Finally, we studied whether assay specificity could be affected by cross-contaminating amplicons generated by the GenoType MTBDRplus assay. *M. tuberculosis* was not detected until at least 10^8 copies of an MTBDRplus amplicon were spiked into 1 ml of sputum, suggesting that false-positive results would be unlikely to occur.

Conventional diagnostic methods for *Mycobacterium tuberculosis* are slow and/or lack sensitivity. A number of new diagnostic approaches have brought incremental improvements to detection and drug susceptibility testing; however, the technical complexity of these assays and their dependence on dedicated laboratory infrastructure have limited their adoption, especially in low-resource, high-burden settings (1, 11, 12, 21). The recently introduced Xpert MTB/RIF (manufactured and marketed by Cepheid, Sunnyvale, CA) assay simultaneously detects the presence of *M. tuberculosis* and its susceptibility to the important first-line drug rifampin (RIF) (7). A sample processing system and an automated heminested real-time PCR assay are integrated into a single disposable cartridge. The assay can be performed directly from a clinical sputum sample or from a decontaminated sputum pellet and can generally be completed in less than 2 h (7).

The Xpert MTB/RIF assay detects *M. tuberculosis* and RIF resistance by PCR amplification of the rifampin resistance-determining region (RRDR) of the *M. tuberculosis rpoB* gene and subsequent probing of this region for mutations that are associated with RIF resistance. Approximately 95% of RIF-

resistant tuberculosis cases contain mutations in this 81-bp region (16). Our previous work has established that the Xpert MTB/RIF assay has a limit of detection (LOD), defined as the minimum number of bacilli that can be detected with 95% confidence) of 131 CFU per ml of clinical sputum (7). The assay was also able to identify RIF resistance in samples containing 23 common clinically occurring *rpoB* mutations. None of the 20 nontuberculosis mycobacteria (NTM) species tested, including the NTM species commonly described as causing human disease were falsely identified as *M. tuberculosis* (7), suggesting high specificity. Several small studies using clinical samples demonstrated 98% to 100% sensitivity overall, 72% sensitivity in smear-negative patients, and a specificity of 100% (7).

In the present study, we expand upon our previous work and report on several critical analytical assay performance characteristics, including dynamic range, sensitivity, specificity, RIF resistance detection in heterogeneous samples, and resiliency against cross-contamination by other nucleic acid amplification techniques (NAATs).

MATERIALS AND METHODS

Diagnostic system. The Xpert MTB/RIF assay and the GeneXpert instrument have recently been described in detail (7). In brief, the assay consists of a single-use multichambered plastic cartridge preloaded with the liquid buffers and lyophilized reagent beads necessary for sample processing, DNA extraction, and heminested real-time PCR. Clinical sputum samples or decontaminated sputum pellets are treated with an NaOH and isopropanol-containing sample reagent (SR). The SR is added at a 2:1 ratio to the sputum sample or sputum pellet and incubated for 15 min at room temperature. The treated sample is transferred into the cartridge, the cartridge is loaded into the GeneXpert instrument, and an automatic process completes the remaining assay steps. The assay cartridge also contains lyophilized *Bacillus globigii* spores which serve as an internal sample

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processing and PCR control. The spores are automatically resuspended and processed during the sample processing step, and the resulting *B. globigii* DNA is amplified during the PCR step. The standard user interface indicates the presence or absence of *M. tuberculosis*, the presence or absence of RIF resistance, and a semiquantitative estimate of *M. tuberculosis* concentration (high, medium, low, and very low). Assays that are negative for *M. tuberculosis* and also negative for the *B. globigii* internal control are reported as invalid.

The PCR assay amplifies a 192-bp segment of the *M. tuberculosis rpoB* gene in a heminested real-time PCR. The internal control heminested *B. globigii* assay is multiplexed with the *M. tuberculosis* assay. *M. tuberculosis* is detected using five overlapping molecular beacon probes (probes A to E) that are complementary to the entire 81-bp RIF resistance-determining "core" region of the wild-type *rpoB* gene (5, 7, 14). Mutations in the *rpoB* gene target inhibit hybridization of one or more of the *rpoB*-specific molecular beacons, reducing or eliminating the signal from the corresponding probes. *M. tuberculosis* is identified when at least two of the five *rpoB*-specific molecular beacons give a positive signal with cycle threshold (C_T) values that are ≤38 and that differ by no more than two cycles. *B. globigii* DNA is detected when the single *B. globigii* molecular beacon produces a C_T of <38 cycles.

The difference in C_T between the first (early C_T) and last (later C_T) *M. tuberculosis*-specific molecular beacon (ΔC_T Max) is the basis of *rpoB* mutation and RIF resistance detection. RIF resistance is identified if the ΔC_T Max is >3.5 cycles. RIF susceptibility is identified if the ΔC_T Max is ≤3.5 cycles. A sample is considered RIF indeterminate when the last probe returns a C_T of >38 and the first probe has a C_T value of >34.5 cycles because the assay terminates at cycle 38, and a ΔC_T Max of >3.5 cannot be measured. For the purpose of the current study, *rpoB* mutations that completely inhibit probe hybridization (and thus caused one or more molecular beacons to show C_T values of >38) were defined as causing probe "dropouts," whereas *rpoB* mutations that permit partial probe hybridization and produce a measurable ΔC_T Max of >3.5 cycles were considered "delays."

Dynamic range testing. The laboratory *M. tuberculosis* strain H37Rv obtained from the American Type Culture Collection (ATCC) (Manassas, VA) was cultured and quantified as previously described (7). Excess discarded sputum samples were collected under institutional review board (IRB)-approved protocols from patients not suspected of tuberculosis as previously described (7). *M. tuberculosis* cells were spiked into 1-ml aliquots of *M. tuberculosis*-negative sputum samples to final concentrations ranging from 10^2 to 10^7 CFU/ml and were tested with the Xpert MTB/RIF assay according to the manufacturer's instructions.

Analytic sensitivity panel. The 79 clinical *M. tuberculosis* isolates analyzed in the sensitivity study included 70 isolates from a collection of 211 highly characterized *M. tuberculosis* strains established by the United Nations Children's Fund/UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (3). Rifampin MIC was previously established by the agar proportion method on Lowenstein-Jensen medium, using a breakpoint of 40 μg/ml to establish resistance (13). *M. tuberculosis* DNA was isolated as described previously (17). The remaining nine clinical *M. tuberculosis* strains were selected from a panel of pansusceptible *M. tuberculosis* isolates obtained in a previous study (6). The phylogenetic lineage of each *M. tuberculosis* isolate within the collection was identified by testing genomic DNA for nine single-nucleotide polymorphisms (SNPs), which were used to classify each isolate into one of 10 SNP cluster groups and SNP cluster subgroups (6). The isolates were selected to include at least five examples of any unique SNP cluster group or subgroup. The details for each isolate, including geographic source, phylogenetic group, drug susceptibility profile, and drug resistance target genotype, are listed in Table S1 in the supplemental material.

Analytic specificity panel. The 89 nontuberculosis bacteria, fungi, and viruses analyzed in the specificity study were obtained from the ATCC and BEI repositories (NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH), the Clinical Microbiology laboratory at the university hospital of the University of Medicine and Dentistry of New Jersey (UMDNJ), Zeptometrix Corporation (Buffalo, NY), or other collaborators (Table 1). Samples obtained from UMDNJ were verified to be of the appropriate species or genera as described in reference 2.

Sensitivity and specificity panel testing. The sensitivity and specificity studies were performed using production-lot cartridges preloaded with production-lot enzyme beads and reagent beads. However, unlike the commercially available assay, these studies used open cartridges that were not sealed with a sonically welded plastic top and were not preloaded with liquid reagents or the sample processing control bead containing *B. globigii* spores. The open cartridges preserved access to the cartridge chambers and permitted manual placement of test

DNA into cartridge chamber 10, which would normally receive the DNA-containing eluent of lysed bacterial cells.

Five hundred microliters of TET buffer (50 mM Tris [pH 8.35], 0.1 mM EDTA, and 0.1% mM Tween 20) was added to cartridge chamber five. A 100-μl sample mixture containing either sensitivity or specificity panel DNA (or RNA for RNA viruses), 90 copies of genomic *B. globigii* DNA, and TET buffer was added directly into chamber 10. *M. tuberculosis* isolates were tested once at 45 genomes per reaction (10 times the Xpert MTB/RIF LOD for DNA) (7), and all other organisms were tested twice at 10^6 genome copies per reaction. Negative controls contained no DNA. Initial DNA concentrations were determined with a NanoDrop ND-1000 (Thermo Scientific) and diluted in TET buffer. When reliable genome size was not available, appropriate concentrations were determined by assuming genome sizes of 4.5 Mb/genome for bacteria and 22.5 Mb/genome for fungi. Testing of mixed samples containing DNA from a wild-type *M. tuberculosis* strain plus an *M. tuberculosis* strain containing either *rpoB* gene mutation 533ccg (ccg mutation at codon position 533) or 531ttg (ttg mutation at codon position 531) was performed at a final concentration of 90 copies per reaction. Strains used for mixture testing are indicated in Table S1 in the supplemental material. Preparations used for mixture testing were confirmed to be of appropriate concentration by quantitative real-time PCR of the *M. tuberculosis lsr2* gene (4). Cartridges were loaded into the GeneXpert instrument and processed with a truncated version of the automated protocol that omitted the sample processing steps, proceeding immediately as though the lysis and elution of a normal sample had already been completed. This protocol preserved the remaining assay steps without change and is equivalent to version 1 of the MTB assay protocol, released on 8 April 2009.

Three organisms (Table 1) were obtained as whole viral particle NATTROL diagnostic standards (Zeptometrix), 100 μl of the viral particle stock was diluted 1:20 in TET buffer, and the mixture was tested with the Xpert MTB/RIF assay according to the manufacturer's instructions.

Amplicon contamination studies. We created a modified MTBDRplus assay amplicon with defined mutations in the *rpoB* target, but unaltered primer binding sites. This distinguished between unintentional amplicon contamination and deliberate experimental contamination. Three smear-positive clinical sputum samples from individuals suspected to have tuberculosis were PCR amplified using the GenoType MTBDRplus kit (Hain Lifescience GmbH) as indicated in the product insert. The 168-bp DNA sequence of the full MTBDRplus amplicon was derived by outward sequencing of the product of the GenoType MTBDRplus assay from within the *rpoB* core. The MTBDRplus amplicon (minus the biotinylated ends) was then reconstructed via overlap extension of two 98-bp oligonucleotides (Hain-AT-do-F [5′-CACGCTCACGTGACAGACC GCCGGGCCCCAGCGCCCACAGTCGGCGCTTGTCGGTCAACCCCGAC AGCGGGTTGTTCTGGACCATGAATTGGCTCAGC-3′] and Hain-AT-do-R [5′-CACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCCACAG TCGGCGCTTGTCGGTCAACCCCGACAGCGGGTTGTTCTGGACCATG AATTGGCTCAGC-3′]). The construct included four mutations in the *rpoB* core region (511ccg, 516gtc, 526gac, and 531tgg) intended to block hybridization of all but one of the Xpert MTB/RIF assay's five molecular beacon probes.

Statistical analysis. The minimum proportion of mutant DNA detectable in a wild-type mixture was determined by binary logistic regression. The data were converted to the percentages of RIF-resistant responses for each ratio of wild type to mutant DNA. Binary logistic regression was fitted through the tested concentrations, and lower and upper 95% confidence intervals (95% CIs) were generated for the curve. The 95% CI for the minimum proportion was determined by where the 95% probability level crossed the upper and lower 95% CIs. In all other cases, 95% CIs were calculated using a Student t test.

RESULTS

Dynamic range. The concentration of *M. tuberculosis* bacteria in clinical sputum samples can vary from over 10^7 to less than 20 CFU/ml (10). We performed analytic studies to examine the CFU relationship between C_T value and cell concentration over the potential range of *M. tuberculosis* cell concentrations. Clinical sputum samples were spiked with known numbers of *M. tuberculosis* bacteria ranging from 10^7 to 10^2 CFU/ml and tested in the assay. The assay correctly identified *M. tuberculosis* in all 28 sputum samples containing 10^7 to 10^3 CFU/ml and identified *M. tuberculosis* in 5 of the 6 samples containing 10^2 CFU/ml. The log-linear relationship between

TABLE 1. Analytic specificity panel

Bacterial, fungal, or viral species	Strain or source[a]	Bacterial, fungal, or viral species	Strain or source[a]
Bacteria		*Proteus mirabilis*	Clinical strain
Acinetobacter baumanii	BEI NR-10146	*Proteus vulgaris*	BEI DD-460
Acinetobacter calcoaceticus	Clinical strain	*Providencia alcalifaciens*	PI 368
Actinomyces israelii	ATCC 12102	*Pseudomonas aeruginosa*	ATCC 27853
Actinomyces meyeri	Clinical strain	*Rhodococcus equi*	ATCC 14187
Bacillus cereus	BEI NR-4198	*Salmonella enterica*	BEI NR-515
Bacillus subtilis	Clinical strain	*Salmonella typhi*	Clinical strain
Bordetella parapertussis	ATCC 151311D	*Serratia marcescens*	Clinical strain
Bordetella pertussis	Cepheid	*Shigella boydii*	Clinical strain
Campylobacter jejuni	BEI NR-3057	*Shigella flexneri*	Clinical strain
Chlamydia pneumoniae	Cepheid	*Staphylococcus aureus*	ATCC 25953
Citrobacter freundii	Clinical strain	*Staphylococcus capitis*	Clinical strain
Corynebacterium diptheriae	PI 581	*Staphylococcus epidermidis*	ATCC 12228
Corynebacterium pseudodiptheriticum	ATCC 10700	*Staphylococcus haemolyticus*	Clinical strain
Corynebacterium xerosis	Clinical strain	*Staphylococcus hominis*	Clinical strain
Enterobacter aerogenes	Clinical strain	*Staphylococcus lugdunensis*	Clinical strain
Enterobacter cloacae	Clinical strain	*Stenotrophomonas maltophilia*	Clinical strain
Enterococcus avium	Clinical strain	*Streptococcus equi*	Clinical strain
Enterococcus faecalis	Clinical strain	*Streptococcus pyogenes*	ATCC 19615
Enterococcus faecium	Clinical strain	*Streptococcus agalactiae*	ATCC 12386
Escherichia coli	Clinical strain	*Streptococcus constellatus*	Clinical strain
Escherichia coli O157H7	ATCC 35150	*Streptococcus mitis*	Clinical strain
Fusobacterium nucleatum	ATCC 25586D	*Streptococcus mutans*	Clinical strain
Haemophilus influenzae	ATCC 49247	*Streptococcus pneumoniae*	Clinical strain
Haemophilus parahemolyticus	ATCC 10014	*Streptococcus uberis*	Clinical strain
Haemophilus parainfluenzae	Clinical strain	*Veillonella parvula*	ATCC 10790D-5
Klebsiella oxytoca	Clinical strain	*Stenotrophomonas maltophilia*	Clinical strain
Klebsiella pneumoniae	Clinical strain	*Yersinia pestis*	BEI NR-2644
Legionella pneumophila	ATCC 33152D		
Leuconostoc mesenteroides	Clinical strain	**Fungi**	
Listeria grayi	ATCC 25401	*Candida albicans*	ATCC 14053D
Listeria monocytogenes	BEI NR-4211	*Cryptococcus neoformans*	A. Casadevall lab
Moraxella catarrhalis	ATCC 8176	*Histoplasma capsulatum*	J. Nosanchuk lab
Morganella morganii	Clinical strain	*Kingella kingae*	ATCC 23332D
Mycoplasma pneumoniae	ATCC 15531D		
Neisseria gonorrhoeae	ATCC 49226	**Viruses**	
Neisseria lactamica	ATCC 23971	Adenovirus[b]	ZM NATADV1-ST
Neisseria meningitidis	Clinical strain	Herpes simplex virus type 1	Cepheid
Neisseria mucosa	ATCC 69695	Herpes simplex virus type 2	Cepheid
Nocardia asteroides	ATCC 19247	Influenza A virus[c]	Cepheid
Nocardia cyriageorgica	ATCC BAA-1516	Influenza B virus[c]	Cepheid
Nocardia farcinica	ATCC 3318	Parainfluenza virus 2[b]	ZM NATPARA2-ST
Pasteurella multocida	ATCC 3160	Parainfluenza virus 3[b]	ZM NATPARA3-ST
Peptostreptococcus anaerobius	ATCC 49031D	Respiratory syncytial virus A[c]	Cepheid
Porphyromonas gingivalis	ATCC 33277D-5	Respiratory syncytial virus B[c]	Cepheid
Prevotella melaninogenica	ATCC 25845D-5	Rhinovirus 6[c]	Cepheid
Propionibacterium acnes	Clinical strain	Rhinovirus 16[c]	Cepheid

[a] The 89 nontuberculosis bacteria, fungi, and viruses analyzed were obtained from the ATCC and BEI repositories (NIH Biodefense and Emerging Infections Research Resources Repository), the University of Medicine and Dentistry of New Jersey (UMDNJ), Zeptometrix Corporation (Buffalo, NY), Presque Isle Cultures (Erie, PA) (PI), or other collaborators. lab, laboratory. Clinical strains were from UMDNJ, and clinical strain DNA was from reference 2.
[b] Whole-particle viral testing, sample from Zeptometrix Corporation.
[c] The genomic source material was RNA.

C_T values and the number of *M. tuberculosis* cells present in each sample was maintained over the entire dynamic range, confirming the ability of the assay to provide semiquantitative estimates of input cell number (Fig. 1A). The *B. globigii* internal control assay also functioned well over the entire range of added *M. tuberculosis* cells. The *B. globigii* assay was positive in all instances with an average cycle threshold of 29.7 (95% CI, 28.9 to 30.4) (Fig. 1B). Interestingly, the C_T values of the *B. globigii* assay did not appear to be affected by the number of *M. tuberculosis* cells added to the sample. We suspect that this decoupling is due to the heminested PCR design of both assays.

Sensitivity. Previous analytic studies examined assay performance using the laboratory *M. tuberculosis* strain H37Rv. We tested the ability of the assay to detect a more phylogenetically and geographically diverse collection of *M. tuberculosis* isolates (Table 2). The studies were performed at a relatively low target concentration (10 times the LOD with DNA) to test sensitivity under challenging conditions. The assay identified all 79 different *M. tuberculosis* strains as *M. tuberculosis* positive. We also tested the ability of the assay to identify different *rpoB* mutations and thereby RIF resistance in a variable clinical strain background. The assay detected RIF resistance in all 37 strains with known mutations in the *rpoB* core. The 42 RIF-
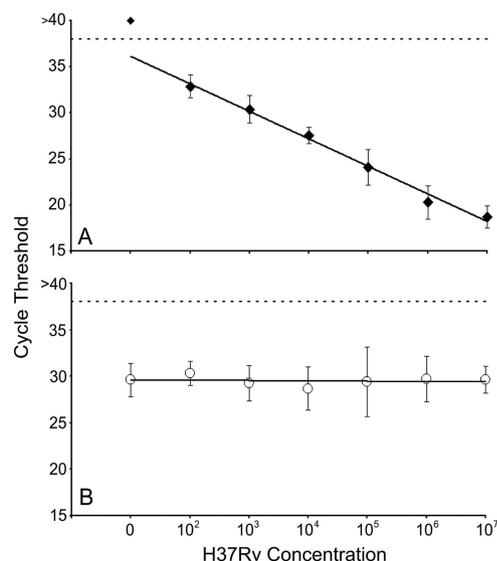
FIG. 1. Dynamic range studies. Log dilutions of *M. tuberculosis* H37Rv cells were added to 1 ml of *M. tuberculosis*-negative sputum to final concentrations ranging from 10^2 to 10^7 CFU/ml ($n = 5$ or 6 per dilution). (A) Average *rpoB* probe B cycle thresholds (C_Ts) were plotted for each *M. tuberculosis* concentration tested. Clinically relevant *M. tuberculosis* concentrations all fall within the linear range of the assay. (B) Average *B. globigii* internal control probe C_Ts were plotted for each concentration of *M. tuberculosis* tested. The *B. globigii* assay gave an average C_T of 29.7 (95% CI, 28.9 to 30.4) for all dilutions and was not influenced by the concentration of *M. tuberculosis* in the sample. Dotted lines indicate maximum valid C_T values for probe positivity.

TABLE 3. Xpert MTB/RIF result for direct testing of genomic DNA

Organism	No. of strains with the following result with the Xpert MTB/RIF assay:		
	M. tuberculosis positive		*M. tuberculosis* negative
	Resistance detected	Resistance not detected	
M. tuberculosis strains[a]			
RIF-resistant	37	0	0
RIF-susceptible	0	42	0
Bacteria[b]	0	0	74
Fungi[b]	0	0	4
Virus[b]	0	0	11
None (negative control)	0	0	52

[a] Strain characteristics are presented in Table 1. RIF, rifampin.
[b] Organism characteristics are presented in Table 1.

susceptible isolates without known *rpoB* core mutations were all identified as RIF susceptible (Table 3). The RIF-susceptible isolates had an average ΔC_T Max of 1.8 (95% CI, 1.74 to 1.92). The 37 RIF-resistant isolates contained 13 unique SNPs, three of which were present only in combination with another SNP (Table 2). Nine of 13 mutations caused at least one of the

molecular beacon probes to drop out completely (C_T of >38). Mutations 516tac, 526cgc, and 533ccg caused a delayed C_T, resulting in ΔC_T Max values of 9.7 (95% CI, 9.2 to 10.2; $n = 2$), 7.7 (95% CI, 7.4 to 8.0; $n = 3$), and 4.23 (95% CI, 3.7 to 4.8; $n = 3$), respectively. The 509agg mutation was present only in combination with the 526cgc mutation. The sample was correctly identified as RIF resistant on the basis of a delayed probe D C_T corresponding to the 526cgc mutation. Interestingly, the 509agg mutation did not cause a dropout or delay of its corresponding probe.

Specificity. A previous study had shown that the Xpert MTB/RIF assay did not cross-react with 20 different NTM species tested at high copy numbers, suggesting that the assay would have high specificity (7). However, normal flora of the upper respiratory tract and respiratory tract pathogens can also be present in sputum samples of individuals suspected to have tuberculosis. We tested a panel of 89 organisms, including

TABLE 2. Characteristics of *M. tuberculosis* test panel isolates

Codon position(s)[a]	Mutation(s)[b]	No. of isolates in SNP cluster group[c]									Total no. of isolates with the mutation(s)
		I	II	IIIa	IIIb	IIIc	IV	V	VIa	VIb	
511	CTG→ccg		1								1
516	GAC→gtc		1						1		2
516	GAC→tac		1						1		2
526	CAC→agc			1							1
526	CAC→cgc		1		1						2
526	CAC→gac		1					1	1		3
526	CAC→tac	1	1		1			1			4
531	TCG→tgg		1					1	1		3
531	TCG→ttg	2	2		1	1	1	4	1	1	13
533	CTG→ccg	1	1					1			3
512, 531	AGC→gcc, TCG→ttg			1							1
515, 516	ATG→att, GAC→tac		1								1
509, 526	AGC→agg, CAC→cgc							1			1
Wild type		5	4	4	4	4	4	6	7	4	42
Total no.		9	15	6	7	5	5	17	10	5	79

[a] Codon position according to the convention of Telenti et al. (15).
[b] The sequence of the codon for the wild type is shown first, and the sequence for the mutant codon is shown in lowercase type.
[c] Single-nucleotide polymorphism cluster groups as determined in references 3 and 6.

many that are found in the respiratory tract (Table 1) to further examine the specificity of the Xpert MTB/RIF assay. Each test was performed with 10^6 genomes per PCR to increase the likelihood of a false-positive test. Our results showed that the Xpert MTB/RIF assay did not mistakenly classify any of these organisms as *M. tuberculosis* (specificity of 100%) (Table 3). None of the organisms except *Nocardia asteroides* produced any C_T of ≤38. *Nocardia cyriageorgica* was detected by a single probe with an average C_T of 38.2. Partial homology between *M. tuberculosis* and *N. asteroides* rpoB primer binding sequences allowed for weak amplification of 10^6 *N. asteroides* genomes and hybridization of two probes with C_T values of 34.9 and 37.2. Although two of the five molecular beacons detected this amplicon, the C_T values of these molecular beacons were consistently >2 cycles apart from each other. This real-time PCR signal did not fulfill the criteria for *M. tuberculosis* detection by the Xpert MTB/RIF assay diagnostic algorithm and thus, were not reported as *M. tuberculosis* by the assay.

Detection of RIF resistance in mixed isolate samples. Patients infected with mixtures of both RIF-susceptible and RIF-resistant populations of *M. tuberculosis* could produce sputum containing a mixture of both strains, complicating resistance detection. We tested the ability of the assay to detect the RIF-resistant fraction of a mixed sample. *M. tuberculosis* DNA with a wild-type *rpoB* sequence was mixed in various ratios with *M. tuberculosis* DNA that contained *rpoB* mutations. Two different *rpoB* mutants were studied. One mutation (*rpoB* 531ttg) caused the molecular beacon complementary to the corresponding wild-type *rpoB* sequence to completely "drop out." The second mutation (*rpoB* 533ccg) caused the molecular beacon complementary to the corresponding wild-type *rpoB* sequence to have a "delayed" C_T, which was sufficient for the Xpert MTB/RIF diagnostic algorithm to identify the mutant as RIF resistant. Our mixing studies demonstrated that the proportion of mutant DNA required for the detection of RIF resistance was dependent on the type of mutation. For the "dropout" 531ttg mutation, the assay could detect the presence of resistance with 95% certainty when the mixture contained at least 65.6% 531ttg DNA (95% CI, 56.9 to 88.4) (Fig. 2A). For the "delay" 533ccg mutation, the assay could not detect the presence of resistance with 95% certainty unless 100% of the DNA was mutant (95% CI, 96.8 to 100%) (Fig. 2B).

Assay response to amplicon contamination. The GenoType MTBDRplus assay is a reverse-hybridization line probe assay that is increasingly used to test for mutations conferring isoniazid and/or rifampin resistance (8). The GenoType MTBDRplus protocol requires substantial manipulation of the assay's PCR product outside closed reaction tubes, increasing the risk for amplicon cross-contamination. The GenoType MTBDRplus and the Xpert MTB/RIF assays amplify a similar region of the *rpoB* gene. Thus, it was theoretically possible that MTBDRplus amplicon contamination of the Xpert MTB/RIF sample could adversely affect the specificity of the Xpert MTB/RIF assay. We sequenced the MTBDRplus amplicon and identified a 168-bp consensus sequence consistent with the approximately 166-bp amplicon length indicated by the GenoType MTBDRplus product insert. We determined that the MTBDRplus amplicon overlapped completely with the Xpert MTB/RIF assay's inner forward primer. However, the amplicon only overlapped the Xpert MTB/RIF assay's outer forward and reverse primers by 13 and 11 bp, respectively (Fig.
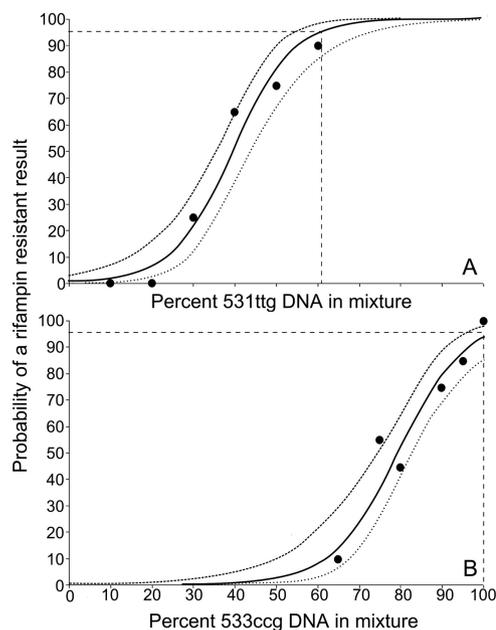

FIG. 2. Minimum detectable fraction of mutant DNA. DNA extracted from RIF-resistant and RIF-susceptible *M. tuberculosis* isolates were mixed to six final concentrations of mutant DNA. Mutant DNA contained either an *rpoB* 531ttg mutation (A) or an *rpoB* 533ccg mutation (B). Sample mixtures equivalent to 20 times the assay limit of detection (LOD) of 4.5 genomes per reaction were added to the chamber receiving eluted DNA in the full cartridge protocol and were processed using a PCR-only protocol. The percentage of samples (*n* = 20) identified as RIF resistant was plotted at each concentration. As determined by logistic regression, there was a 95% probability of detecting RIF resistance when the DNA mixture contained 61.2% or 100% of the total DNA in the sample mixture for a 531ttg and 533ccg mutant, respectively. The curves from top to bottom indicate the upper, middle, and lower bounds of the 95% CI. Dashed lines indicate limit of detection.

3A). We spiked various numbers of a modified MTBDRplus amplicon into *M. tuberculosis*-negative sputum samples (treated with SR), simulating amplicon cross-contamination. Xpert MTB/RIF assays were then performed to test whether the spiked amplicon was falsely identified as tuberculosis. We found that concentrations of modified MTBDRplus less than approximately 10^8 copies per ml of sputum did not produce any false-positive results (Fig. 3B). We postulated that much of the assay specificity could be due to the efficient wash steps occurring within the Xpert MTB/RIF cartridge. Therefore, we also added modified MTBDRplus amplicon directly to the cartridge DNA chamber 10. At least 10^4 copies of MTBDRplus amplicon copies needed to be added to each reaction to cause a false-positive result (C_T < 38), even with the filter and wash steps removed (Fig. 3C). These results suggest that MTBDRplus amplicon cross-contamination is unlikely to adversely affect Xpert MTB/RIF assay specificity.

DISCUSSION

Near-patient molecular assays must be technically simple and robust compared to assays that are performed in specialized laboratories. These assays must also have a clinically relevant dynamic range and show high levels of sensitivity and
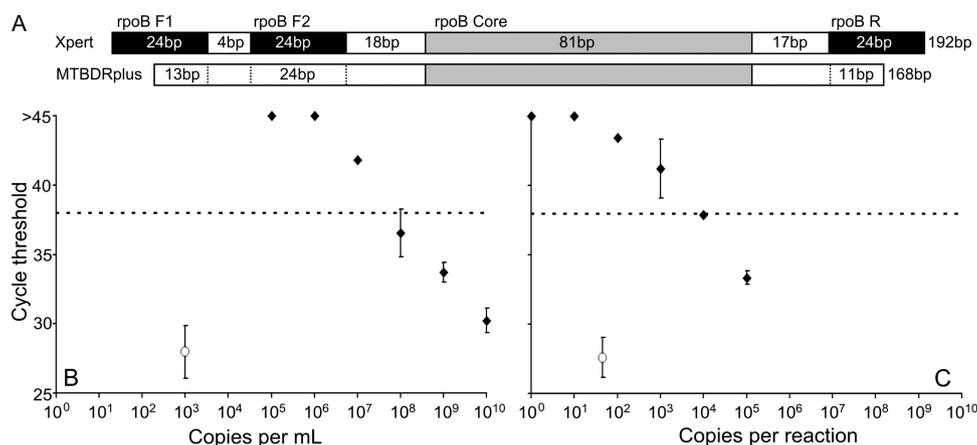
FIG. 3. Potential risk from amplicon contamination. (A) Alignment demonstrating the overlap between the GenoType MTBDRplus PCR amplicon (determined by sequencing) and the priming regions of the Xpert MTB/RIF assay. (B) MTBDRplus simulated amplicon (10^{10} to 10^{5} copies per ml) was spiked into a mixture of sputum and SR prior to transfer to the cartridge sample chamber ($n = 3$ per dilution). At least 10^{8} copies per ml were required to induce a false-positive result. Sputum spiked with 10^{3} CFU of *Mycobacterium bovis* BCG per ml was processed according to the package insert as a control for sputum inhibition (open circle). (C) MTBDRplus amplicon was spiked into TET buffer that also contained *B. globigii* DNA added directly to the elution receiving chamber of an open cartridge and then processed with a PCR-only protocol ($n = 2$ per dilution). At least 10^{4} copies per PCR were required to induce a false-positive result. The control (open circle) was 45 copies of *M. tuberculosis* DNA per reaction. Dotted lines indicate maximum valid C_T values for probe positivity.

specificity. Previous tests using clinical samples had suggested that the Xpert MTB/RIF assay would have an excellent dynamic range and that it would perform well in sensitivity and specificity studies (7); however, these properties had not been systematically examined. The analytical testing that we performed in the current study is a useful complement to clinical investigations because it allows for the simulation and testing of low-probability events in a controlled environment and at defined target concentrations. The Xpert MTB/RIF assay functioned over a wide dynamic range. A wide variety of *M. tuberculosis* strains and common *rpoB* mutations were reliably detected. The specificity against NTM has been established previously (7). Our study shows that specificity is also unlikely to be affected by the presence of nonmycobacterial species or cross-contamination with GenoType MTBDRplus amplicons. Our study also demonstrates that the identification of RIF resistance in mixed samples will depend on the nature of the *rpoB* mutation that is present in the RIF-resistant isolate.

Several prior studies of molecular beacon-based RIF resistance testing suggest that clinical isolates do not usually contain mixtures of RIF-susceptible and RIF-resistant organisms (5, 7, 19). However, mixed infections have been reported (18, 20), and studies of mixed samples are warranted. We found that the ability of the assay to detect mixed samples is dependent on the particular *rpoB* mutation that is present in the mixture. Mutations that effectively block the hybridization of the assay's molecular beacon to the mutant *rpoB* sequence appear to be more easily discernible than those that merely inhibit probe hybridization. For mixtures that contain mutant species with a "dropout" mutation, total fluorescence is almost completely dependent on the fraction of molecular beacon population binding to the available wild-type targets. This allows for a substantial amount of wild-type target to accumulate before the affected molecular beacon reports a C_T within 3.5 cycles of the other unaffected molecular beacons. When the mutant species is a "delay" mutant, the molecular beacon-

mutant target hybrid itself is a substantial contributor to total fluorescence. Under these circumstances, only a small amount of wild-type DNA appears to be required to overcome the delay in C_T that occurs with only mutant DNA present in the sample. Fortunately, most of the common *rpoB* mutations that cause RIF resistance produce probe dropouts rather than delays in the Xpert MTB/RIF assay (7). However, our results suggest that the Xpert MTB/RIF assay should not be used to monitor patients for the emergence of RIF resistance during treatment. Approximately 5% of all RIF resistance is not encoded by mutations within the *rpoB* core region (16), and this last subset of RIF-resistant isolates will not be detectable either in pure culture or in mixed samples by the Xpert MTB/RIF assay.

The GenoType MTBDRplus assay was recently endorsed by the World Health Organization for rapid drug susceptibility testing of *M. tuberculosis*, and it will likely be present in tertiary clinical laboratories where the Xpert MTB/RIF assay might also be deployed (9). We found that the Xpert MTB/RIF assay is relatively resistant to contamination by MTBDRplus amplicons and could safely be used in the same laboratory environment. This is the result of inefficient amplification of the MTBDRplus amplicon, which has only limited overlap with the outer priming regions of the Xpert MTB/RIF assay. Additionally, the most likely route for amplicon entry will be by contaminating the clinical sample or sample-SR mixture. This mixture is filtered and washed within the GeneXpert instrument several times prior to PCR, significantly diluting a potential amplicon contaminant. Other commercial or in-house assays that amplify a larger region of the *rpoB* gene could provide a much more efficient amplification template and could result in increased probability of false-positive results if used without proper contamination controls. The Xpert itself contains amplicons within the sealed cartridge, reducing or eliminating the need for the precautions typically associated with other nucleic acid amplification tests.

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