ORIGINAL ARTICLE

Evaluation of a Rapid Molecular Drug-Susceptibility Test for Tuberculosis

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ABSTRACT

BACKGROUND

Fluoroquinolones and second-line injectable drugs are the backbone of treatment regimens for multidrug-resistant tuberculosis, and resistance to these drugs defines extensively drug-resistant tuberculosis. We assessed the accuracy of an automated, cartridge-based molecular assay for the detection, directly from sputum specimens, of *Mycobacterium tuberculosis* with resistance to fluoroquinolones, aminoglycosides, and isoniazid.

METHODS

We conducted a prospective diagnostic accuracy study to compare the investigational assay against phenotypic drug-susceptibility testing and DNA sequencing among adults in China and South Korea who had symptoms of tuberculosis. The Xpert MTB/RIF assay and sputum culture were performed. *M. tuberculosis* isolates underwent phenotypic drug-susceptibility testing and DNA sequencing of the genes *katG*, *gyrA*, *gyrB*, and *rrs* and of the *eis* and *inhA* promoter regions.

RESULTS

Among the 308 participants who were culture-positive for *M. tuberculosis*, when phenotypic drug-susceptibility testing was used as the reference standard, the sensitivities of the investigational assay for detecting resistance were 83.3% for isoniazid (95% confidence interval [CI], 77.1 to 88.5), 88.4% for ofloxacin (95% CI, 80.2 to 94.1), 87.6% for moxifloxacin at a critical concentration of 0.5 μg per milliliter (95% CI, 79.0 to 93.7), 96.2% for moxifloxacin at a critical concentration of 2.0 μg per milliliter (95% CI, 87.0 to 99.5), 71.4% for kanamycin (95% CI, 56.7 to 83.4), and 70.7% for amikacin (95% CI, 54.5 to 83.9). The specificity of the assay for the detection of phenotypic resistance was 94.3% or greater for all drugs except moxifloxacin at a critical concentration of 2.0 μg per milliliter (specificity, 84.0% [95% CI, 78.9 to 88.3]). When DNA sequencing was used as the reference standard, the sensitivities of the investigational assay for detecting mutations associated with resistance were 98.1% for isoniazid (95% CI, 94.4 to 99.6), 95.8% for fluoroquinolones (95% CI, 89.6 to 98.8), 92.7% for kanamycin (95% CI, 80.1 to 98.5), and 96.8% for amikacin (95% CI, 83.3 to 99.9), and the specificity for all drugs was 99.6% (95% CI, 97.9 to 100) or greater.

CONCLUSIONS

This investigational assay accurately detected *M. tuberculosis* mutations associated with resistance to isoniazid, fluoroquinolones, and aminoglycosides and holds promise as a rapid point-of-care test to guide therapeutic decisions for patients with tuberculosis. (Funded by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, and the Ministry of Science and Technology of China; ClinicalTrials.gov number, NCT02251327.)

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MULTIDRUG-RESISTANT TUBERCULOSIS (defined by resistance to isoniazid and rifampin) is a consequence of ineffective treatment, both at the individual level, when antibiotics are improperly selected or taken, and at the programmatic level, when the use of standard regimens is based on algorithms that may not include drug-susceptibility testing. Incorrect diagnosis and treatment of drug-resistant tuberculosis is associated with morbidity, mortality, and ongoing transmission of infection. For uncomplicated multidrug-resistant tuberculosis, the World Health Organization (WHO) recently endorsed a treatment regimen of 9 to 12 months, as a potential alternative to conventional regimens of 18 to 24 months.[1] Fluoroquinolones and second-line injectable drugs — aminoglycosides and capreomycin — are core components of this shortened regimen. Rapid methods for the detection of susceptibility and resistance to fluoroquinolones and second-line injectable drugs are needed to identify patients who are microbiologically eligible for the shortened regimen. Detection of *Mycobacterium tuberculosis* drug resistance with the use of conventional culture-based phenotypic drug-susceptibility testing is slow and biohazardous and requires substantial laboratory infrastructure and training.

Xpert MTB/RIF (Cepheid) is an integrated, automated, cartridge-based system, used with GeneXpert instrumentation, for the rapid molecular detection of *M. tuberculosis* and mutations associated with rifampin resistance. Xpert MTB/RIF is widely used in tuberculosis programs and has contributed to the global increase in detection of rifampin-resistant tuberculosis.[2] However, for patients with rifampin-resistant tuberculosis, Xpert MTB/RIF provides no further information to guide the selection of appropriate antibiotics or to promptly identify and triage to equipped health care centers those patients who have extensively drug-resistant tuberculosis (defined as multidrug-resistant tuberculosis that is additionally resistant to fluoroquinolones and second-line injectables). *M. tuberculosis* resistance to fluoroquinolones, aminoglycosides, and isoniazid is associated with approximately 25 mutations in six genes and promoter regions.[3-10] We recently described a new cartridge, for use with the GeneXpert platform, for the rapid molecular detection of these mutations.[11] This assay can provide results from unprocessed sputum samples in just over 2 hours, with minimal hands-on technical time. Here, we describe a clinical study to assess the diagnostic accuracy of this investigational assay for the detection of resistance to fluoroquinolones, aminoglycosides, and isoniazid.

METHODS

STUDY DESIGN AND OVERSIGHT

We conducted a blinded, multicenter, prospective diagnostic accuracy study. The investigational assay was the index test, and phenotypic culture-based drug-susceptibility testing and DNA sequencing, considered separately, were the reference standards for resistance detection. The primary objective of the study was to determine the sensitivity and specificity of the investigational assay for the detection of *M. tuberculosis* resistance to isoniazid, moxifloxacin, ofloxacin, amikacin, and kanamycin.

The study was designed, implemented, and supervised by the Tuberculosis Clinical Diagnostics Research Consortium (TB-CDRC), which is made up of academic investigators at participating sites. Members of the TB-CDRC performed statistical analyses, wrote the manuscript, and made the decision to submit the manuscript for publication. All the authors vouch for the accuracy and completeness of the data and analyses and for the fidelity of the study to the protocol, which (with the statistical analysis plan) is available with the full text of this article at NEJM.org.

The investigational assay cartridges, Xpert MTB/RIF cartridges, and 10-color GeneXpert instruments were donated by Cepheid. Cepheid personnel had no role in study design, implementation, data analysis, manuscript writing, or the decision to submit the study findings for publication.

STUDY POPULATION

Adults in Seoul, South Korea, and Zhengzhou, China, who had symptoms of pulmonary tuberculosis were enrolled in the study. Participants were enrolled prospectively into one of two groups — the drug-resistance-risk group and the case-detection group. The inclusion and exclusion criteria for each group are provided in Table S1 in the Supplementary Appendix, available at NEJM.org. All participants provided written informed consent. The ethics committees at the

enrolling sites and Johns Hopkins University approved this study.

STUDY PROCEDURES

For each participant, one investigational assay and one Xpert MTB/RIF test were performed directly on the same sputum specimen; in addition, one smear microscopy test, one mycobacterial liquid culture, and one solid culture were performed after digestion and decontamination of sputum. Participants provided up to two expectorated sputum specimens 1 hour to 4 days apart. If the volume of the first specimen exceeded 3.5 ml, it was homogenized with glass beads (Fisher Scientific) and split into two portions: 1.5 ml for the investigational assay and Xpert MTB/RIF and the remainder for smear and cultures. If the volume of the first specimen was between 1.5 and 3.5 ml, it was used for the investigational assay and Xpert MTB/RIF, and the second sputum specimen was used for smear and culture. Full details of the study design and procedures are provided in the protocol.

XPERT MTB/RIF AND INVESTIGATIONAL ASSAY

The investigational assay cartridge was assembled by Cepheid, using components synthesized or sourced by Cepheid. Sample reagent was added to sputum (in a 2:1 dilution), and 2.0 ml of the resulting mixture was added to one Xpert MTB/RIF and one investigational cartridge.[12] Xpert MTB/RIF and investigational assays were performed with the use of standard four-module GeneXpert instruments. For the investigational assay, the instrument was calibrated to detect 10 fluorescence channels, with the use of research-version software that enabled 10-color melting-temperature analysis. For each probe targeting a resistance-associated genetic region, the melting temperature or temperatures were determined and interpreted as wild-type, mutant (if the measured temperature was different from the known wild-type melting temperature), or heteroresistant (if both wild-type and mutant melting temperatures were present).[11] A specimen was considered to be resistant to a drug if a mutant melting temperature was detected in any of the gene targets associated in high frequency with resistance — namely, *katG* and the *inhA* promoter for isoniazid, *gyrA* for the fluoroquinolones ofloxacin and moxifloxacin, and *rrs* for kanamycin and amikacin.[3,4,8,11,13-18] A specimen with a wild-type or uninterpretable melting temperature in a high-frequency gene target was considered to be susceptible or of indeterminate susceptibility, respectively, to the corresponding drug, unless there was a mutant melting temperature in the low-frequency gene target — namely, *gyrB* for fluoroquinolones and the *eis* promoter for kanamycin.[3,4,8,11,13,15,19,20] The personnel performing the investigational assay were unaware of the other test results. The investigational assay was conducted in real time, but the results were not released to clinicians and therefore did not affect decisions regarding treatment.

MYCOBACTERIAL CULTURES

Sputum was digested with *N*-acetyl-L-cysteine and sodium hydroxide (final concentration, 2%) and processed with the use of standard methods.[21] Smear microscopy was performed with Ziehl–Neelsen staining on the concentrated pellet.[22] A volume of 0.5 ml of the resuspended pellet was inoculated into liquid culture (BACTEC MGIT, BD Microbiology Systems), and 0.2 ml was inoculated onto Löwenstein–Jensen medium. Cultures that were positive for growth of acid-fast bacilli underwent confirmation of the presence of *M. tuberculosis* complex by means of paranitrobenzoic acid (PNB) and thiophen-2-carboxylic acid hydrazide (TCH) testing,[22] MPT64 antigen detection (Capilia TB, Tauns Laboratories), or polymerase chain reaction (MolecuTech MTB-ID V3, YD Diagnostics).

PHENOTYPIC DRUG-SUSCEPTIBILITY TESTING

Indirect drug-susceptibility testing was performed from the first positive *M. tuberculosis* culture with the use of the BACTEC MGIT 960 system. The critical concentrations used for each drug were 0.1 μg per milliliter for isoniazid, 0.5 and 2 μg per milliliter for moxifloxacin, 2 μg per milliliter for ofloxacin, 1 μg per milliliter for amikacin, and 2.5 μg per milliliter for kanamycin.[23]

DNA SEQUENCING

The molecular drug resistance of cultured isolates was characterized by Sanger DNA sequencing (Table S2 in the Supplementary Appendix). DNA was prepared from the same culture that was used for phenotypic drug-susceptibility testing. Each of the six gene targets included in the assay

was sequenced to detect mutations in target and nontarget regions. Sequencing was the reference standard for the detection of heteroresistance, defined as the presence, in a single sample, of both wild-type and mutant sequences for a given gene locus.[11]

STATISTICAL ANALYSIS

The primary objective of the study was to determine the sensitivity and specificity of the investigational assay for the detection of resistance to isoniazid, fluoroquinolones (ofloxacin and moxifloxacin), and aminoglycosides (kanamycin and amikacin). In the primary analyses, the reference standards for phenotypic and molecular drug-susceptibility testing were considered separately, and sensitivity and specificity were calculated for the detection of resistance to each drug.[24] The main analysis population for drug-susceptibility testing, which was made up of participants who were culture-positive for *M. tuberculosis* and who had interpretable results of reference-standard drug-susceptibility testing, included participants who were prospectively enrolled into the drug-resistance-risk and case-detection groups. Secondary analyses of diagnostic accuracy for tuberculosis case detection included only participants in the case-detection group. To explore the diagnostic accuracy of the investigational assay for the testing of patients who are already known to have rifampin-resistant tuberculosis, we conducted a post hoc analysis of a "reflex-test population" that was made up of participants who were included in the main analysis of drug-susceptibility testing and in whose sputum the Xpert MTB/RIF test indicated the presence of rifampin-resistant *M. tuberculosis*. Reference-standard drug-susceptibility testing was performed independently and regardless of the investigational-assay results. Invalid or indeterminate investigational-assay results were enumerated but not included in sensitivity and specificity calculations. The binomial exact method was used to calculate 95% confidence intervals.

RESULTS

PARTICIPANTS

A total of 405 participants were enrolled in the study from June 2014 through June 2015 (Fig. 1). Four enrolled participants produced insufficient sputum for tests and were excluded late in the study. Thus, 401 participants were eligible for inclusion in the study. A total of 93 participants were excluded from the analyses of drug-susceptibility testing, including 79 for whom cultures were negative for *M. tuberculosis*, 2 for whom specimens were mislabeled, 10 for whom *M. tuberculosis* DNA was of insufficient quality or quantity for sequencing, and 2 for whom MGIT drug-susceptibility testing cultures were contaminated. Thus, 308 culture-positive participants were included in the main analysis population for drug-susceptibility testing.

DISTRIBUTION OF PHENOTYPIC AND GENOTYPIC RESISTANCE

Phenotypic drug-susceptibility testing identified 194 of 308 participants (63.0%) as having infections that were resistant to one or more drugs, including 55 participants with multidrug-resistant tuberculosis, 54 with tuberculosis that was multidrug-resistant in addition to being resistant to fluoroquinolones or aminoglycosides, and 39 with extensively drug-resistant tuberculosis (Table 1). DNA sequencing identified 25 different mutations among the six gene targets; 15 of the mutations occurred in isolates from both China and South Korea (Table S3 in the Supplementary Appendix). The most common mutations were *katG* S315T, *gyrA* D94G, *rrs* A1401G, *inhA* C(–15)T, and *gyrA* A90V.

OPERATIONAL CHARACTERISTICS OF THE INVESTIGATIONAL ASSAY

The investigational assay provided no drug-susceptibility testing information for 4 of 308 participants (1.3%) (2 had invalid results, and 2 had no *M. tuberculosis* detected) (Table S4 in the Supplementary Appendix). Among the remaining 304 participants, the investigational-assay results were indeterminate for 18 (1.0%) of 1824 total gene targets. The assay sensitivities for tuberculosis case detection were 96 of 98, or 98.0% (95% confidence interval [CI], 92.8 to 99.8), for the investigational assay and 97 of 99, or 98.0% (95% CI, 92.9 to 99.8), for Xpert MTB/RIF.

INVESTIGATIONAL ASSAY VERSUS PHENOTYPIC DRUG-SUSCEPTIBILITY TESTING

The sensitivity and specificity, respectively, of the investigational assay for the detection of pheno-
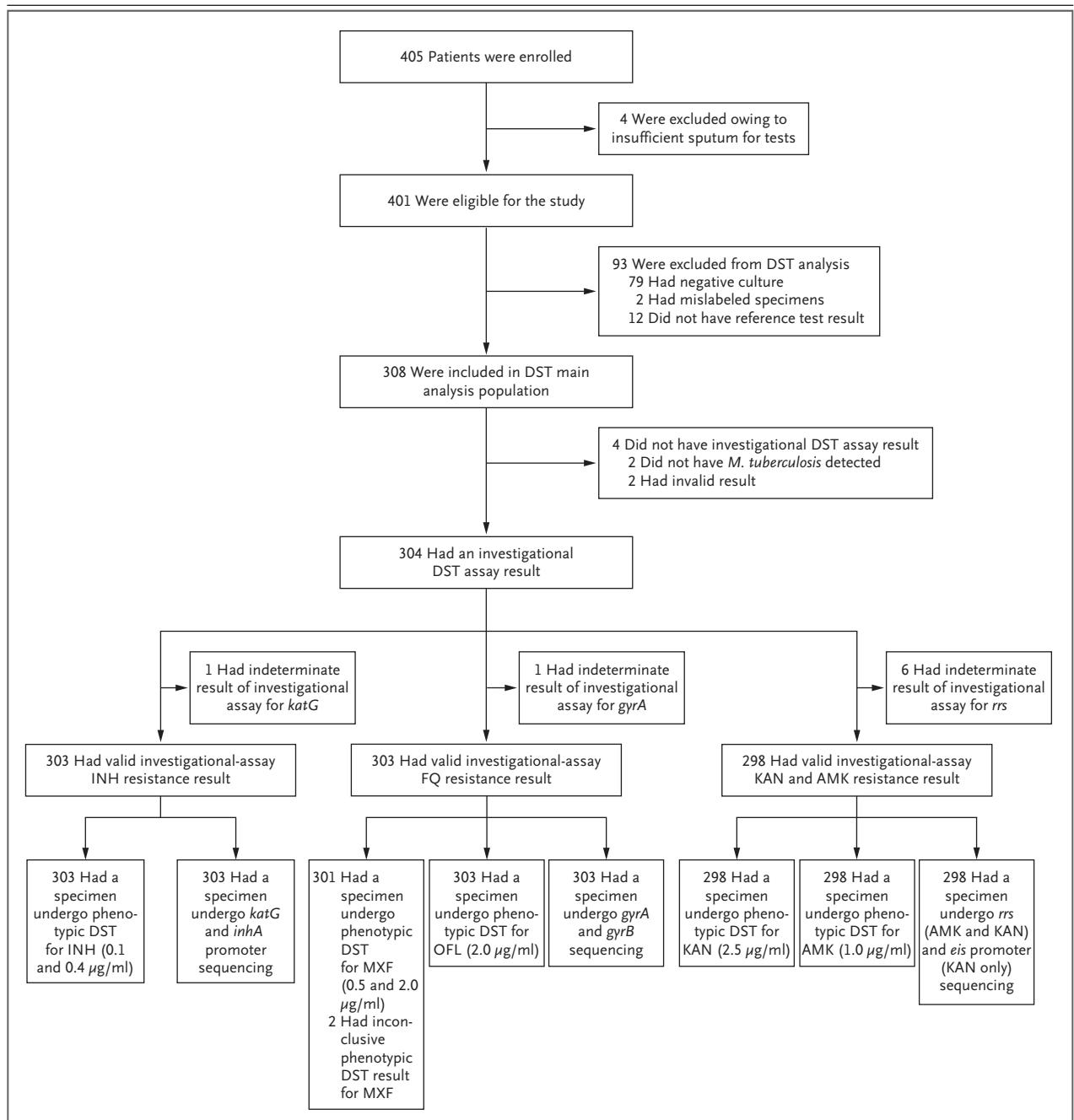
Figure 1. Participant Enrollment and Testing in the Main Analysis Population.

Among the 12 patients who were excluded from the analysis of drug-susceptibility testing (DST) because of a lack of a reference-test result, 10 had DNA that was of insufficient quality or quantity for sequencing, and 2 had uninterpretable MGIT phenotypic DST results because of contamination. Complete DST reference-standard results were not achievable for 4.3% of participants (14 of 322) whose culture was positive for *Mycobacterium tuberculosis*. Among the 308 participants in the main analysis population for DST, 152 were excluded from the reflex-test analysis population (146 with an Xpert MTB/RIF result indicating that rifampin resistance was not detected, 4 with an Xpert MTB/RIF result indicating that *M. tuberculosis* was not detected, and 2 with an indeterminate Xpert MTB/RIF result with regard to rifampin resistance); the reflex-test analysis population therefore included 156 participants. AMK denotes amikacin, FQ fluoroquinolone, INH isoniazid, KAN kanamycin, MXF moxifloxacin, and OFL ofloxacin.

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Table 1. Demographic and Clinical Characteristics of Participants at Enrollment, and Drug-Resistance Status Based on Phenotypic Drug-Susceptibility Testing.

Characteristic	Case-Detection Group*	Drug-Resistance-Risk Group†	All Participants
Demographic and clinical characteristics‡			
Enrolled in China — no./total no. (%)	86/111 (77)	196/290 (68)	282/401 (70)
Enrolled in South Korea — no./total no. (%)	25/111 (23)	94/290 (32)	119/401 (30)
Male sex — no./total no. (%)	72/111 (65)	230/290 (79)	302/401 (75)
Median age (range) — yr	48 (21–82)	47 (18–85)	47 (18–85)
Previous tuberculosis — no./total no. (%)	0/111 (0)	184/290 (63)	184/401 (46)
Receiving tuberculosis treatment at enrollment — no./total no. (%)	96/111 (86)	285/290 (98)	381/401 (95)
All study tests performed from one sputum specimen — no./total no. (%)	72/111 (65)	228/290 (79)	300/401 (75)
Results of testing for *Mycobacterium tuberculosis* — no./total no. (%)			
Study culture positive	99/111 (89)	223/290 (77)	322/401 (80)
Smear microscopy positive for acid-fast bacilli	67/111 (60)	196/290 (68)	263/401 (66)
Smear microscopy negative for acid-fast bacilli	32/111 (29)	27/290 (9)	59/401 (15)
Study culture negative	12/111 (11)	67/290 (23)	79/401 (20)
Drug-resistance status — no./total no. (%)§			
Isoniazid resistance only	13/98 (13)	14/210 (7)	27/308 (9)
Rifampin resistance only	0	2/210 (1)	2/308 (0.6)
Fluoroquinolone resistance only	2/98 (2)	4/210 (2)	6/308 (2)
Aminoglycoside resistance only	1/98 (1)	1/210 (0.5)	2/308 (0.6)
Multidrug resistance only	2/98 (2)	53/210 (25)	55/308 (18)
Multidrug resistance with fluoroquinolone resistance, aminoglycoside susceptibility	0	46/210 (22)	46/308 (15)
Multidrug resistance with aminoglycoside resistance, fluoroquinolone susceptibility	1/98 (1)	7/210 (3)	8/308 (2.5)
Extensively drug resistant	0	39/210 (19)	39/308 (13)
Other polyresistance	2/98 (2)	7/210 (3)	9/308 (3)
No resistance to isoniazid, rifampin, fluoroquinolones, or aminoglycosides	77/98 (79)	37/210 (18)	114/308 (37)

* Participants in this group had suspected or confirmed new pulmonary tuberculosis and had received antituberculosis drugs for less than 3 days.
† Participants in this group either had confirmed pulmonary tuberculosis with documented rifampin resistance and had received antituberculosis drugs for 31 days or less or had a history of tuberculosis plus ongoing signs or symptoms of pulmonary tuberculosis and suspected drug resistance.
‡ Demographic and clinical characteristics are reported for the 401 study-eligible participants.
§ Drug-resistance status is reported for the 308 participants in the main analysis population for drug-susceptibility testing.

typic resistance were 83.3% and 99.2% for isoniazid, 88.4% and 96.6% for ofloxacin, 87.6% and 94.3% for moxifloxacin at a critical concentration of 0.5 μg per milliliter, 96.2% and 84.0% for moxifloxacin at a critical concentration of 2.0 μg per milliliter, 71.4% and 98.4% for kanamycin, and 70.7% and 99.6% for amikacin (Table 2). Almost all isolates that were found by phenotypic drug-susceptibility testing to be resistant but found by the investigational assay to be susceptible (i.e., to have a wild-type melting temperature) were also found to be wild-type by sequencing (Fig. 2A). All but one of the isolates that were found by phenotypic drug-susceptibility testing to be susceptible but were found by the investigational assay to be resistant (i.e., to

Table 2. Sensitivity and Specificity of the Investigational Assay, with Phenotypic Drug-Susceptibility Testing as the Reference Standard, in the Main Analysis Population for Drug-Susceptibility Testing.

Drug	Investigational-Assay Result + Phenotypic Drug-Susceptibility Test Result*				Sensitivity		Specificity	
	R+R	R+S	S+R	S+S	no./total no.	% (95% CI)	no./total no.	% (95% CI)
	no. of specimens							
Isoniazid†	150	1	30	122	150/180	83.3 (77.1–88.5)	122/123	99.2 (95.6–100.0)
Ofloxacin‡	84	7	11	201	84/95	88.4 (80.2–94.1)	201/208	96.6 (93.2–98.6)
Moxifloxacin, 0.5 μg/ml‡§	78	12	11	200	78/89	87.6 (79.0–93.7)	200/212	94.3 (90.3–97.0)
Moxifloxacin, 2.0 μg/ml‡	51	40	2	210	51/53	96.2 (87.0–99.5)	210/250	84.0 (78.9–88.3)
Kanamycin¶	35	4	14	245	35/49	71.4 (56.7–83.4)	245/249	98.4 (96.0–99.6)
Amikacin¶	29	1	12	256	29/41	70.7 (54.5–83.9)	256/257	99.6 (97.9–100.0)

* The numbers of specimens with each combination of results for the investigational assay and the MGIT drug-susceptibility test are shown. R+R indicates specimens found resistant by both methods, R+S specimens found resistant by the investigational assay and susceptible by phenotypic drug-susceptibility testing, S+R specimens found susceptible by the investigational assay and resistant by phenotypic drug-susceptibility testing, and S+S specimens found susceptible by both methods.

† One specimen was excluded because of an indeterminate investigational-assay result for *katG* (it was found to be susceptible to isoniazid by phenotypic drug-susceptibility testing).

‡ One specimen was excluded because of an indeterminate investigational-assay result for *gyrA* (it was found to be susceptible to ofloxacin and moxifloxacin [0.5 μg per milliliter] by phenotypic drug-susceptibility testing).

§ Two specimens were excluded because of missing moxifloxacin (0.5 μg per milliliter) phenotypic drug-susceptibility testing results (one was found resistant and one was found susceptible by the investigational assay).

¶ Six specimens were excluded because of indeterminate investigational-assay results for *rrs* (by phenotypic drug-susceptibility testing, five were found susceptible and one was found resistant to amikacin and kanamycin).

have a mutant melting temperature detected) were found by DNA sequencing to have corresponding resistance mutations (Fig. 2B).
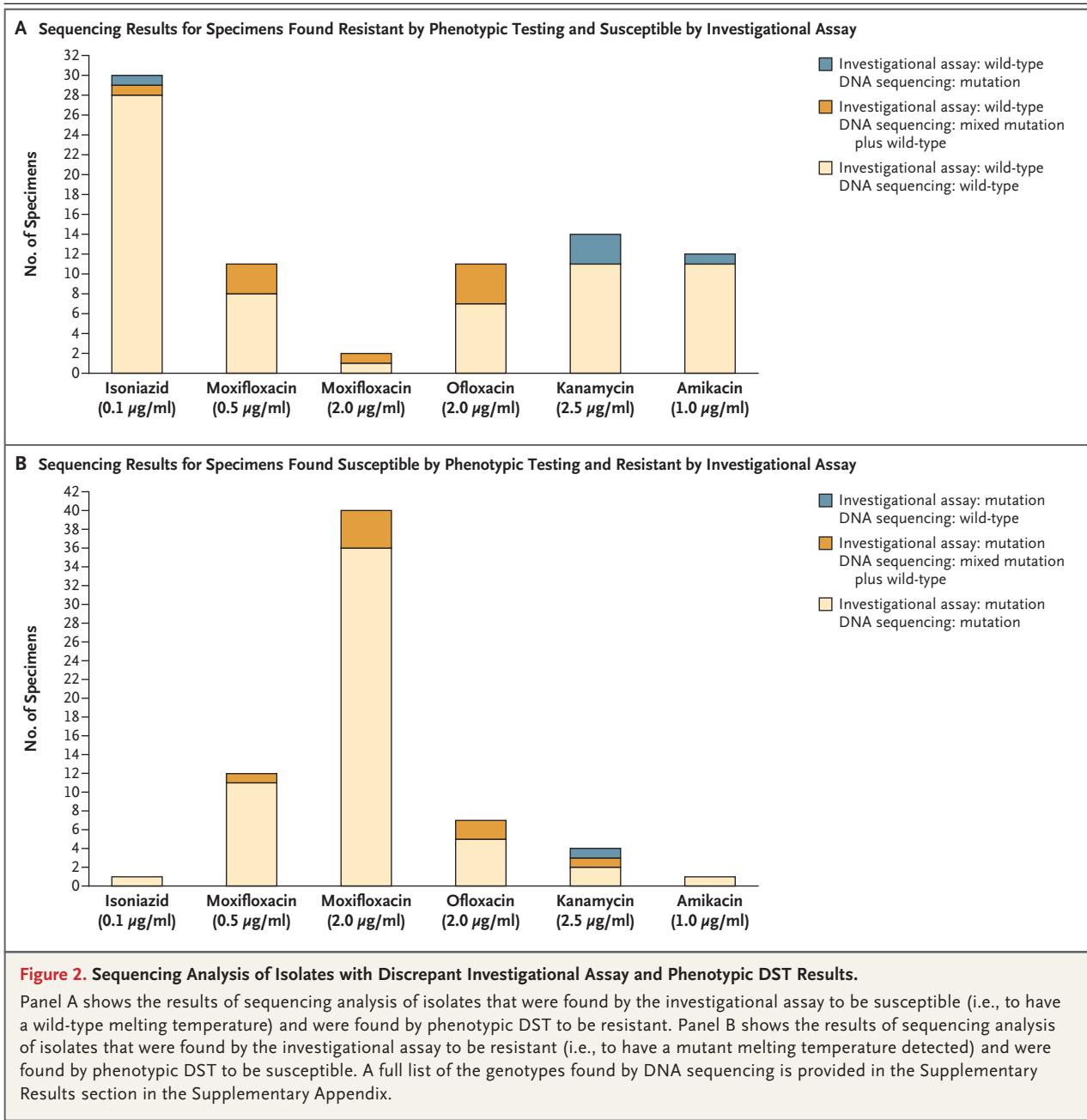
INVESTIGATIONAL ASSAY VERSUS DNA SEQUENCING

The sensitivity and specificity, respectively, of the investigational assay for the detection of resistance mutations were 98.1% and 100.0% for isoniazid, 95.8% and 100.0% for fluoroquinolones, 92.7% and 99.6% for kanamycin, and 96.8% and 100.0% for amikacin (Table 3, and Table S5 in the Supplementary Appendix). Among the 13 specimens that had mutations that were missed by the investigational assay, 10 (76.9%) were identified as heteroresistant by DNA sequencing. The assay successfully detected the mutant population in 16 of 26 (61.5%) sequencing-confirmed heteroresistant bacillary populations, the majority of which were found to be resistant by phenotypic drug-susceptibility testing (Table S6 in the Supplementary Appendix). The diagnostic accuracies of the investigational assay in the reflex-test analysis population are provided in Table S7 in the Supplementary Appendix, and the diagnostic accuracies according

to sputum smear microscopy status and enrollment site are provided in Tables S8 and S9 in the Supplementary Appendix.

IDENTIFICATION OF PATIENTS ELIGIBLE FOR THE SHORTENED TREATMENT REGIMEN

One potential use of the investigational assay would be to determine the eligibility of patients for a shortened treatment regimen for multidrug-resistant tuberculosis. Among patients with multidrug-resistant tuberculosis, the investigational assay correctly identified 48 of 53 patients (90.6%; 95% CI, 79.3 to 96.9) who had tuberculosis without phenotypic resistance either to fluoroquinolones or to aminoglycosides (i.e., patients who were microbiologically eligible for the shortened regimen) and 81 of 92 patients (88.0%; 95% CI, 79.6 to 93.9) who had tuberculosis with phenotypic resistance to fluoroquinolones, aminoglycosides, or both (i.e., patients who were not microbiologically eligible for the shortened regimen). Accordingly, the predictive value of a positive investigational-assay result (resistance detected) for microbiologic unsuitability for the shortened regimen was 81 of 86

A Sequencing Results for Specimens Found Resistant by Phenotypic Testing and Susceptible by Investigational Assay

Legend:
- Investigational assay: wild-type / DNA sequencing: mutation
- Investigational assay: wild-type / DNA sequencing: mixed mutation plus wild-type
- Investigational assay: wild-type / DNA sequencing: wild-type

X-axis categories: Isoniazid (0.1 μg/ml), Moxifloxacin (0.5 μg/ml), Moxifloxacin (2.0 μg/ml), Ofloxacin (2.0 μg/ml), Kanamycin (2.5 μg/ml), Amikacin (1.0 μg/ml)

B Sequencing Results for Specimens Found Susceptible by Phenotypic Testing and Resistant by Investigational Assay

Legend:
- Investigational assay: mutation / DNA sequencing: wild-type
- Investigational assay: mutation / DNA sequencing: mixed mutation plus wild-type
- Investigational assay: mutation / DNA sequencing: mutation

X-axis categories: Isoniazid (0.1 μg/ml), Moxifloxacin (0.5 μg/ml), Moxifloxacin (2.0 μg/ml), Ofloxacin (2.0 μg/ml), Kanamycin (2.5 μg/ml), Amikacin (1.0 μg/ml)

Figure 2. Sequencing Analysis of Isolates with Discrepant Investigational Assay and Phenotypic DST Results.
Panel A shows the results of sequencing analysis of isolates that were found by the investigational assay to be susceptible (i.e., to have a wild-type melting temperature) and were found by phenotypic DST to be resistant. Panel B shows the results of sequencing analysis of isolates that were found by the investigational assay to be resistant (i.e., to have a mutant melting temperature detected) and were found by phenotypic DST to be susceptible. A full list of the genotypes found by DNA sequencing is provided in the Supplementary Results section in the Supplementary Appendix.

(94.2%; 95% CI, 87.0 to 98.1). The predictive value of a negative investigational-assay result (no resistance detected) for microbiologic eligibility for the shortened regimen was 48 of 59 (81.4%; 95% CI, 69.1 to 90.3).

DISCUSSION

In this study, we assessed the clinical diagnostic accuracy of a cartridge-based, automated assay for the rapid detection, directly from sputum specimens with the use of the GeneXpert platform, of *M. tuberculosis* mutations that are associated with resistance to isoniazid, fluoroquinolones, and aminoglycosides. This assay identified known mutations in genetic regions associated with resistance to these drugs with reasonable certainty; the sensitivity estimates were lower when phenotypic culture-based drug-susceptibility testing was used as the reference standard.

Table 3. Sensitivity and Specificity of the Investigational Assay, with DNA Sequencing as the Reference Standard, in the Main Analysis Population for Drug-Susceptibility Testing.*

Drug	Investigational-Assay Result + DNA Sequencing Result†				Sensitivity		Specificity	
	M+M	M+NM	NM+M	NM+NM	no./total no.	% (95% CI)	no./total no.	% (95% CI)
	no. of specimens							
Isoniazid‡	151	0	3	149	151/154	98.1 (94.4–99.6)	149/149	100.0 (97.6–100.0)
Fluoroquinolones§	91	0	4	208	91/95	95.8 (89.6–98.8)	208/208	100.0 (98.2–100.0)
Kanamycin¶	38	1	3	256	38/41	92.7 (80.1–98.5)	256/257	99.6 (97.9–100.0)
Amikacin¶	30	0	1	267	30/31	96.8 (83.3–99.9)	267/267	100.0 (98.6–100.0)

* There are no known silent mutations that occur within the gene regions tested by the investigational assay for resistance. In the study, no silent mutations in these regions were detected either by the investigational assay or by sequencing.
† The numbers of specimens with each combination of results for the investigational assay and DNA sequencing are shown. M+M indicates specimens found to have a mutation by both methods, M+NM specimens found by the investigational assay to have a mutation and found by DNA sequencing to have no mutation, NM+M specimens found by the investigational assay to have no mutation and found by DNA sequencing to have a mutation, and NM+NM specimens found by both methods to have no mutation.
‡ One specimen was excluded because of an indeterminate investigational-assay result for *katG* (also found indeterminate for *katG* by sequencing).
§ Ofloxacin and moxifloxacin are grouped as fluoroquinolones, since *gyrA* and *gyrB* mutations confer resistance to both drugs. One specimen was excluded because of an indeterminate investigational-assay result for *gyrA* (no *gyrA* or *gyrB* mutation was detected by sequencing).
¶ Six specimens were excluded because of an indeterminate investigational-assay result for *rrs* (five with no *rrs* mutation found by sequencing and one with an *rrs* mutation found by sequencing).

Among patients with rifampin-resistant tuberculosis, the predictive value of a positive investigational-assay result for resistance to fluoroquinolones, aminoglycosides, or both was 94%. Although we did not test this strategy, we speculate that a positive test result could be used to triage patients away from the new shortened treatment regimen for multidrug-resistant tuberculosis and toward treatment centers with the capacity for comprehensive drug-susceptibility testing and with experience treating highly drug-resistant tuberculosis.

The WHO has set targets for the diagnostic sensitivity and specificity of next-generation molecular drug-susceptibility tests, with sequencing used as the reference standard, of 95% and 98%, respectively.[24] The investigational assay described here met the sensitivity target for isoniazid, fluoroquinolones, and amikacin and missed the sensitivity target for kanamycin by approximately 2 percentage points. The investigational assay met the specificity target for all tested drugs.

Not surprisingly, the sensitivity of the investigational genotypic assay to detect resistance was lower when culture-based phenotypic drug-susceptibility testing was considered as the reference comparator. Most isolates that were found to be resistant by phenotypic drug-susceptibility testing but were found to be wild-type (and thus susceptible) by the investigational assay were also found to be wild-type by DNA sequencing. Our results are consistent with those reported in studies in which other molecular assays were used for *M. tuberculosis* drug-susceptibility testing.[25-30] There are at least two potential causes of the phenotypic–genotypic discrepancies — alternative molecular mechanisms of resistance, many of which are still unknown, and limitations of the critical-concentration methods used for phenotypic testing, such that up to 5% of wild-type *M. tuberculosis* strains are categorized as drug-resistant.[31]

Phenotypic–genotypic discrepancy was the driver of the lower-than-ideal predictive value of a negative investigational test (no resistance detected) for microbiologic eligibility for the shortened regimen. If the investigational assay were used as the sole test for triaging patients with rifampin-resistant tuberculosis onto or away from the shorter treatment regimen for multidrug-resistant tuberculosis, approximately 20% of patients who have tuberculosis with phenotypic resistance to fluoroquinolones, aminoglycosides, or both would be inappropriately triaged to receive the shorter regimen. However, this is predicted to be a shortcoming of any molecular test that interrogates the set of common resistance-associated genetic loci targeted by the investiga-

tional assay. Currently, there are no other rapid, point-of-care tests to facilitate evidence-based treatment selection for patients with rifampin-resistant tuberculosis.

We identified sequencing-confirmed resistance mutations in some phenotypically susceptible isolates. This phenomenon was most commonly observed for fluoroquinolones, especially moxifloxacin, and resulted in the investigational assay having a lower specificity than culture when the moxifloxacin critical concentration of 2.0 μg per milliliter was used. This situation was almost always associated with *gyrA* A90V or S91P mutations, which confer low-level moxifloxacin resistance, with minimum inhibitory concentrations (MICs) of 0.25 to 1.0 μg per milliliter.[16,32] Although these MICs are higher than those of wild-type *M. tuberculosis*, isolates with *gyrA* A90V or S91P mutations are potentially treatable with increased moxifloxacin dosing,[33,34] and these mutations can be distinguished from other *gyrA* mutations with the use of the investigational assay.[11] Heteroresistance was detected by the investigational assay in some phenotypically susceptible isolates. Mixed populations of susceptible and resistant bacilli can be clinically relevant, since the unmasking of drug resistance can occur during treatment.[35,36] Thus, molecular diagnostics can provide important information for optimizing treatment that is not revealed by dichotomous results of critical concentration–based phenotypic drug-susceptibility testing and does not become available in a timely manner when agar-proportion-method testing is used.

Our study has several limitations. First, most participants with culture-confirmed tuberculosis had high sputum bacillary burdens, as evidenced by the high proportion of specimens that were found to be positive on smear microscopy; the results of our study will need to be confirmed in more patients with smear-negative tuberculosis and with a reference standard of multiple cultures. However, the completion of all tests from one sputum specimen for most participants minimized any potential effect of between-specimen heterogeneity. Second, the geographic representation of participants and *M. tuberculosis* strains was limited. However, DNA sequencing confirmed that the infecting organisms in the study population contained a diverse set of mutations that are prevalent in other geographic areas. Third, although we did not evaluate capreomycin,

a cyclic peptide second-line injectable drug, the *rrs* A1401G mutation detected by the investigational assay accounts for the majority of capreomycin resistance when a molecular mechanism is identified.[8] Finally, phenotypic drug-susceptibility testing was performed only at critical concentrations; MICs were not established, and therefore the full breadth of phenotypic–genotypic relationships was not assessed.

Our results compare favorably with published results for the newest versions of the Genotype MTBDR line probe assays (Hain Lifescience), although we did not perform Genotype MTBDR assays.[28,30,37] The investigational assay and Genotype MTBDRsl, version 2.0, share most genetic targets for the detection of fluoroquinolone and aminoglycoside resistance.[28,37] The line-probe assays, which are recommended by the WHO to assess candidacy for the shortened treatment regimen for multidrug-resistant tuberculosis, require a minimum of 4 to 6 hours before a result is obtained, which typically precludes same-day therapeutic decision-making; are largely confined to reference centers that meet laboratory-infrastructure and assay training requirements; and are inadequately sensitive when used to test smear-negative sputum specimens from patients with tuberculosis.[30,38] In contrast, a result of the investigational assay can be obtained in 2 hours, the assay was found to preserve sensitivity when applied to smear-negative sputum, and it incorporates the same single-step specimen processing as the Xpert MTB/RIF, which can be implemented with minimal staff training and biosafety requirements.[2] Existing GeneXpert instruments have the potential to be upgraded to run both the investigational assay and the Xpert MTB/RIF assay by upgrading software and performing a 10-color calibration, allowing both assays to be run contemporaneously with one instrument. These features should permit the investigational assay to be used in peripheral sectors of the global health care system, where more rapid identification of extended drug resistance may improve therapeutic decision-making.

APPENDIX

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