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A Novel Reporter Phage To Detect Tuberculosis and Rifampin Resistance in a High-HIV-Burden Population

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Improved diagnostics and drug susceptibility testing for *Mycobacterium tuberculosis* are urgently needed. We developed a more powerful mycobacteriophage (Φ^2GFP10) with a fluorescent reporter. Fluorescence-activated cell sorting (FACS) allows for rapid enumeration of metabolically active bacilli after phage infection. We compared the reporter phage assay to GeneXpert MTB/RIF for detection of *M. tuberculosis* and rifampin (RIF) resistance in sputum. Patients suspected to have tuberculosis were prospectively enrolled in Durban, South Africa. Sputum was incubated with Φ^2GFP10, in the presence and absence of RIF, and bacilli were enumerated using FACS. Sensitivity and specificity were compared to those of GeneXpert MTB/RIF with an *M. tuberculosis* culture as the reference standard. A total of 158 patients were prospectively enrolled. Overall sensitivity for *M. tuberculosis* was 95.90% (95% confidence interval (CI), 90.69% to 98.64%), and specificity was 83.33% (95% CI, 67.18% to 93.59%). In acid-fast bacillus (AFB)-negative sputum, sensitivity was 88.89% (95% CI, 73.92% to 96.82%), and specificity was 83.33% (95% CI, 67.18% to 93.59%). Sensitivity for RIF-resistant *M. tuberculosis* in AFB-negative sputum was 90.00% (95% CI, 55.46% to 98.34%), and specificity was 91.94% (95% CI, 82.16% to 97.30%). Compared to GeneXpert, the reporter phage was more sensitive in AFB smear-negative sputum, but specificity was lower. The Φ^2GFP10 reporter phage showed high sensitivity for detection of *M. tuberculosis* and RIF resistance, including in AFB-negative sputum, and has the potential to improve phenotypic testing for complex drug resistance, paucibacillary sputum, response to treatment, and detection of mixed infection in clinical specimens.

Mycobacterium tuberculosis remains a leading cause of death worldwide (1–3). While the overall global tuberculosis (TB) incidence has declined over the past decade, there are increasing numbers of drug-resistant TB cases, and TB incidence in the countries of southern Africa where HIV is endemic has reached levels not seen in the United States or Western Europe since the turn of the last century (3–6).

Drug-resistant TB remains a challenge to TB control due to increased mortality, poor treatment outcomes, and a lack of rapid comprehensive drug susceptibility testing (DST) (7–9). Where the epidemics of drug-resistant TB and TB-HIV have converged, most notably in southern Africa, the major diagnostic challenge is to perform comprehensive DST in paucibacillary specimens (10–12).

GeneXpert MTB/RIF (GeneXpert) is an automated real-time PCR system for the detection of *M. tuberculosis* and rifampin (RIF) resistance. The strengths of GeneXpert include rapidity, ease of use and interpretation, biohazard containment, and diagnostic accuracy in acid-fast bacillus (AFB) smear-positive sputum (13, 14). However, GeneXpert has significant limitations, including the inability to detect second-line or complex TB drug resistance, distinguish between viable and nonviable *M. tuberculosis*, and detect and distinguish minority *M. tuberculosis* subpopulations (mixed or heteroresistant) (15–17). Importantly, GeneXpert has decreased sensitivity in AFB smear-negative sputum, which is common in TB-HIV (16).

Phenotypic diagnostics have the potential to perform DST for drugs with complex or uncharacterized genetic mechanisms of resistance, quantify level of resistance, distinguish viable from nonviable *M. tuberculosis*, and detect mixed infection (18). Phenotypic assays are rapidly adapted to new drugs and have the potential to measure treatment response. To date, however, with few exceptions, phenotypic assays have been slow and/or cumbersome to implement. Without rapid, comprehensive DST, patients may start suboptimal regimens leading to amplification of TB drug resistance (19–21).

Our group has extensive experience with the construction of genetically modified mycobacterium-specific viruses (mycobacteriophages) for the genetic manipulation of *M. tuberculosis* (18, 21, 22). We recently described the construction of a new, more powerful reporter phage, Φ^2GFP10, which uses a more efficient promoter and a more powerful fluorescence reporter to al-

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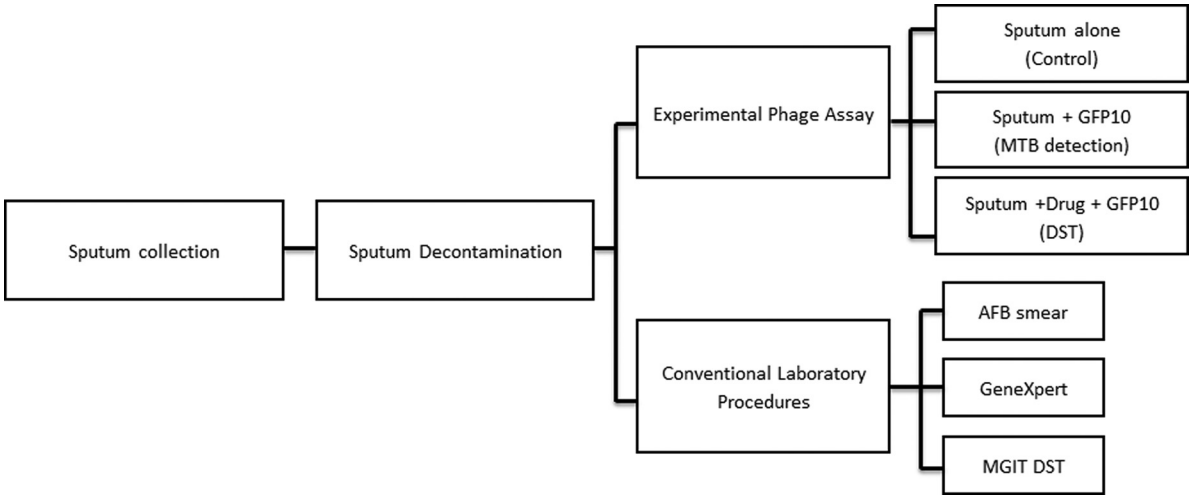

FIG 1 Schematic representation of study procedures. After sputum was collected at the clinic, it was brought to the experimental lab, decontaminated, and split for testing by reporter phage (Φ^2GFP10) and conventional testing, including GeneXpert MTB/RIF. The gold standard was a combination of MGIT and agar culture; all DST was done in solid media.

low direct visualization of individual metabolically active bacilli using fluorescence microscopy or fluorescence-activated cell sorting (FACS), including in clinical sputum (23). The Φ^2GFP10 phage allows for rapid detection of *M. tuberculosis* and phenotypic DST in clinical sputum samples, including down to paucibacillary concentrations ($<10^4$ *M. tuberculosis* bacilli/ml). We performed this study to assess the accuracy of the Φ^2GFP10 phage to detect *M. tuberculosis* bacilli and assess phenotypic drug resistance in the sputum of TB suspects with and without HIV infection.

MATERIALS AND METHODS

Study population. Adult TB suspects were enrolled at an outpatient municipal chest clinic (Prince Cyril Zulu Communicable Disease Clinic) and a regional TB referral hospital (King DinuZulu Hospital Complex) in Durban, South Africa from November 2012 through May 2013. Study staff screened consecutive patients presenting with suspected TB based on eligibility criteria. Adult patients were eligible to participate if they were identified by clinic staff as pulmonary TB suspects, were not currently taking TB medications, and had a documented sputum AFB smear result and/or a Gene Xpert MTB/RIF result in the clinic system. Initially, consecutive patients with a clinic system result of AFB smear-positive or GeneXpert-positive were preferentially enrolled to improve the efficiency of the study ($n = 132$). Subsequently, consecutive AFB smear-negative patients were enrolled ($n = 41$). Study staff interviewed the

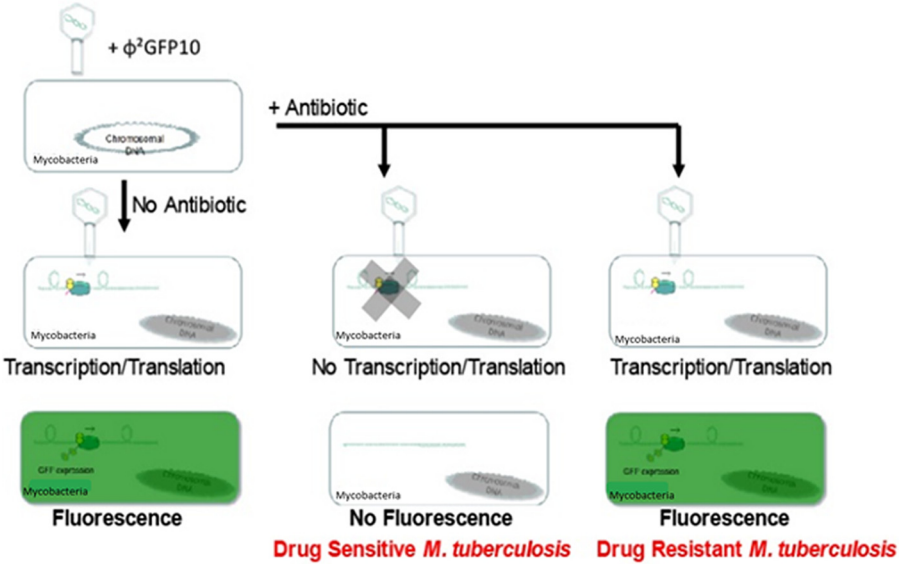

FIG 2 Schematic showing phenotypic drug susceptibility testing using reporter phage. Initially, mycobacteria are infected with reporter phage (Φ^2GFP10). In the absence of antibiotics, the virus transcribes a fluorescent reporter gene (*mVenus*), and green fluorescent protein (GFP) is produced. In the presence of an antibiotic to which *M. tuberculosis* is susceptible, transcription and translation is inhibited and fluorescence is markedly inhibited. In drug-resistant *M. tuberculosis*, inoculation with an antibiotic to which the organism is resistant does not cause attenuation of the fluorescent signal.

patients using a standardized questionnaire. Demographic, symptom, and clinical data were recorded, including prior TB history. HIV counseling and testing was offered to all patients as per routine clinical practice. CD4 T-cell counts were obtained, and antiretroviral therapy was offered at the discretion of the treating physician.

All data were collected prospectively. Approval for the study was obtained from ethics review boards at the University of KwaZulu-Natal, Albert Einstein College of Medicine, and the KwaZulu-Natal Department of Health and the eThekwini Municipality. Written informed consent was obtained from all participants.

Laboratory methods. After enrollment, a single sputum sample was obtained from each participant. The quality of the sputum was assessed by the study staff, descriptively and in terms of volume. Sputum was transported using universal precautions to the KwaZulu-Natal Research Institute for TB/HIV biosafety level 3 (BSL3) laboratory within 6 h after collection. The sputum was decontaminated and liquefied using standard *N*-acetyl-L-cysteine-sodium hydroxide methods, centrifuged, and resuspended in buffered solution. Ziehl-Neelsen (ZN) staining was performed. AFB bacillary burden was graded as negative, scanty, 1+, 2+, or 3+ according to standard definitions. Subsequently, the sample was divided for parallel testing with standard TB culture techniques, GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA), and the Φ^2GFP10 phage assay. Technicians certified by the Health Professions Council of South Africa performed all laboratory testing (Fig. 1).

Standard TB culture techniques included Middlebrook 7H10 agar plates and Bactec mycobacterial growth indicator tube (MGIT) 960 broth (Becton Dickinson, Sparks, MD, USA). Middlebrook agar plates were read at 3 weeks and 6 weeks for *M. tuberculosis* growth. MGIT tubes were monitored for 42 days for *M. tuberculosis* growth. MGIT cultures that were contaminated were decontaminated and recultured. All positive cultures by MGIT were identified as *M. tuberculosis* isolates by examining for characteristic cording and repeat positive ZN staining. Additional evidence that the samples were not nontuberculous mycobacteria (NTM) species included a GeneXpert result identifying *M. tuberculosis* and by drug susceptibility pattern.

GeneXpert MTB/RIF was performed on sputum according to the manufacturer's protocol. Briefly, the sample reagent was mixed at a three to one ratio with sputum and homogenized. If the sample volume was <1 ml, sterile phosphate buffer (Merck, USA) was added to bring the final volume to 1 ml. Two milliliters of homogenized mixture was transferred into an GeneXpert MTB/RIF assay cartridge. Results were obtained after the end of the run, and qualitative and quantitative outputs were recorded. Microbiology lab staff was blinded to clinical information and the initial AFB status.

Φ^2GFP10 phage assay. The Φ^2GFP10 phage expressing the mVenus fluorescent protein from the P_L promoter of the L5 mycobacteriophage was used for *M. tuberculosis* detection and DST on patient samples (23). A high level of fluorescence in the sputum + phage well indicates the presence of metabolically active *M. tuberculosis* isolates; the persistence of fluorescence in the presence of antibiotic indicates *M. tuberculosis* isolates which are resistant to the antibiotic of interest (Fig. 2).

The aliquot of the decontaminated sputum sample for the Φ^2GFP10 phage assay, collected during the week (Wednesday to Friday), was resuspended in 7H9 medium and incubated at 37°C prior to assay. On the following Monday, each specimen was incubated with rifampin and loaded alongside two no-drug controls on a 96-well plate. After overnight incubation the Φ^2GFP10 phage was added to the rifampin-treated well and one of the no-drug controls. The additional no-drug well and a phage-only well were used as negative controls. Laboratory grown *M. tuberculosis* culture was used as a positive-control in each plate. The plate was read on a BD Accuri C6 Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) after 24 h using automated settings. Fluorescence was measured by enumerating fluorescent

TABLE 1 Demographic and clinical characteristics of enrolled participants

Demographic	TB patient data[a]
Gender	
Male	82 (52.9)
Female	73 (47.1)
Median age (range) (yr)	33 (18–63)
HIV status	
Positive	79 (51.0)
Negative	45 (29.0)
Unknown	34 (20.0)
Taking antiretroviral therapy[b]	
Yes	28 (35.4)
No	51 (64.6)
CD4 T-cell count[b] (no. of cells/mm^3)	
Known	38 (48.1)
Unknown	41 (51.9)
Median CD4 count (IQR)	237 (127–384)
Previous TB treatment	
Yes	44 (28.4)
No	110 (71.2)
Unknown	1 (0.6)
Smear status	
Negative	63 (40.6)
Scanty	9 (5.8)
1+	12 (7.7)
2+	25 (16.1)
3+	46 (29.7)
Liquid culture status[c]	
Positive	119 (75.3)
Negative	39 (24.7)
MDR-TB	
Yes	30 (19.0)
No	128 (81.0)
TB symptoms	
Cough	127 (95.5)
Sweats	99 (74.4)
Weight loss	110 (83.3)
Any[d]	131 (98.5)

[a] Data shown are number (%) unless otherwise specified.
[b] Among HIV-infected patients.
[c] Three patients were MTB positive on solid medium culture only, due to contamination of liquid culture.
[d] Cough, sweats, and/or weight loss.

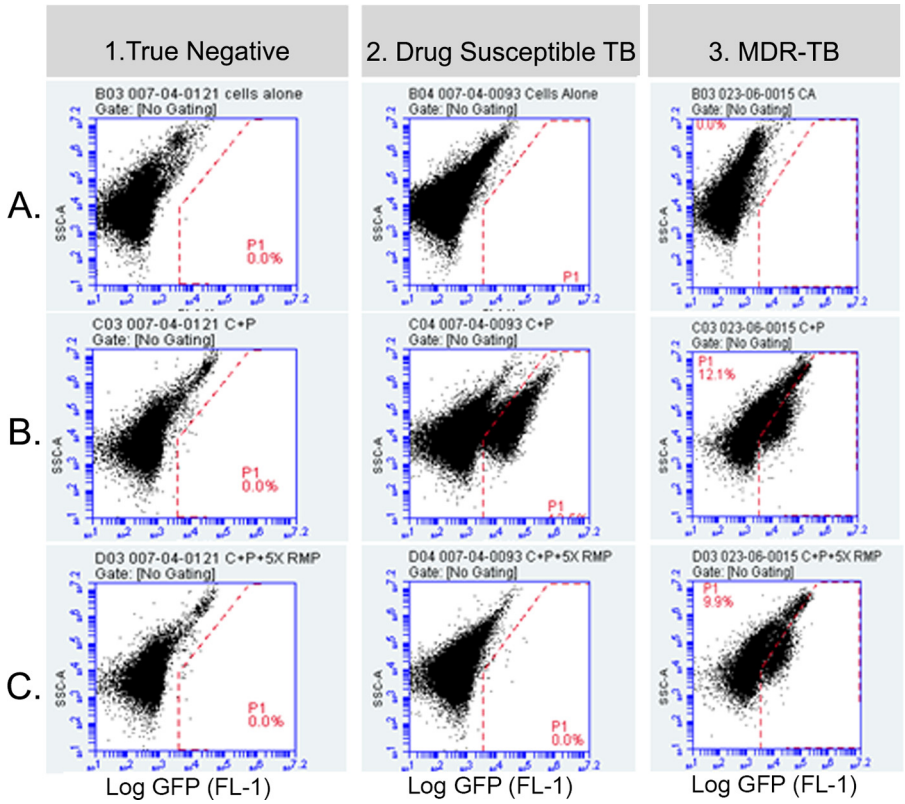

FIG 3 Flow cytometry (FACS) analysis of 3 representative clinical sputum samples. Flow cytometry plots showing side scatter (SSC) (*y* axis) plotted against green fluorescence protein (Log GFP) (*x* axis). Columns represent sputum from 3 patients. Column 1 is sputum from a patient who did not have TB (true negative). Column 2 is sputum from a patient with drug-susceptible TB (drug-susceptible TB). Column 3 is sputum from a patient with multidrug-resistant TB (MDR-TB). Row A, sputum alone; Row B, sputum + reporter phage; Row C, sputum + reporter phage + RIF. FACS gate is the red dashed line. For the true negative, there is no increase in gated events after inoculation with reporter phage (1B) because there is no viable *M. tuberculosis* in sputum. For the drug-susceptible TB patient, fluorescent events increase after the addition of reporter phage (2B) but decrease with RIF (2C) indicating RIF-susceptibility. For the MDR-TB patient, there is an increase in gated events after reporter phage (3B) and no substantial decrease after RIF (3C) indicating RIF resistance.

events by flow cytometry gating and cutoffs for positive results were defined numerically.

Fluorescence-activated cell sorting (FACS) gating. FACS gates and FACS thresholds were constructed based on preliminary experiments in TB culture and spiked sputum using mycobacteria, which constitutively expressed green fluorescent protein (24). Briefly, the FACS gate was used to count the number of gated fluorescent events in each experiment, which fell into the size and fluorescent intensity characteristic of mycobacteria. *M. tuberculosis* bacilli were detected based on increased numbers of fluorescent events that fell within a FACS gate constructed using side scatter (SSC) and fluorescent intensity in the FL-1 channel. Optimal experimental conditions were determined by various incubation times, detergents, and drug concentrations while analyzing FACS results. Relatively high FACS SSC thresholding was needed in sputum to improve the fluorescent signal to noise ratio. A standardized, invariant FACS gating strategy was used for all samples to acquire and analyze results throughout the study.

Outcomes. The primary outcome was comparison of the Φ^2GFP10 reporter phage result to that of a composite of liquid culture and solid agar culture performed in the experimental laboratory on the same sputum sample. If either MGIT or agar was positive for detection of *M. tuberculosis* isolates, the reference standard was considered positive. To determine rifampin resistance, drug susceptibility testing was done on 7H10 according to standard protocols and techniques. A secondary outcome was comparison to GeneXpert MTB/RIF performed on the same sputum sample. Outcomes were stratified by sputum bacillary load grade (smear status) and by HIV status.

Statistical methods. Receiver operating characteristic (ROC) curves were calculated by plotting the sensitivity against 1-specificity for all values of gated fluorescent events (representing fluorescent *M. tuberculosis*). Separate cut points were determined for detection of *M. tuberculosis* and detection of RIF resistance. For detection of *M. tuberculosis*, cut points were chosen to optimize sensitivity while preserving specificity. For detection of RIF resistance, cut points were chosen to optimize specificity while preserving sensitivity. Sensitivity, specificity, and 95% confidence intervals (CI) were calculated using standard methods. ROC curves were calculated using SPSS (Version 21.0; IBM Corp., Armonk, NY).

Infection control methods. Running a sample through the flow cytometer may create an aerosol. Therefore, the flow cytometer was kept under a biosafety hood in a BSL3 facility. Preliminary experiments with nonpathogenic mycobacteria revealed no contamination outside the biosafety hood using this approach. All

BSL3 procedures and protocols were observed. The flow cytometer used in these experiments was exclusively used with mycobacteria. Periodic decontamination was performed as per manufacturer's recommendations.

RESULTS

A total of 173 patients were prospectively enrolled (148 at the municipal chest clinic and 25 at a TB referral hospital). Sputum was collected from all 173 patients and transported to the lab for analysis (median sputum volume, 2 ml; interquartile range [IQR], 1 to 3 ml). Of these, 10 samples did not have the phage assay performed (FACS analysis was not performed in a timely fashion), and 5 did not have interpretable *M. tuberculosis* culture results. Phage assay was performed for 158 samples, which were paired with a reference *M. tuberculosis* culture sample and were included in the present study. Of these, 155 had an interpretable GeneXpert MTB/RIF result. Complete demographic and clinical data were collected on 155/158 patients. The mean time to results for the phage assay was 7.5 days (range, 5 to 9 days) versus 18.2 days for MGIT (IQR, 6.7 to 42 days) among patients who did not have initial contamination.

Patients were more likely to be male (52.9%) than female (47.1%), younger (median age, 33 years), and had a substantial burden of HIV disease (63.7% of patients were HIV tested). Many had not had previous HIV testing, and of those tested only a minority were on antiretroviral therapy (ART) (35.4%), probably reflecting the fact that many patients were presenting at a primary care setting (Table 1).

The reporter phage assay allowed us to accurately distinguish between true-negative patients (who did not have TB), drug-susceptible TB patients, and multidrug-resistant TB (MDR-TB) patients (RIF resistant) (Fig. 3). We evaluated the accuracy of the Φ^2GFP10 reporter phage assay by calculating ROC curves and area under the curve (AUC) by means of c-statistic. A standardized invariant FACS gate had excellent test characteristics in detecting *M. tuberculosis* (c-statistic, 0.936) and RIF-resistant *M. tuberculosis* (c-statistic, 0.935). We looked at paucibacillary samples in two ways, by AFB smear status and by time to TB culture positivity (TTP) in liquid growth medium (25). AFB smear-negative and high-TTP samples test characteristics were excellent for detection of *M. tuberculosis* and RIF resistance (see Fig. S1 and S2 in the supplemental material).

In AFB smear-positive patients, the sensitivity of the Φ^2GFP10 reporter phage was 95.90% (95% CI, 90.69% to 98.64%), and in AFB smear-negative patients, it was 88.89% (95% CI, 73.92% to 96.82%). Specificity was not estimable in smear-positive patients since all had TB by MGIT culture; specificity was 83.33% (95% CI, 67.18% to 93.59%) in AFB smear-negative patients. With respect to detecting RIF-resistant *M. tuberculosis*, the sensitivity in smear-positive sputum was 85.00% (95% CI, 62.08% to 96.62%), and the specificity was 84.85% (95% CI, 73.89% to 92.47%). In smear-negative sputum, the sensitivity for detecting RIF-resistant *M. tuberculosis* was 90.00% (95% CI, 55.46% to 98.34%), and the specificity was 91.94% (95% CI, 82.16% to 97.30%). When we stratified by high TTP as a proxy for bacillary load, the reporter phage test characteristics were similar to the reported values for AFB smear-negative sputum. The majority of HIV-infected patients were AFB smear-negative (58.3%). In the HIV-coinfected strata, sensitivity of the reporter phage was 84.21% (95% CI, 72.13% to 92.50%) to detect *M. tuberculosis* and 90.00% (95% CI, 68.26% to 98.47%) to detect rifampin resistance (Table 2).

TABLE 2 Sensitivity and specificity of reporter phage assay (Φ^2GFP10) to detect *Mycobacterium tuberculosis* and rifampin resistance compared to combined reference standard and Gene Xpert MTB/RIF[a]

Detection sensitivity and specificity	Reporter phage assay data	Gene Xpert MTB/RIF data
Detection of MTB		
All patients	122/158	119/155
Sensitivity	95.90 (90.69–98.64)	94.12 (88.25–97.59)
Specificity	83.33 (67.18–93.59)	97.22 (85.42–99.54)
AFB smear positive	92/94	91/92
Sensitivity	98.84 (93.67–99.81)	100.0 (95.94–100)
Specificity	[b]	[b]
AFB smear negative	30/64	28/63
Sensitivity	88.89 (73.92–96.82)	75.00 (55.12–89.26)
Specificity	83.33 (67.18–93.59)	97.14 (85.03–99.52)
HIV positive	53/79	53/79
Sensitivity	84.21 (72.13–92.50)	92.45 (81.77–97.86)
Specificity	81.82 (59.70–94.70)	95.65 (77.98- 99.27)
Detection of rifampin resistance		
All patients	26/158	24/155
Sensitivity	86.67 (69.26–96.16)	83.33 (62.60–90.97)
Specificity	88.28% (81.41–93.29)	95.16 (94.19–99.75)
AFB smear positive	19/94	19/92
Sensitivity	85.00 (62.08–96.62)	94.74% (73.90–99.12)
Specificity	84.85 (73.89–92.47)	98.63 (92.57–99.77)
AFB smear negative	7/64	5/63
Sensitivity	90.00 (55.46–98.34)	40.00 (6.49–84.60)
Specificity	91.94 (82.16–97.30)	98.28 (90.73–99.71)
HIV positive	17/79	17/79
Sensitivity	90.00 (68.26–98.47)	82.35 (56.55–95.99)
Specificity	88.14 (77.06–95.07)	100.0 (94.17–100)

[a] Combined reference standard, MGIT 960 + agar culture and DST performed in experimental lab on the same sputum sample as for the reporter phage assay. Data shown are number of patients/total no. of patients or percent (95% CI).
[b] No specificity value since all AFB smear positives were combined reference MTB positive.

DISCUSSION

This study demonstrates the excellent test characteristics of a next-generation reporter mycobacteriophage, Φ^2GFP10, in detection of *M. tuberculosis* and rifampin resistance in clinical sputum. This study was performed in a high-burden HIV setting with a substantial burden of HIV-infected participants and AFB smear-negative sputum. The reporter phage performed extremely well in HIV-infected patients and in AFB smear-negative samples, highlighting its potential role in this critical setting. Compared to GeneXpert MTB/RIF, the reporter phage had improved sensitivity in AFB smear-negative sputum; however, specificity was lower. The reporter phage also provided additional information compared to that of GeneXpert and liquid TB culture. Using the reporter phage in combination with GeneXpert would yield an additional 4 diagnosed cases of MDR-TB in this study. One case found to be RIF resistant by Xpert and Φ^2GFP10 was initially drug-susceptible on conventional DST. When conventional DST was repeated, it was found to in fact be MDR-TB.

Comparative advantages for the phage assay to liquid *M. tuberculosis* culture include superior time to results for TB detection.

For drug susceptibility testing, the TB culture may require up to an additional 42 days after *M. tuberculosis* detection to obtain solid agar DST results (26). GeneXpert has excellent test characteristics in AFB smear-positive sputum (16). However, in a systematic review, the pooled sensitivity of GeneXpert in AFB smear-negative sputum was 67% (27), current DST with GeneXpert is limited to Rifampin, and in general genotypic DST is not accurate where the genetic mechanism of drug resistance is uncharacterized, incompletely characterized, complex, or in the presence of mixed infection. In addition, genotypic tests do not distinguish viable from nonviable cells and, therefore, are suboptimal to measure treatment response.

Limitations include those associated with the phage assay and with the study design. The current reporter phage assay includes reduced specificity and equipment requirements. Decreased specificity may be due to viral host promiscuity and/or choice of cut point. The reporter phage may infect nontuberculous mycobacteria (NTM) as well as *M. tuberculosis*. To address this limitation, we have developed a control condition to detect NTM (4-nitrobenzoic acid) going forward (28). To improve the specificity, we might increase the FACS cut point (increase the number of FACS gated events required to classify the sample as positive) with a trade-off of decreased sensitivity. This higher specificity would be useful in a "rule out" test, for example, in determining second-line resistance in samples found to be rifampin resistant by GeneXpert. In this study, we used a clinical FACS (BD Accuri C6), which is inexpensive and in wide clinical use. Ultra-inexpensive flow cytometers are being developed, which may be amenable to the reporter phage assay (29). An important operational limitation to this assay is the requirement for a BSL3 laboratory and biosafety cabinet since we are interrogating the metabolic activity of live cells. Compared to GeneXpert, the assay takes substantially longer for a result but is faster than *M. tuberculosis* culture.

There are also limitations related to study design. We enriched for TB drug resistance by enrolling at a TB referral center as well as a municipal chest clinic. This strategy improved efficiency but prevented estimation of positive and negative predictive value. A single sputum sample was used for each diagnostic test, which improved our ability to compare tests but may have reduced the sensitivity of each individual test by reducing the quantity of sputum tested.

In summary, the Φ^2GFP10 reporter phage showed high sensitivity for detecting *M. tuberculosis* and RIF resistance, including in AFB smear-negative sputum, an important clinical subset in HIV endemic populations. These results represent a significant advance over earlier-generation reporter phages and are promising given the potential for the reporter phage to detect phenotypic resistance to drugs other than RIF. The current lack of a rapid TB diagnostic with comprehensive DST is a critical obstacle to improve patient outcomes and reduce the transmission of drug-resistant *M. tuberculosis* strains. Advanced phenotypic assays such as Φ^2GFP10 may have an important role to play.

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We declare no conflicts of interest.

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