Early Detection of Emergent Extensively Drug-Resistant Tuberculosis by Flow Cytometry-Based Phenotyping and Whole-Genome Sequencing

Max R. O'Donnell,[a,b,c] Michelle H. Larsen,[d] Tyler S. Brown,[e] Paras Jain,[d] Vanisha Munsamy,[g] Allison Wolf,[a] Lorenzo Uccellini,[a] Farina Karim,[g] Tulio de Oliveira,[f] Barun Mathema,[b] William R. Jacobs,[d] Alexander Pym[g]

[a]Division of Pulmonary, Allergy, and Critical Care Medicine, Columbia University Medical Center, New York, New York, USA
[b]Department of Epidemiology, Mailman School of Public Health, Columbia University Medical Center, New York, New York, USA
[c]Centre for AIDS Programme of Research in South Africa (CAPRISA), Durban, South Africa
[d]Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York, USA
[e]Infectious Diseases Division, Massachusetts General Hospital, Boston, Massachusetts, USA
[f]KwaZulu-Natal Research Innovation and Sequencing Platform (KRISP), College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa
[g]Africa Health Research Institute (AHRI), Durban, South Africa

ABSTRACT A critical gap in tuberculosis (TB) treatment is detection of emergent drug resistance. We hypothesized that advanced phenotyping with whole-genome sequencing (WGS) will detect low-frequency *Mycobacterium tuberculosis* drug resistance. We assessed a reporter mycobacteriophage (Φ^2GFP10) *in vitro* to detect drug-resistant subpopulations and predict *M. tuberculosis* bactericidal activity in this pilot study. Subsequently, we prospectively studied 20 TB patients with serial Φ^2GFP10, Xpert MTB/RIF, and *M. tuberculosis* culture through end of treatment. WGS was performed, and single nucleotide polymorphisms (SNPs) were examined to detect mixed infection in selected *M. tuberculosis* isolates. Resistant *M. tuberculosis* isolates were detected at 1:100,000, and changes in cytometry-gated events were predictive of *in vitro M. tuberculosis* bactericidal activity using the Φ^2GFP10 assay. Emergent drug resistance was detected in one patient by Φ^2GFP10 at 3 weeks but not by conventional testing (*M. tuberculosis* culture and GeneXpert). WGS revealed a phylogeographically distinct extensively drug-resistant tuberculosis (XDR-TB) genome, identical to an XDR-TB isolate from the patient's spouse. Variant lineage-specific SNPs were present early, suggesting mixed infection as the etiology of emergent resistance with temporal trends providing evidence for selection during treatment. Φ^2GFP10 can detect low-frequency drug-resistant *M. tuberculosis* and with WGS characterize emergent *M. tuberculosis* resistance. In areas of high TB transmission and drug resistance, rapid screening for heteroresistance should be considered.

KEYWORDS South Africa, extensively drug-resistant tuberculosis, heteroresistance, phage, tuberculosis, whole-genome sequencing

Mycobacterium tuberculosis has overtaken HIV infection as a leading cause of death due to single infectious etiology worldwide (1–5). While overall global tuberculosis (TB) incidence has declined, our best estimates show drug-resistant TB rapidly increasing in both absolute numbers and as a proportion of all incident TB cases (2, 6, 7). Meanwhile TB incidence in HIV-endemic countries in southern Africa has reached levels not seen in the United States or Western Europe since the turn of the last century (2, 8–11).

Phenotypic diagnostics have potential to perform drug susceptibility testing (DST) for drugs with complex or uncharacterized genetic mechanisms of resistance, to

quantify the level of resistance, to distinguish viable from nonviable *M. tuberculosis*, to measure treatment response, and to detect mixed infection (12, 13). Our group has extensive experience with the construction of genetically modified mycobacterium-specific viruses (mycobacteriophages) for the genetic manipulation of *M. tuberculosis* (14–16). We recently described the construction and diagnostic capabilities of a new, more powerful reporter phage, Φ^2GFP10, which uses a more efficient promoter and more powerful fluorescence reporter to allow direct visualization of individual metabolically active bacilli using fluorescence-activated cell sorting (FACS), including in clinical sputum (17). The Φ^2GFP10 phage allows for rapid *M. tuberculosis* detection and phenotypic DST in clinical sputum samples, including paucibacillary concentrations.

We performed preliminary *in vitro* experiments to determine the limits of detection of the Φ^2GFP10 reporter phage for subpopulations of drug-resistant bacteria and to determine the correlation of inhibition of fluorescence expression after Φ^2GFP10 infection in the presence of antibiotics with *M. tuberculosis* bactericidal activity. The correlate experiments in clinical samples were to look for heteroresistance in clinical TB samples and measure dynamic changes in mycobacterial viability in the sputum of TB patients on treatment. As an unanticipated occurrence, one TB patient developed amplification of drug resistance, which was detected by the reporter phage assay prior to detection by conventional means (*M. tuberculosis* culture and GeneXpert MTB/RIF). In the present study, we describe an approach utilizing phage infection, cell sorting, and whole-genome sequencing (WGS) to characterize emergent drug resistance in TB patients on treatment.

RESULTS

Detecting low-frequency drug-resistant *M. tuberculosis* subpopulations using a mycobacteriophage assay. To determine the limits of detection for the Φ^2GFP10 reporter phage, we designed a mixed-infection experiment using a drug-susceptible TB strain (mc^{2}6230) and a rifampin-resistant TB strain (mc^{2}7902). A liquid broth of *M. tuberculosis* in log-phase growth for both strains was prepared. The numbers of bacilli per ml were estimated using optical density measurements and a standard correlation scale. Initial mixing conditions of 1:1,000, 1:10,000, and 1:100,000 RR-TB to DS-TB wells were prepared. To determine the accuracy of the Φ^2GFP10 reporter phage to recapitulate the initial mixing proportions, we prepared a rifampin and no-drug well (as described in Materials and Methods), as well as appropriate positive and negative controls. Φ^2GFP10 phage infection, followed by flow cytometry and FACS gating, was performed as described in Materials and Methods. The number of FACS-gated events in the rifampin condition was used to approximate the number of rifampin-resistant bacilli, and the number of FACS-gated events in the no-drug condition approximated the number of total bacilli (drug susceptible and resistant). Using the rifampin condition as the numerator and the no-drug condition as the denominator, we determined the percentage of RR-TB in each experiment, which was extremely close to the initial condition down to the 1:100,000 dilution ($R^2 = 0.992$) (Fig. 1).

Predicting *M. tuberculosis* bactericidal activity using a mycobacteriophage assay. We hypothesized that fluorophage infection could be used as a rapid surrogate marker of potentially viable cells. To evaluate this hypothesis, we performed *in vitro* experiments to determine the potential of the mycobacteriophage assay to predict subsequent bactericidal activity. In liquid *M. tuberculosis* culture in the presence or absence of antimycobacterial agents, we compared dynamic changes in numbers of FACS-gated events after phage infection (representing metabolically active mycobacteria) with subsequent CFU in the presence of various antibiotics taken simultaneously from liquid culture at serial daily time points from time zero through day 7. Decreased enumerated FACS-gated events after phage infection was highly correlated mycobacterial killing, as measured by quantitative decreases in the *M. tuberculosis* CFU ($R = 0.96$, $P < 0.001$) (Fig. 2A).

Dynamic changes in FACS-gated *M. tuberculosis* in the sputa of TB patients on treatment. To evaluate the ability of the reporter phage assay to detect dynamic
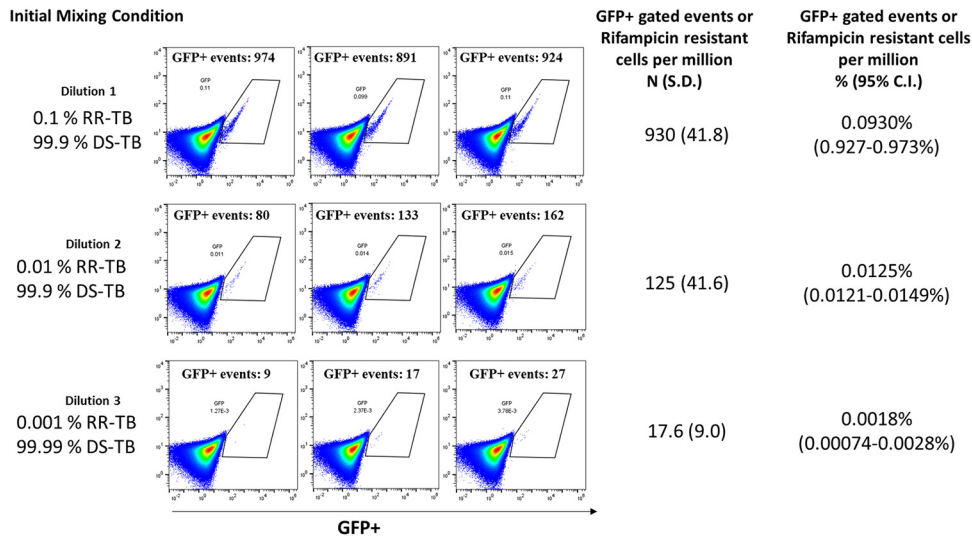
FIG 1 Recapitulation of initial mixing conditions of rifampin-resistant tuberculosis (RR-TB) with drug-susceptible tuberculosis (DS-TB) after phage infection and flow cytometry. Flow cytometry detects rare rifampin-resistant tuberculosis subpopulations. To estimate the limits of detection for the Φ2GFP10 reporter phage assay to detect resistant bacilli in mixed drug-susceptible/drug-resistant populations, we mixed attenuated drug-susceptible tuberculosis (mc²6230) with attenuated rifampin-resistant tuberculosis (RR-TB) (mc²7902), followed by serial dilutions, to create three mixed populations with 0.1, 0.01, and 0.001% RR-TB, respectively. We then incubated each initial mixing condition with Φ²GFP10 using previously described methods in the presence or absence of rifampin (17). FACS using experimentally constructed gates as previously described (17) were used to enumerate fluorescent events in the "Φ²GFP10 + *M. tuberculosis*" and "Φ²GFP10 + *M. tuberculosis* + rifampin conditions." As previously shown (17), fluorescence in the presence of rifampin corresponds to rifampin resistance. We estimated the percentage of rifampin-resistant *M. tuberculosis* in each mixing condition by using the number of FACS-gated events in the "Φ2GFP10 + *M. tuberculosis* + rifampin" well as the numerator and the number of FACS-gated events in the "Φ2GFP10 + *M. tuberculosis*" well as the denominator. In this way, we were able to show that we were able to recapitulate the initial mixing conditions down to 1 drug-resistant *M. tuberculosis* organism per 100,000, with a high degree of correlation between phage-derived percentages and initial mixing conditions ($R^2 = 0.992$).

changes on treatment, we recruited 20 participants with microbiologically confirmed drug-susceptible TB. The participants were followed with weekly study visits during the first month of standard TB chemotherapy, again at 8 weeks, and then clinically through the end of treatment, including serial symptom screening, adherence by self-report and pill counts, and HIV testing. In patients undergoing treatment, the numbers of fluorescent events after Φ²GFP10 infection and cell sorting decreased during treatment, correlating with inverse time to positivity and reflecting an *M. tuberculosis* treatment response.

All 20 participants were positive at baseline as determined by the GeneXpert MTB/RIF for the presence of *M. tuberculosis*, but all were negative for rifampin resistance. Drug susceptibility to rifampin and six other first- and second-line drugs was confirmed using standard protocols. Fifty-six percent of the patients whose HIV status was known were HIV infected, and 4 patients (20%) had previously received TB treatment.

We analyzed the kinetics FACS-gated events using the mycobacteriophage assay on serial samples from the 20 participants described above. When the number of FACS-gated events representing viable phage-infected bacteria expressing green fluorescent protein (GFP) was compared to the inverse of time to positivity in liquid TB growth media (a measure of mycobacterial burden), there was a high degree of correlation ($R = 0.89$, $P = 0.033$) (Fig. 2B), suggesting that the Φ²GFP10 assay may be a useful predictor of bacterial growth kinetics and, indirectly, sputum bacillary load.

Emergent drug resistance on treatment. In one patient (PID 151) at week 4 of treatment the overall numbers Φ²GFP10 gated events substantially increased in the presence or absence of antimycobacterial agents (Fig. 2C). These findings suggested emergent drug resistance despite complete medication adherence by pill count and
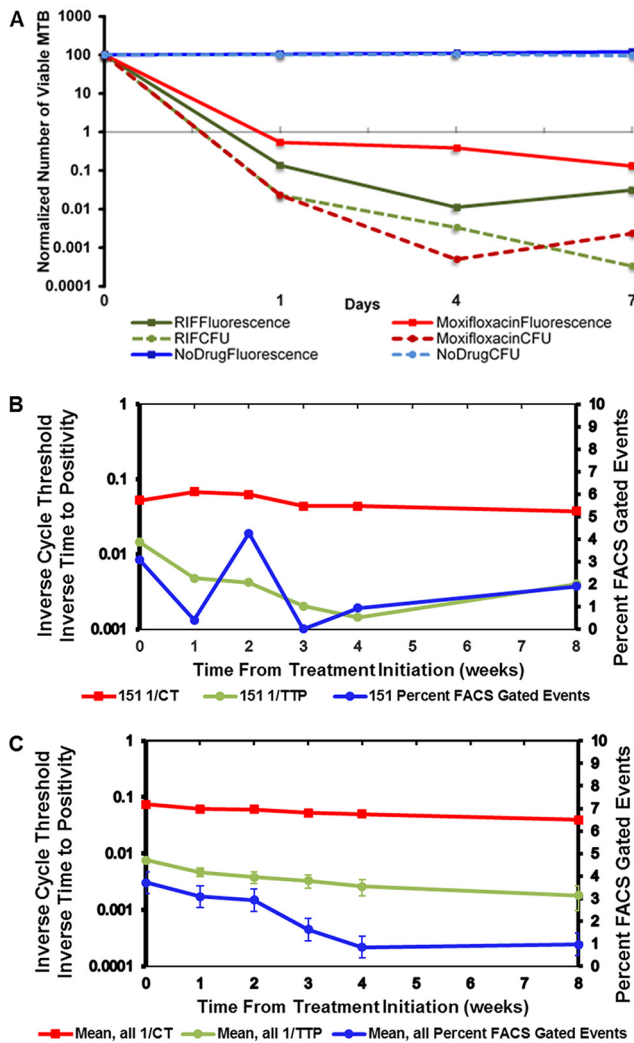
FIG 2 Fluoromycobacteriophage measures dynamic killing of *M. tuberculosis* in response to antibiotics *in vitro* and in *ex vivo* sputum samples from TB patients on treatment. (A) *In vitro* attenuated drug-susceptible *M. tuberculosis* (mc²6230) was inoculated with rifampin (RIF), moxifloxacin, or no drug. Φ²GFP10 was added 16 h prior to each time point, and the number of fluorescent *M. tuberculosis* organisms was measured by flow cytometry (FACS) and by counting CFU in parallel. The data are normalized by dividing the *M. tuberculosis* counts by the baseline *M. tuberculosis* counts and plotted against a logarithmic scale. The data show that untreated *M. tuberculosis* remained viable in substantial numbers through day 7 by both FACS and CFU, while the levels of drug-susceptible *M. tuberculosis* treated with moxifloxacin or rifamycin decreased markedly. (B) *Ex vivo* sputum samples from AFB smear-positive TB patients (*n* = 20) from the initiation of treatment through 8 weeks of treatment. Sputa were sampled weekly through week 8. GeneXpert, culture DST, and phage assay (Φ2GFP10) were performed at baseline and at weeks 1, 2, 3, 4, and 8. Φ²GFP10 was added 16 h prior to each time point; the number of fluorescent *M. tuberculosis* was measured by flow cytometry (FACS), and the bacillary burden was estimated using the time to positivity in liquid *M. tuberculosis* growth media. The percentage FACS-gated events compared events in FACS-gated to all FACS events. The cycle threshold was derived from GeneXpert RT-PCR measurements. The time to positivity was the time to *M. tuberculosis* culture positivity in MGIT liquid culture media. The standard error of the mean is shown. Gated events and inverse times to positivity were highly correlated (*R* = 0.89, *P* = 0.033). (C) Detection of treatment failure by reporter phage. Emergent drug resistance was detected at week 4 in one patient (PID 151) by the reporter phage but not until week 8 by the GeneXpert MTB/RIF and the conventional proportion method DST. On conventional DST, the patient showed resistance to isoniazid, rifampin, ofloxacin, kanamycin, capreomycin, amikacin, and ethambutol at week 8 despite having drug-susceptible *M. tuberculosis* at treatment initiation. Baseline drug-susceptible TB with emergent XDR-TB in this patient was confirmed by independent DST done on separate clinical isolates collected by the provincial TB control system. The percentage of FACS-gated events, the time to positivity, and the cycle threshold were defined as described above.

self-report. Subsequent drug-susceptibility testing on the isolate using Φ^2GFP10 demonstrated an XDR-TB resistance pattern (resistance to isoniazid, rifamycin, kanamycin, and ofloxacin) (see Fig. S1 in the supplemental material). Conventional drug susceptibility testing and GeneXpert MTB/RIF revealed drug-resistant isolates only at week 8. While the overall cycle threshold (C_T) values from the GeneXpert MTB/RIF were concordant with time to positivity and phage-gated events in predicting response to treatment (Fig. S2 and 3), analysis of the C_T values from the GeneXpert MTB/RIF did not demonstrate any probe-specific reductions in C_T values, confirming that the GeneXpert MTB/RIF was unable to detect this drug-resistant bacteria at earlier time points (Fig. S4).

The rapid amplification of resistance from drug susceptible to XDR in PID 151 in the absence of second-line drug exposure suggested mixed infection with a susceptible and resistant *M. tuberculosis* strain rather than acquired drug resistance. To exclude the possibility of laboratory cross contamination, we first obtained the drug susceptibility profile of the sample sent to the local diagnostic laboratory after 2 months of therapy. This independent sample was also reported as resistant to the six drugs tested (rifampin, isoniazid, ethambutol, streptomycin, ofloxacin, and kanamycin).

Genome sequencing to distinguish heteroresistance from evolved drug resistance. Using WGS data, we confirmed that PID 151 did not develop XDR-TB *de novo* during treatment but instead developed XDR-TB via primary or secondary polyclonal infection with a genetically distinct XDR-TB isolate. During follow-up, we discovered PID 151's cohabiting spouse had been admitted to a TB referral hospital for treatment of XDR-TB. WGS-based phylogenetic reconstruction shows that isolates collected from PID 151 at early treatment time points (baseline; weeks 1, 2, and 4) clustered with drug-susceptible isolates from sub-Saharan Africa belonging to *M. tuberculosis* phylogeographic lineage 2 and isolates from later time points (weeks 3 and 8 and the spouse's *M. tuberculosis* isolate) clustered with lineage 4 and with historical drug-resistant isolates from KwaZulu-Natal, South Africa. (Fig. 3).

We then analyzed mixed variant calls as described in Materials and Methods. Sequences from weeks 1 to 8 exhibit a significant proportion of mixed variant calls, likely introduced by the presence of multiple *M. tuberculosis* subpopulations in the sequenced isolates, whereas no mixed variant calls were identified in the baseline sample. The overall numbers of mixed variant calls decreased as a proportion of all variants during the course of treatment (from 33% to 5% by Week 8). The number of lineage 4-specific SNPs that were called for each serial isolate increased progressively from four in the baseline isolate to 425 in the week 3 isolate, consistent with a dynamic polyclonal infection in which the lineage 4 isolate replaced the lineage 2 isolate under antibiotic pressure. Taken together, these lines of evidence suggest that PID 151 was infected with a drug-susceptible lineage 2 isolate and superinfected with an XDR-TB isolate, most likely from her spouse, which was subsequently selected for during antibiotic treatment for drug-susceptible TB.

When we looked for the presence of lineage-specific SNPs as variant reads, these were found at baseline (4) and increased from week 1; by week 3, lineage 4-specific SNPs had reached the maximum possible number (i.e., 425). Sequences from weeks 1 to 8 exhibit a significant proportion of mixed variant calls, likely introduced by the presence of multiple *M. tuberculosis* subpopulations in the sequenced isolates, whereas no mixed variant calls were identified in the baseline sample. Taken together, these lines of evidence suggest that PID 151 was infected with a drug-susceptible lineage 2 isolate and superinfected with an XDR-TB isolate, most likely from her spouse, which was subsequently selected for during antibiotic treatment for drug-susceptible TB.

DISCUSSION

It is increasingly recognized that mixed infection with *M. tuberculosis* is prevalent, particularly in settings with a high *M. tuberculosis* force of infection and HIV coinfection (18–20). Mixed infection with heteroresistant *M. tuberculosis* can rapidly lead to treatment failure and increased mortality due to positive selection for resistant *M. tuberculosis* subpopulations with infective antibiotics (21). We report here on the sequential
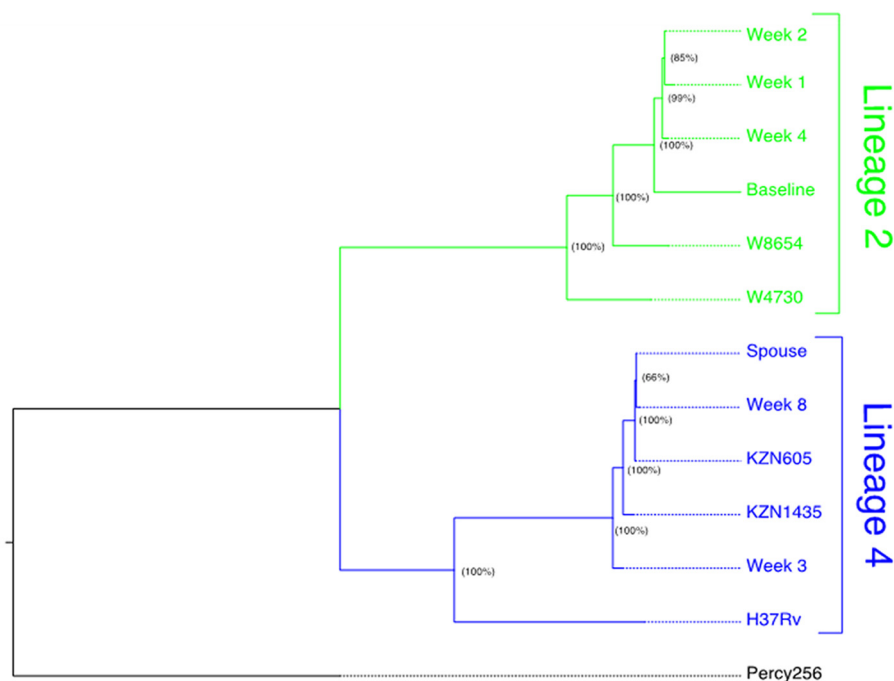
FIG 3 Genotyping demonstrates distinct *M. tuberculosis* lineages in a patient (PID 151) who acquired extensively drug-resistant tuberculosis (XDR-TB) on treatment (A) and analysis of heterozygous single-nucleotide polymorphisms reveals low frequency mixed infection from early in treatment (B). (A) Maximum-likelihood phylogenetic reconstruction. Whole-genome sequencing (WGS) data were obtained for baseline and week 1, 2, 3, 4, and 8 *M. tuberculosis* cultures from the patient who developed XDR-TB on treatment, as identified by the reporter phage assay (patient 151). Contact investigation determined that the patient's spouse was previously diagnosed with XDR-TB, and we subsequently obtained WGS data for an *M. tuberculosis* isolate from the patient's spouse. Phylogenetic reconstruction determined that initial isolates from patient 151 (baseline, week 1, and week 4) cluster distinctly with representative archival isolates from *M. tuberculosis* phylogeographic lineage 2 (W8654 and W4730) and isolates collected later during treatment (week 3 and week 8), as well as the isolate obtained from the patient's spouse, cluster distinctly with archival MDR-TB and XDR-TB isolates collected in KwaZulu-Natal, South Africa (KZN1435 and KZN605). Node labels indicate bootstrap support (proportion of 100 bootstrap replicates) supporting each clade. (B) Whole-genome sequencing to detect mixed infection in early time points. To evaluate for potential evidence of polyclonal infection, we characterized sequence read heterozygosity at SNPs. We defined mixed variant calls as SNPs where reads matching the reference accounted for >25% of the total read depth. The numbers and proportions of all unfiltered SNPs meeting these criteria for each isolate are included column 2. We used representative archival strains to define nonoverlapping subsets of SNPs that are unique to either *M. tuberculosis* phylogeographic lineage 2 or lineage 4. Isolates phylogenetically grouped in lineage 2 in panel A share 362 unique SNPs; those grouped in lineage 4 share 425 unique SNPs. As a proxy estimate for the extent of admixture between the pretreatment L2 isolate and the L4 spousal isolate, we tabulated the number of L4-specific SNPs called for each isolate (column 3) and quantitated the average read depth at lineage 4-specific SNPs (column 4) for each isolate obtained during treatment. The normalized averaged read depth, i.e., the average read depth at lineage 4-specific SNPs divided by the average read depth for all isolates in a sample, is provided in column 6.

emergence of TB drug resistance in a TB patient on treatment due to heteroresistance. Use of a novel reporter mycobacteriophage assay using a cell sorting strategy identified drug-resistant *M. tuberculosis* subpopulations of interest at substantially earlier time points compared to conventional methods. Our findings were confirmed and extended by WGS results of baseline and subsequent *M. tuberculosis* isolates, demonstrating an initial fully susceptible lineage 4 isolate that was replaced over time with an extensively drug-resistant lineage 2 isolate, confirming mixed infection as the likely etiology of the emergent drug resistance. As part of developing this clinical study, we performed proof-of-concept *in vitro* work demonstrating the correlation of phage-measured inhibition of fluorescence by antibiotics with subsequent *M. tuberculosis* killing, as measured by quantitative culture, and *in vitro* limits of mixed infection detection using the phage assay.

Although the reporter phage assay does not need to be linked to WGS, this may be

an appealing approach. A phenotypic/genotypic approach could use high-throughput reporter phage screening to identify potentially *M. tuberculosis* heteroresistance to increase the efficiency of WGS by enriching for heteroresistance, potentially reducing false positives in WGS, and allow for recognition of heteroresistance due to antimycobacterial agents with mechanisms of drug resistance that are incompletely genetically characterized (e.g., bedaquiline and delaminid).

Fittingly, the original work on mixed TB infection in the human host was done using mycobacteriophage typing (22). An early report from 1976 examined 87 TB patients with *M. tuberculosis* isolated from two or more different organs and found evidence of different TB strains by differential phage infection in 3/87 (23). Modern study of mixed *M. tuberculosis* infection dates early studies using restriction fragment length polymorphisms (RFLP) with insertion sequence 6110 probes to fingerprint *M. tuberculosis* isolates (24, 25). RFLP was superseded by PCR-based methods and now whole-genome sequencing. With each progressively higher resolution technique, more frequent mixed infections have been revealed. Whole-genome sequencing and deep sequencing have revealed much more diverse heterogeneity than previously realized and herald a growing understanding that mixed infection and mixed infection with drug-resistant bacilli—heteroresistance—has both pathogen evolutionary and clinical implications.

The limitations of this study include epidemiologic, microbiologic, and sequencing limitations. From an epidemiologic standpoint, a larger, better-designed study is required to establish the role of combined phage phenotyping and whole-genome sequencing to clinically characterize heteroresistance. Microbiologic limitations include bias introduced by *M. tuberculosis* culturing, which may be responsible for some small inconsistencies seen in our results (e.g., week 3 isolate clustering with lineage 4). Sequencing directly from clinical sputum would obviate this source of bias but is currently technically challenging. Sequencing limitations include lack of high depth coverage at all SNP positions of interest; however, high-quality reads revealing emergent lineage-specific SNPs provides convincing evidence for early heteroresistance.

In high-burden TB settings, drug-resistant TB can and does emerge during treatment and may be due to the amplification of drug resistance, mixed infection (heteroresistance), or intercurrent infection. There are currently no widely accepted or feasible means to detect heteroresistance early as it emerges early in treatment. In the present study, we detected emergent heteroresistance using an advanced phenotypic reporter phage assay and whole-genome sequencing to detect heteroresistance and demonstrated presence of mixed infection in early in treatment. Larger studies are needed to define the role and utility of such an approach.

MATERIALS AND METHODS

Laboratory methods. (i) Phage infection and flow cytometry gating. Methods describing the generation of the Φ^2GFP10 phage have been previously published (26, 27). Briefly, the phage construct consists of an optimized mycobacteriophage backbone with a constitutive L5 promoter upstream from an mVenus reporter gene which produces GFP. After phage infection in a viable *M. tuberculosis* bacillus GFP expression is a biomarker for metabolic activity detectable by fluorescence microscopy, flow cytometry, or a fluorescent plate reader. In the presence of an effective antimycobacterial agent, translation is disrupted, GFP is not expressed, and fluorescence is not detected.

After Φ^2GFP10 phage infection, the *M. tuberculosis* was characterized and enumerated using flow cytometry after infection (BD Accuri C6 flow cytometer [Becton Dickinson, Franklin Lakes, NJ]) using automated settings. Appropriate flow cytometry gates were experimentally derived. Viable *M. tuberculosis* were measured by enumerating fluorescent events by flow cytometry gating, and cutoffs for positive results were defined as previously described (26, 27).

(ii) Drug susceptibility testing with the phage assay. Methods describing drug susceptibility testing using the phage assay have been previously described (26, 27). Briefly, *M. tuberculosis* culture was set out in a 96-well plate with no-antibiotic controls and antibiotics of interest (isoniazid, rifampin, ofloxacin, and kanamycin). After overnight incubation, the Φ^2GFP10 phage was added to all antibiotic-treated wells and one of the no-drug controls. A laboratory-grown *M. tuberculosis* culture was used as a positive control in each plate. Flow cytometry was performed after 24 h. Fluorescence in the "*M. tuberculosis* culture + phage" condition versus the negative control indicates the presence of metabolically active *M. tuberculosis*. Fluorescence in the "*M. tuberculosis* culture + phage + antibiotic" condition indicates *M. tuberculosis* that is resistant to the antibiotic of interest.

(iii) Measuring bactericidal activity of antimycobacterial drugs *in vitro* using Φ^2GFP10 phage. To estimate the ability of Φ^2GFP10 phage to predict bactericidal activity of antimycobacterial drugs, we

compared the number of viable bacteria determined after Φ^2GFP10 phage infection to the number of CFU in the presence of antibiotics. Attenuated drug-susceptible *M. tuberculosis* (mc²6230) was inoculated with rifampin (RIF), moxifloxacin, or no drug. Φ^2GFP10 was added 16 h prior to each time point, and the number of fluorescent *M. tuberculosis* organisms was measured by flow cytometry (FACS) and by counting the CFU in parallel. Data were normalized by baseline *M. tuberculosis* counts and plotted against a logarithmic scale.

Clinical methods. As a pilot study to determine the phage assay's ability to detect changes in *M. tuberculosis* populations during treatment, consecutive TB suspects were enrolled at an outpatient municipal chest clinic in Durban, South Africa. Adult pulmonary TB suspects were eligible to participate if they were not currently taking TB medications and had a documented positive sputum acid-fast bacillus (AFB) smear result in the clinic system. Demographic, symptom, and clinical data were recorded by study staff, including prior TB history. HIV counseling and testing was offered to all patients. Extensively drug-resistant tuberculosis (XDR-TB) was defined as resistance to isoniazid, rifampin, any of the fluoroquinolones, and any of the second-line injectable agents. Written informed consent was obtained from all participants.

The primary objective in the clinical pilot study was to measure dynamic changes in Φ^2GFP10 reporter phage FACS-gated events during treatment in clinical sputum of TB patients. Early morning sputum samples, adherence, and clinical data were obtained at baseline and at weeks 1, 2, 3, 4, and 8. *M. tuberculosis* culture (BD MGIT), *M. tuberculosis* drug susceptibility testing using solid agar testing, Gene Xpert MTB RIF, and an Φ^2GFP10 assay were performed at all time points. Patients were followed through end of TB treatment. Approval for the study was obtained from ethics review boards at the University of KwaZulu-Natal, Albert Einstein College of Medicine, Columbia University Medical Center, the KwaZulu-Natal Department of Health, and the eThekwini Municipality.

Whole-genome sequencing and data analysis. Single *M. tuberculosis* colonies were selected from agar growth media and genomic *M. tuberculosis* DNA was isolated from clinical samples using standard methods. Library preparation and WGS were performed as previously described on the Illumina MiSeq (28). Raw sequencing reads were trimmed for low-quality bases, filtered for duplicate reads, and aligned to the H37Rv *M. tuberculosis* reference genome (NC_000962.3) using the Burrows-Wheeler Aligner. Variant calls were obtained using SAMtools. Variants in the highly variable PE/PPE gene family, variants in repeat regions or mobile elements, and variants within 15 bp of an insertion or deletion were excluded from analysis. The mean read depth at all SNPs ranged from 6.8 to 43.02. High-quality SNPs were defined as those with PHRED-scaled quality scores of >100. Mixed variant calls were defined as any SNP where reads matching the reference accounted for >25% of the total read depth. Lineage-specific SNPs were defined with respect to well-characterized clinical isolates from KwaZulu-Natal (KZN605 for lineage 4 and W8654 for lineage 2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC .01834-18.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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