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Colorimetric Phage-Based Assay for Detection of Rifampin-Resistant *Mycobacterium tuberculosis*▽

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Tests based on bacteriophage replication enable rapid screening of *Mycobacterium tuberculosis* for drug resistance. We describe a novel broth-based colorimetric method for detecting phage replication. When clinical isolates were tested by this novel method, high concordance was observed with both the traditional phage assay and gene mutation analysis for detection of resistance to rifampin.

The emergence of drug-resistant tuberculosis that cannot be cured using standard drug regimens is a serious threat to the control of the disease. Early detection of drug resistance is desirable both for the patient and to aid control measures. Molecular tests are available for rapid detection of resistance to rifampin. However, cost restraints and the lack of infrastructure have prevented their implementation in resource-poor settings. Monitoring for drug resistance in most countries where tuberculosis is endemic remains culture based and is slow and time-consuming. We have previously reported a rapid, low-cost, phage-based assay for screening isolates for susceptibility to antituberculosis drugs (12). Continued replication and production of progeny phage in the presence of drug are indicative of a drug-resistant strain of bacteria. A 96-microwell plate version of the assay is reported to have high sensitivity and specificity for detecting resistance to rifampin (4, 7, 9) Progeny phage is detected by the traditional method of plating aliquots on a lawn of fast-growing indicator bacteria and observing plaque formation (1). We describe here a novel colorimetric end point for the assay using the redox dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Poole, United Kingdom) and its application to a panel of 96 clinical isolates of *Mycobacterium tuberculosis*.

Bacteriophage stocks. The bacteriophage used in these studies, mycobacteriophage D29, infects a wide range of mycobacteria, including members of the *M. tuberculosis* complex (3). Stocks may be easily maintained in the laboratory by propagation on a lawn of fast-growing mycobacteria such as *Mycobacterium smegmatis* mc^2155 (William R. Jacobs, Jr., Howard Hughes Medical Institute, Albert Einstein College of Medicine, New York, NY). Plates were prepared by adding 10% (vol/vol) stationary-phase *M. smegmatis* culture to 1.5% agar in Luria-Bertani broth (Becton Dickinson, Sparks, MD) in 90-mm petri dishes (6). The plates were inoculated by spreading 100 μl of mycobacteriophage D29 (London School of Hygiene & Tropical Medicine, London, United Kingdom) at approximately 4×10^3 PFU/ml in Middlebrook 7H9 broth with 1 mM calcium chloride. Following overnight incubation at 37°C, 10 ml of Middlebrook 7H9 broth with 10% OADC (oleic acid-albumin-dextrose-catalase) and 1 mM calcium chloride was added to the plate. Following a further overnight incubation at 37°C, the broth was collected and filtered though a sterile 0.4-μm filter to remove bacteria and debris. The filtrate containing the phage stock was stored at 4°C with the addition of 0.1% sodium azide (Sigma-Aldrich, Poole, United Kingdom). Phage stocks containing 10^9 to 10^{10} PFU/ml were prepared by this method. The titer of the phage stock was established by plating serial dilutions on a lawn of *M. smegmatis* followed by overnight incubation at 37°C. Enumeration of the resulting PFU enabled back-calculation of the concentration of PFU in the stock suspension.

Phage drug resistance assay. Testing of the two detection methods was performed in parallel on duplicate samples at final drug concentrations of 2 and 10 μg/ml. All manipulations involving *M. tuberculosis* were performed in a class I microbiological safety cabinet. Bacteria from Lowenstein-Jensen agar slopes were suspended in Middlebrook 7H9 broth supplemented with OADC (Becton Dickinson, Sparks, MD) and 1 mM calcium chloride, as described previously (6). Bacterial suspension samples (75 μl) were placed in the wells of a 96-microwell plate (Greiner Labortechnik, Stonehouse, United Kingdom) containing equal volumes of drug at the appropriate dilution and a zero-drug control. Following overnight incubation at 37°C, 50 μl of a stock suspension of mycobacteriophage D29 was added to give a final concentration of 3×10^7/ml, and the infection was allowed to proceed. After incubation at 37°C for 90 min, any residual extracellular bacteriophage was killed during the latent phase of the infection. Different virucidal agents were used for the two detection methods.

MTT assay. For the colorimetric MTT detection method, 0.3% sulfuric acid (75 μl) was added to each well (2). Plates were left for 5 min at room temperature to allow the acid to kill

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any extracellular phage and then neutralized by the addition of 25 μl of 0.24 M sodium hydroxide. To detect bacteriophage replication, test samples (50 μl) were transferred to a fresh 96-well microplate, with each well containing 200 μl of a stationary-phase culture of *M. smegmatis* mc^{2}155 diluted 1:5 in supplemented Middlebrook 7H9 broth. Following overnight incubation at 37°C, each well received 20 μl of MTT solution (2 μg/ml in water). The color of each well was recorded after an additional 16 h of incubation. MTT is a tetrazolium dye that upon reduction changes in color from yellow to purple. Growth of *M. smegmatis* in the wells results in a color change, whereas for samples where bacterial proliferation is prevented by the lytic action of the phage, no such change is observed. Purple coloration in those samples treated with rifampin indicates that phage replication has not proceeded and that the isolate is susceptible to the drug. Yellow coloration in samples exposed to drug indicates successful phage replication and a rifampin-resistant isolate. Growth of *M. smegmatis* provides a sufficient substrate for easy visual determination, and further treatment to solubilize the formazan dye products was not required.

Plaque assay. For the plaque assay, 100 ml of 30 mM ferrous ammonium sulfate (Sigma-Aldrich, Poole, Dorset, United Kingdom) was added to each well to inactivate extracellular phage as described previously (8). Indicator plates containing a lawn of *M. smegmatis* mc^{2}155 were prepared as described above. The indicator plates were stored in the refrigerator and dried for 1 h at 37°C prior to use, to remove surface moisture (6). Ten-microliter aliquots of each sample were placed on the agar surface, and plaques were observed following overnight incubation at 37°C. A strain was classified as susceptible if plaques in the lawn were observed for the zero-drug control samples but not for the drug-treated samples. Strains were designated as resistant if plaques were observed for the drug-treated samples. Up to 12 samples were spotted onto the indicator plate, eight petri dishes being required for a 96-well assay.

Sequence analysis. All isolates were also investigated for mutations predictive of resistance in an 81-bp region of the *rpoB* gene as described by Telenti et al. (11). Sequencing was performed at the Genome Research Centre at the London School of Hygiene & Tropical Medicine using a BigDye terminator cycle sequencing kit (version 3.1; Applied Biosystems, Foster City, CA). The primers used were those described by Ohno et al. (10). The sequences obtained were compared to those of the *M. tuberculosis* H37Rv RNA polymerase beta subunit gene (*rpoB*; GenBank accession no. U12205) using the DNAStar MegAlign program (DNAStar, Madison, WI).

The results are shown in Table 1. Ten isolates were found to be resistant, and 70 were found to be susceptible by all three methods. The MTT phage test demonstrated high concordance (97.9%) with both the plaque phage test and sequence analysis, when tested with rifampin at 10 μg/ml. When a 2-μg/ml concentration of drug was used, the level of agreement was reduced to 90.6% with the plaque test and 89.6% with sequence analysis. If the results from the sequence analysis are considered the "gold standard" for assessing resistance, the estimated sensitivity, specificity, positive predictive, and negative predictive values when rifampin was used at 10 μg/ml were 90.9, 98.8, 90.9 and 98.8%, respectively, for the MTT test and 90.9, 96.5, 76.9, and 98.9%, respectively, for the plaque test. In comparison to sequence analysis, the MTT test produced fewer apparent false-positive results than the plaque test. This suggests that the colorimetric phage assay may offer improved accuracy over plaque-based methods. However, further testing and thorough investigation of discrepant results will be required to substantiate this supposition. For implementation in resource-poor laboratories, new technology should be robust and affordable. In this respect, the microwell phage assay does not require investment in sophisticated equipment other than that utilized for handling cultures of *M. tuberculosis*. For processing a batch of 30 isolates, the cost of reagents was estimated to be less than $1 per isolate tested (tax and overhead costs excluded; goods were purchased via the London Universities Purchasing Consortium). The microwell phage assay enables high-throughput screening of *M. tuberculosis* isolates for drug resistance. For more rapid detection of drug-resistant disease, it may be possible to use a phage-based test to detect drug resistance directly in clinical specimens. Preliminary reports from Peru suggest that resistance to rifampin may be detected in sputum specimens found positive for acid-fast bacilli by smear microscopy (5). However, assessment of the utility of such an approach awaits further data on its sensitivity and specificity.

In conclusion, the MTT colorimetric method is convenient to use and, when used with rifampin at 10 μg/ml, may provide a rapid means of differentiating rifampin-susceptible and -resistant isolates of *M. tuberculosis*. The method avoids preparation of indicator plates, minimizing both reagent and labor requirements. The multiwell format permits semiautomation by use of multichannel pipettes and facilitates the handling of large numbers of samples. Further studies are required to further validate this assay and to investigate its potential for detecting resistance to other antituberculosis drugs.

TABLE 1. Susceptibility profiles of 96 clinical isolates of *M. tuberculosis* tested by the MTT colorimetric phage test, a traditional plaque-based test, and sequence analysis of an 81-bp region of the *rpoB* gene

Isolate(s)	Susceptibility profile[a]				Sequence analysis
	MTT phage test		Plaque phage test		
	2 μg/ml[b]	10 μg/ml	2 μg/ml	10 μg/ml	
12375	R	R	R	R	Asp516Tyr
527	R	R	R	R	His526Asp
16122	R	R	R	R	His526Asp
16446	R	R	R	R	His526Asp
17034	R	R	R	R	Ser531Leu
889	R	R	R	R	His526Tyr
33	R	R	R	R	Ser531Leu
521	R	R	R	R	Ser531Leu
10816	R	R	R	R	Ser531Leu
12108	R	R	R	R	Ser531Leu
17020	S	S	S	S	His526Asn
17053	R	R	R	R	WT
12015	R	S	R	R	WT
11720	S	S	R	R	WT
4 isolates	R	S	R	S	WT
5 isolates	S	S	R	S	WT
3 isolates	R	S	S	S	WT
70 isolates	S	S	S	S	WT

[a] R, resistant; S, susceptible; WT, wild type.
[b] Final concentration of drug.

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