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Rapid Film-Based Determination of Antibiotic Susceptibilities of *Mycobacterium tuberculosis* Strains by Using a Luciferase Reporter Phage and the Bronx Box

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Detecting antibiotic resistance in *Mycobacterium tuberculosis* is becoming increasingly important with the global recognition of drug-resistant strains and their adverse impact on clinical outcomes. Current methods of susceptibility testing are either time-consuming or costly; rapid, reliable, simple, and inexpensive methods would be highly desirable, especially in the developing world where most tuberculosis is found. The luciferase reporter phage is a unique reagent well-suited for this purpose: upon infection with viable mycobacteria, it produces quantifiable light which is not observed in mycobacterial cells treated with active antimicrobials. In this report, we describe a modification of our original assay, which allows detection of the emitted light with a Polaroid film box designated the Bronx Box. The technique has been applied to 25 *M. tuberculosis* reference and clinical strains, and criteria are presented which allow rapid and simple discrimination among strains susceptible or resistant to isoniazid and rifampin, the major antituberculosis agents.

A recent World Health Organization (WHO) study documents that drug-resistant tuberculosis (TB) is now a worldwide phenomenon (23, 41). Since multiply drug-resistant TB is difficult to eradicate with the WHO-recommended short-course treatment regimens (12, 23, 41), its presence complicates efforts to reduce the estimated 8 million new cases and prevent the 3 million deaths from TB predicted to occur each year (28). Inappropriate therapy of drug-resistant *Mycobacterium tuberculosis* may lead to increased levels of resistance to multiple drugs (17), increased costs for retreatment regimens (17), increased opportunities for spread to contacts and health care workers (9, 19, 26), and increased mortality (24, 37). Nonetheless, early recognition of drug-resistant *M. tuberculosis* can lead to effective interventions, even in those who are human immunodeficiency virus (HIV) infected (37). Accordingly, the Centers for Disease Control and Prevention (CDC) have recommended that the detection of drug-resistant *M. tuberculosis*, and institution of appropriate therapy, be expedited (6).

However, practical antibiotic susceptibility testing (AST) of *M. tuberculosis* remains problematic, especially in the developing world. Conventional methods such as the agar-based proportion technique require 3 to 6 weeks for results, while the BACTEC radiometric method requires only 1 to 2 weeks but utilizes radioactive substrates and expensive technology (11). Similar time-to-results as that for the BACTEC method have been reported for E-test (38) and redox-indicator-dye methods (42). Novel methods for AST such as genotypic assays (20, 36) and flow cytometry (15) are more rapid, requiring 1 to 2 days, but are technology intensive. Finally, relatively rapid assessments of mycobacterial viability (in the presence of effective antibiotics) have been performed by quantifying mycobacterial ATP stores (22) or by measuring the ability of viable mycobacteria to support bacteriophage replication (39) or to synthesize the sensitive reporter gene product, firefly luciferase (2, 7).

We have developed a method which combines elements of these phenotypic assays, utilizing bacteriophage to introduce the firefly luciferase gene (*Fflux*) into any viable isolate of *M. tuberculosis*, thus yielding visible light in the presence of cellular ATP and the exogenous substrate luciferin (5, 14, 29). Effective antimycobacterial drugs will abrogate light production in this system either by decreasing ATP stores, by impairing luciferase protein production, or by interfering with productive phage infection; AST can be performed within 2 to 3 days (5, 14, 29). The greatest cost issue with this method is the requirement for a luminometer to quantify the light output. Alternative methods for detecting luciferase activity have been described, i.e., using X-ray film for colonies of transformed *Escherichia coli* (40) or *Candida albicans* (34) and polaroid film for recombinant HIV-infected (13) or pseudorabies (16) virus-infected mammalian cells. Film has also been used to detect nontuberculous mycobacterial strains constitutively expressing luciferase after infection with a lysogenic recombinant phage (31). In the present work, we describe conditions for photographic detection of light from recombinant lytic phage-in-

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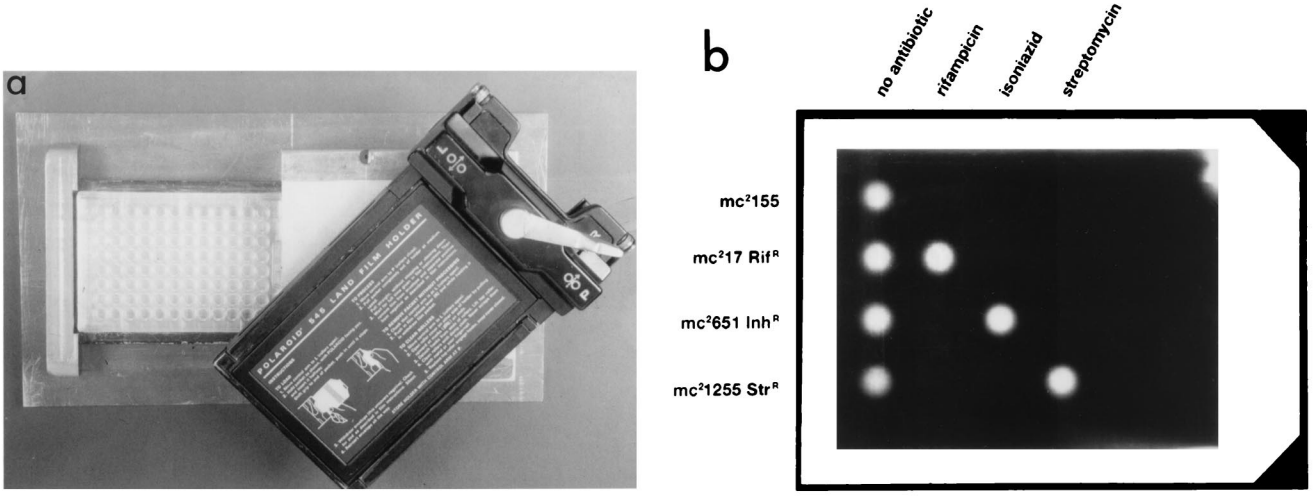

FIG. 1. (a) Picture of the Bronx Box. (b) Result of a Bronx Box AST experiment with *M. smegmatis* strains mc^{2}155 (fully susceptible), mc^{2}651 (INH resistant), mc^{2}17 (rifampin resistant), and mc^{2}1255 (streptomycin resistant). Each strain (200 μl of a culture grown to an optical density at 600 nm of 0.5) was treated with medium alone (no antibiotic) or antibiotics (rifampin, 100 μg/ml; INH, 25 μg/ml; streptomycin, 100 μg/ml) for 12 h. A total of 20 μl of phAE85 was added to each sample, and after a further 6-h incubation, 100 μl of flash luciferin buffer was added and the plate was loaded in the Bronx Box for a 12-h exposure. Spots of light correspond to wells containing viable mycobacteria.

fected *M. tuberculosis* by using a custom-made light-tight box which accommodates a 96-well plate and a polaroid film cassette and their application for rapid AST of clinical *M. tuberculosis* isolates.

MATERIALS AND METHODS

Mycobacteria. BCG-Pasteur (ATCC 35734), *M. tuberculosis* Erdman (ATCC 35801), *Mycobacterium smegmatis* mc^{2}155 (33), and spontaneous drug-resistant mutants of *M. smegmatis*—mc^{2}17 (rifampin resistant) (unpublished), mc^{2}651 (isoniazid [INH]-resistant) (3), and mc^{2}1255 (streptomycin resistant) (25)—are from our reference collection. BCG::*FFlux* is BCG-Pasteur transformed with pKB15, an integrating plasmid which leads to expression of *Fflux* from the P_L promoter of phage L5 (21). All other clinical strains were obtained from the clinical mycobacteriology laboratories of the Montefiore Medical Center of the Albert Einstein College of Medicine, Bronx, N.Y. (n = 17) or the Kings County Hospital Center, Brooklyn, N.Y. (n = 6). These clinical strains can be subdivided into (i) reference strains submitted for proficiency testing (date of submission in brackets) by the CDC (n = 5, strains A and B [Feb. 1998], I [Aug. 1996], K [Jan. 1996], and S [June 1997]) or by the College of American Pathologists (n = 1, strain E05 [1997]), with well-validated AST results, and (ii) primary clinical isolates (n = 17) obtained by the participating hospitals. AST was performed for the clinical isolates by the BACTEC 460 method, and if resistance was found, it was confirmed by the agar-proportion method (11). Mycobacteria were grown in Middlebrook 7H9 medium (Difco, Detroit, Mich.) supplemented with 0.5% bovine serum albumin, fraction V (Boehringer Mannheim, Indianapolis, Ind.), 0.9% NaCl, 0.2% glycerol, and 0.2% glucose (MADC) and was further supplemented with 0.05% Tween-80. BCG::*Fflux* was grown in MADC with Tween and 50 μg of hygromycin/ml. Reagents were obtained from Sigma Chemical Co., St. Louis, Mo.

Luciferase reporter phage. Luciferase reporter phage phAE85, constructed as described by Carrière et al. (5), was utilized for these experiments. It was propagated on lawns of *M. smegmatis* mc^{2}155, as mentioned previously, and was maintained at a titer of $>10^{11}$ PFU/ml in SM buffer (0.58% NaCl, 0.2% $MgSO_4$, 0.01% gelatin, 50 mM Tris [pH 7.5]).

The Bronx Box. This is a custom-made light-tight aluminum box manufactured by one of us (L.F.) which accommodates a Microlite 1 96-well plate (Dynex, Chantilly, Va.) and a Polaroid 545 Land Film Holder cassette loaded with Polapan 57 3000 ASA film (Polaroid, Cambridge, Mass.) (Fig. 1a). This device can be placed within a zippered black pouch with sleeves and armholes for loading and removing the film in complete darkness; alternatively, the box can be sealed with regular opaque laboratory tape or placed within a drawer during development. These steps are not essential but result in sharper pictures.

Antibiotics. INH, rifampin, streptomycin, and ethambutol (all from Sigma) were diluted in water (or methanol for rifampin), and then filtered and diluted in sterile distilled water to a 10× working concentration as follows: INH, 1 or 2 μg/ml; rifampin, 20 μg/ml; streptomycin, 20 μg/ml; ethambutol, 75 μg/ml.

Luciferin buffer evaluation. The optimized luciferin buffer ("flash luciferin") for luminometer readings from luciferase-expressing mycobacteria was previously shown to consist of 50 mM sodium citrate (SC) (pH 4.5) with 0.33 mM luciferin (modified from reference 14). In this work, we developed an optimized "glow luciferin" buffer for photographic detection, consisting of 50 mM SC (pH 5.3), 8 mM dithiothreitol (DTT), and 12 mM $MgSO_4$ (stored at 4°C up to 1 week) supplemented with 0.2 mM luciferin (adjusted to pH 5 to 6, stored at −20°C, and freshly thawed before use). All reagents were obtained from Sigma. All buffers were initially evaluated by using 10 μl of BCG::*Fflux* with 100 μl of each buffer loaded into wells of a Microlite 1 plate (Dynex) and assayed on a Dynatech ML3000 luminometer (Dynex) (cycle mode, 10 s reading every 15 s).

AST testing (liquid media). A smooth liquid culture of mycobacteria was obtained after cultivation in MADC with Tween for 5 to 21 days until a turbidity of at least 2 McFarland units (corresponding to $>5 \times 10^7$ CFU/ml) (Remel, Lenexa, Kan.) was attained. Since Tween interferes with phage infection (14, 29), 1 to 2 ml of cells were centrifuged twice (1,800 × *g*, 15 min, 20°C) with 5 ml of wash medium (MADC with 1% glycerol) and resuspended in the original volume of MADC without Tween. Then, 180 μl of the washed mycobacteria was added to separate wells of a sterile Microlite 1 plate containing 20 μl of MADC or 10× concentrations of INH and rifampin, and in certain experiments streptomycin and ethambutol. The plate was covered with a lid, sealed with Parafilm, and incubated for 48 h at 37°C. Then, 20 μl of phage was added to each well, and after 4 to 6 h at 37°C, duplicate 25-μl aliquots from each well were removed for quantitative luciferase assays on a LUMAC 2010a luminometer (Celsis, Monmouth Park, N.J.). A total of 100 μl of flash luciferin buffer was injected, and the relative light units (rlu) were measured after 10 s of integration. An inhibition index for each antibiotic was calculated as [(rlu after antibiotic treatment)/(rlu without antibiotics)] × 100, an index of <10% has been found to correlate with a strain being susceptible, while an index of >10% is considered resistant (29, 30). For the Bronx Box experiments, 100 μl of glow luciferin buffer was added to 125 μl of phage-infected mycobacteria (with or without antibiotics) in each of the wells of the Microlite 1 plate. The plate was covered with an acetate plate sealing membrane (no. 3501; Dynex) and loaded into the chamber of the Bronx Box, superimposed with Polaroid Polapan 57 film in its cassette. After a 3-h or overnight (16 h) exposure, the film was developed and spots of light were visualized.

Direct AST testing from solid media. Some mycobacteria were derived directly from Löwenstein-Jensen slants (BBL, Cockeysville, Md.) or Middlebrook 7H10 agar (Difco) plates supplemented with oleic acid albumin dextrose complex (BBL). Several colonies were scraped and transferred to a 15-ml polystyrene centrifuge tube containing sterile 4-mm-diameter glass beads (Fisher Scientific, Pittsburgh, Pa.) and 1.5 ml of MADC. After vortexing for 20 s, any large clumps of cells remaining were allowed to settle for 10 min, and 1 ml of the supernatant (approximate turbidity of 2 McFarland units) was directly utilized for AST assays, as described above, without the need for washing. Notably, the 10× concentration of INH was reduced to 1 μg/ml, and the duration of antibiotic action was extended to 72 h.

RESULTS

The Bronx Box assay can assess drug susceptibilities of mycobacteria. The ability of the Bronx Box assay to detect light from luciferase reporter phage-infected mycobacteria was first evaluated with the nonpathogenic rapid-growing mycobacte-

rium *M. smegmatis* and phAE85. Further, the availability of known drug-resistant mutant strains of *M. smegmatis* allowed us to test whether the Bronx Box assay could be used to reliably identify the presence of drug resistance. In fact, with minimal modification of our prior luminometer-based protocols (14, 29), with our flash luciferin buffer, clearly visible spots were obtained from wells containing each of the *M. smegmatis* strains infected with phAE85 (Fig. 1b). In addition, spots of equivalent intensity were seen from each of the drug-resistant strains in the presence of the antibiotic to which they were resistant; no spots were seen in wells containing a mycobacterium treated with an antibiotic to which it was susceptible (Fig. 1b). In these assays, approximately 2×10^7 CFU of mycobacteria were utilized per assay. In adapting this protocol to the slower-growing, pathogenic mycobacteria, and to improve our sensitivity of detection, several further modifications were considered.

Optimization of luciferin buffer. Preliminary experiments with our luminometer-optimized reporter phage assays showed that after addition of the luciferin substrate, the light output peaked rapidly, within seconds, and then declined to a new baseline of only 10 to 25% of the peak which then decayed more gradually (see Fig. 2, SC 4.5). It was apparent that a photographic detection method with this type of buffer would be unable to measure the peak due to the manipulation time required for loading the film. Therefore, a method was sought for either (i) predictably delaying the onset of the peak, (ii) elevating the level of the "steady-state" baseline light output value, or optimally (iii) doing both in extending the duration of the peak beyond the initial seconds. There is evidence from cell-free luciferase readout systems that extended light output can be achieved by modifications of the reaction buffer (1, 18), so we assessed these methods in our in vivo mycobacterial cell assay.

First, we confirmed preliminary work which identified a pH of 4.5 as optimal for luminometer-based detection, and then we measured the light output over a 1- to 2-h period rather than just over the first few seconds. The low pH apparently stabilizes the luciferin substrate and allows it to enter the mycobacterial cell in its uncharged form (1). However, a higher pH might (i) allow the photon generation to occur closer to its most efficient pH (i.e., 8 to 9) (1) and (ii) preserve mycobacterial cell viability (8), enhancing the duration of light output, even though the luciferin would be less readily available intracellularly. We used a strain of BCG constitutively expressing firefly luciferase (BCG::*Fflux*) as the source of intracellular luciferase for these experiments and fixed the luciferin concentration at 0.2 mM. As previously seen, peak light output decreased with a rising pH above pH 4.5 with the following buffer: 50 mM SC at pH 4.1, 4.6, 5.3, and 6; 25 mM HEPES at pH 6.4, 7.2, and 8; 50 mM Gly-gly at pH 7.8, and 50 mM Tris at pH 7.0 and 7.8. Nonetheless, this work confirmed that at a higher pH, light output was generated, though of lower amplitude and at a significantly delayed onset (data not shown). We therefore sought to boost this delayed peak by adding other previously described modifiers of the luciferase reaction, Mg, DTT, and ATP (1, 18), to the pH 7.8 buffered luciferin solution. The results in Fig. 2 show that this approach is successful, with each of these components required for extending the light output and increasing its amplitude. Next, we considered the effects of these reagents on the lower pH buffer systems. Surprisingly, while ATP was beneficial with Tris buffer at pH 7.8, it actually interfered with optimal light output if added to either HEPES buffer (pH 6.4) (not shown) or SC buffer (pH 5.3) (Fig. 2).

Testing of luciferin buffers in the Bronx Box. The best buffers from the experiments described above were compared to

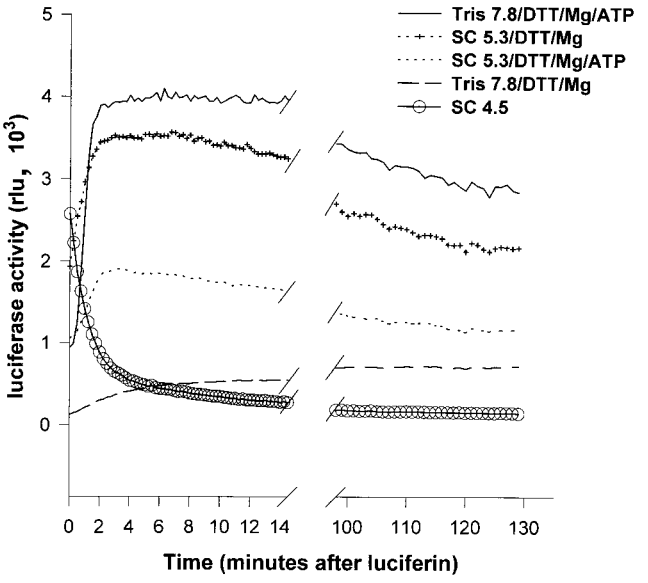

FIG. 2. Kinetics of luciferase-generated light production depends on the composition of luciferin buffer. A total of 10 μl of BCG::*Fflux* (constitutively expressing luciferase) was treated with 90 μl of 0.2 mM luciferin in the following buffers (from top to bottom in the figure): 15 mM Tris (pH 7.8) with 12.5 mM DTT–15 mM $MgSO_4$–6 mM ATP, 50 mM SC (pH 5.3) with 12.5 mM DTT–15 mM $MgSO_4$, 50 mM SC (pH 5.3) with 12.5 mM DTT–15 mM $MgSO_4$–6 mM ATP, 15 mM Tris (pH 7.8) with 12.5 mM DTT–15 mM $MgSO_4$, and 50 mM SC (pH 4.5). Photons (rlu) were measured every 15 s for 2 h with a Dynatech ML3000 luminometer (cycle mode).

the standard flash luciferin in the Bronx Box with dilutions of BCG::*Fflux* as the source of luciferase. After a 3-h exposure of film, the brightest spots and the highest sensitivities were obtained with either SC buffer at pH 5.3 with DTT and Mg or with Tris at pH 7.8 containing DTT, Mg, and ATP. Notably, a further overnight exposure (reloading the Bronx Box after the first 3 h) showed residual spots, albeit much lighter than on the 3-h film, suggesting that not all of the emitted light is exhausted in the first 3 h and that for optimal sensitivity, overnight exposure should be used. In all further experiments, the SC pH 5.3 buffer with DTT and Mg was utilized (and designated glow luciferin buffer) inasmuch as the Tris buffer (with ATP) required an additional and labile component, the ATP, which might potentially confound our AST assays. The superior sensitivity of the glow luciferin buffer to the flash luciferin was also confirmed using Erdman and BCG mycobacteria infected with phAE85 for 3 h and then treated with the respective buffers. Finally, certain other conditions were tested for their effect on light output directly in the Bronx Box with BCG::*Fflux*. The roles of supplemental calcium (1 to 10 mM) (10, 18), pyrophosphate (1 to 100 mM) (18), and a range of luciferin concentrations (0.1 to 1 mM) were evaluated, showing nonsignificant differences from the above-noted optimized glow luciferin buffer.

Adapting the Bronx Box assay for *M. tuberculosis*. In order to work with *M. tuberculosis*, a biosafety level 3 pathogen, a modification of the Bronx Box protocol was required, either the film box had to be entirely contained within a biosafety hood or the plate containing the mycobacteria had to be sealed before being removed from the hood. Given that the latter was more practical, the oxygen requirement of the reaction had to be considered. Therefore, an O_2-permeable transparent acetate sealing membrane (Dynex no. 3501) was adhered over the plate prior to loading in the Bronx Box. There was no attenuation of the

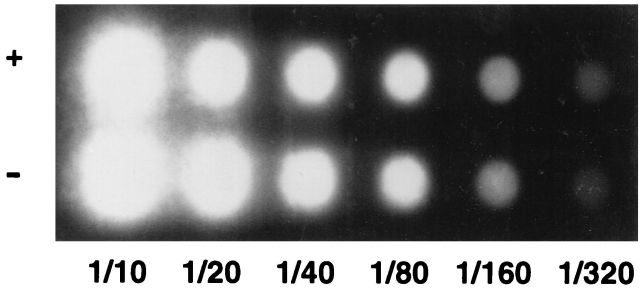

FIG. 3. Sensitivity of detection of luciferase in the Bronx Box with (+) and without (−) acetate membrane covering. A twofold dilution series, starting from a 1 to 10 dilution of a 2 McFarland unit culture of BCG::*Fflux* (100 μl in each of 6 wells, from approximately 2×10^6 (left) to 0.7×10^5 CFU (right) were each treated with 100 μl of glow luciferin buffer (see text), loaded in the Bronx Box, and exposed for 3 h.

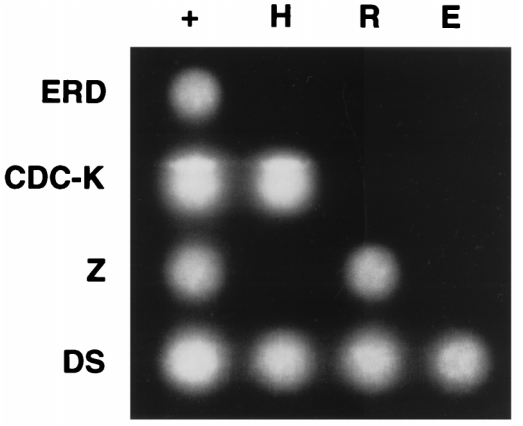

FIG. 4. Bronx Box AST of clinical *M. tuberculosis* strains. A total of 125 μl of mycobacteria was treated with medium alone (+) or with INH (0.2 μg/ml) (H), rifampin (2 μg/ml) (R), or ethambutol (2.5 μg/ml) (E) for 48 h. The mycobacteria were then treated with 100 μl of glow luciferin buffer, loaded in the Bronx Box, and exposed for 3 h. The mycobacteria tested were the Erdman susceptible reference strain (ERD), a CDC INH-resistant strain (CDC-K), and clinical monoresistant (Z) and multidrug resistant (DS) strains.

light signal when identical wells containing the nonpathogen BCG::*Fflux* were tested with or without the membrane (Fig. 3).

Sensitivity of detection with the Bronx Box assay. With our luminometer, the threshold of detection for obtaining obvious spots is about 2,000 rlu per 25 μl of cells when 125 μl of cells is utilized for the Bronx Box assay (e.g., Fig. 3). Further, there is a linear correlation between the intensity of the spots and the rlu readings obtained by a luminometer. In order to establish the minimum number of cells required to produce a spot on the Polaroid film in the Bronx Box assay, we tested serial twofold dilutions of BCG-Pasteur and *M. tuberculosis* Erdman infected with phAE85. After 6 h of phage infection, glow luciferin buffer was added, and the film was exposed for an additional 3 h or overnight. A 16-fold dilution of each of the strains was visualized as a spot at 3 h, corresponding to 1.7×10^6 CFU of each mycobacterium. The sensitivity of the assay for detecting BCG::*Fflux* is 20-fold greater (Fig. 3), reflecting the fact that each cell in this population constitutively and synchronously expressed high levels of luciferase. In order to further boost the sensitivity, 20,000 ASA Polaroid film was tested; surprisingly, it produced no greater sensitivity of detection, perhaps because the spectral frequency of the emitted light was not able to be optimally detected by this film.

Applying the Bronx Box assay for AST of *M. tuberculosis*. In preliminary experiments using the non-pathogens *M. smegmatis* (Fig. 1b) and *M. bovis* BCG (not shown), the presence of spots after phage infection in the Bronx Box assay could be abolished by 1 to 2 days of pretreatment with effective antibiotics (INH, rifampin, or streptomycin). This effect corresponded to a decrease of >90% in the emitted light (rlu) in these antibiotic-containing wells, as measured by a luminometer. We then extended these assays to clinical strains of *M. tuberculosis* with various antibiotic susceptibilities. Although a range of antibiotic concentrations can be readily tested in this system, we chose those breakpoint concentrations which were previously shown to be useful in discriminating susceptible from resistant organisms in this (5, 29, 30) and similar systems (2, 8). Of note, the concentration of INH was increased from 0.1 to 0.2 μg/ml to account for the larger inoculum of cells being used than in our prior studies. A typical result with four different strains is shown in Fig. 4. In total, 25 unique strains were tested in 58 experiments and produced spots after phAE85 infection. The AST results with these strains are summarized in Table 1, showing that the accuracy of the assay for INH and rifampin, the major anti-tuberculosis agents, was nearly 100%. The one exception was a strain from a patient who relapsed after prolonged therapy with rifampin, ethambutol, and ofloxacin. The relapse isolate was identified as being rifampin susceptible by the BACTEC method but was repeatedly identified as being rifampin resistant by the Bronx Box assay. Molecular analysis of a subsequent isolate from the same patient (30a) revealed a genotypic correlate of rifampin resistance (an amino acid 533 Leu to Pro mutation) which has been associated previously with phenotypic resistance (35). Thus, there is both clinical and genotypic (by sequencing) evidence that corroborates the phenotypic resistance suggested for this strain by the Bronx Box assay.

Bronx Box assay for direct AST from agar. Although the strains tested as described above were derived from broth cultures at a density of 2 McFarland units, a more direct approach was evaluated whereby freshly growing tuberculous strains on Löwenstein-Jensen or 7H10 slants were inoculated into media to produce a 2 McFarland unit suspension and distributed immediately for AST experiments. Two modifications of the liquid protocol were found to be useful: (i) 3 days of incubation rather than 2 days was optimal to allow full antibiotic efficacy, and (ii) the INH concentration could be reduced to the more standard 0.1 μg/ml. Phage was added and antibiotic susceptibility measured as before with the Bronx Box assay. Once again, spots were observed in the no antibiotic well and in wells containing antibiotic to which the organism was found to be resistant but were not observed in wells containing effective antibiotics. The results for nine strains tested with the above protocol are summarized in Table 1. Four strains were primary

TABLE 1. Sensitivity and specificity of the Bronx Box assay for detecting antibiotic resistance in unique *M. tuberculosis* isolates

Growth platform	INH		Rifampin	
	% Sensitivity[a]	% Specificity[b]	% Sensitivity[a]	% Specificity[b]
Liquid medium	100 (8/8)	100 (13/13)	100 (8/8)	93 (14/15)
Solid medium	100 (3/3)	100 (6/6)	100 (3/3)	100 (6/6)
Either medium[c]	100 (9/9)	100 (16/16)	100 (10/10)	94 (16/17)

[a] Values in parentheses are number of resistant strains correctly identified/number of resistant strains tested.

[b] Values in parentheses are number of susceptible strains correctly identified/number of susceptible strains tested.

[c] Some strains were tested on both media.

clinical isolates grown on Löwenstein-Jensen slants, while the other five strains were previously tested and were grown on Middlebrook 7H10 plates. Concordance with the expected susceptibilities was 100% for these strains for INH and rifampin (Table 1).

DISCUSSION

The WHO states that TB control is lacking precisely in countries where the disease is most prevalent (41). The presence of TB, and especially drug-resistant TB, in these developing nations is a concern for all, since we are in an era of global commerce and migrations. In the United States, for example, a major contributor to the rise in TB cases in the 1990s has been the influx of foreign-born cases (4). Thus attention to TB control throughout the world should be a priority for the developed world as much as for the developing world.

Part of the problem in TB control in developing nations is inadequate resources to perform drug susceptibility test and ensure appropriate therapy for all patients. We have previously shown that the luciferase reporter phage assay is useful for determining antibiotic susceptibilities of clinical strains of *M. tuberculosis*, with sensitivity (5) and specificity (28), in a timely and a relatively "low-tech" manner. To further expedite the use of this assay in developing countries where most tuberculosis occurs, we have developed a photographic detection apparatus, designated the Bronx Box, which greatly simplifies the detection protocol and removes the requirement for the most costly component in the protocol, a luminometer. The semiquantitative output which results is simple to interpret and accurate compared to that from the luminometer or conventional AST assays. Furthermore, we show here that our method can be applied directly to primary isolates of *M. tuberculosis* grown on solid agar, available in 3 to 4 weeks from the time of sample acquisition (11) and still the most common means of cultivating *M. tuberculosis* worldwide (23, 41).

Significantly, our method uses concentrations of antibiotics which have been validated in the other major liquid AST methodology, the BACTEC method (32). We are thus able to correctly identify even low-level resistant strains (some of the MICs for the reference strains are close to the breakpoint) and in the case of the one rifampin-resistant "misdiagnosis," may actually be more sensitive for early emergence of resistance. In fact, if the assay is applied earlier to primary cultures, slower-growing resistant subpopulations could be detected more readily than by other growth-based methods. Furthermore, subtle differences from wild-type patterns of susceptibility can be evaluated by applying a range of antibiotic concentrations in calculating an MIC (42). Though not common in our data set, the issue of intermediate susceptibility needs to be considered, whether for a heterogeneous population of strains or for a homogeneous population which has a slightly elevated MIC relative to that for wild-type isolates. We have observed that phage-induced light production (measured by a luminometer) is reduced by at least 90% after antibiotic treatment of strains considered to be susceptible by conventional assays (29). Conversely, resistant strains in the presence of ineffective antibiotics produce at least 10% of the light observed without any antibiotic (29). In order to be certain that this 10% threshold can be visualized by the Bronx Box assay, we are currently including an additional control, a 1:10 dilution of the no-antibiotic, phage-infected cells. If this well produces any spot at all, we are confident that we will be able to visualize any relevant resistance, should it be present, as spots of similar or greater intensity in the antibiotic-containing wells.

While not reported here, other antimycobacterial agents can be tested or screened for activity by using photographic detection. Our preliminary data with ethambutol and streptomycin in this data set, while still being optimized, reveal a sensitivity and specificity of 50 and 97% for ethambutol resistance and 89 and 67% for streptomycin resistance, respectively. Notably these antibiotics have been difficult to standardize for AST (41) and have undergone modification of their breakpoints even with conventional methods (32).

Our efforts to improve the sensitivity of photographic detection were largely empirical. Though it is not clear why DTT and Mg affect the kinetics of luciferase-generated light output, it can be postulated that the DTT stabilizes the luciferin and Mg stabilizes the ATP (18). Although it has been shown that high ATP levels can interfere with light production (1), it is still surprising that at low pH, modest levels of extrinsic ATP can dampen light production in our in vivo luciferase detection system. Understanding these phenomena may help guide us in developing superior reaction conditions. In the meantime, there are ongoing efforts in our laboratory to improve the sensitivity of the film detection system by using more sophisticated optics and newer phages. In addition, we hope to further simplify the protocol (i) to be able to add phage and luciferin at the same time and (ii) to be able to add phage and antibiotics at the same time, with a lysogenic phage (10, 31). We plan to extend the methods directly to processed sputum samples. Finally, we aim to verify that a phage-infected mycobacterial culture is far less virulent than an uninfected culture, given that excess phage in the assay ultimately will lyse all the mycobacterial cells, thus providing an important extra measure of laboratory safety.

In summary, the Bronx Box adaptation of the luciferase reporter phage assay is a rapid, reliable means for performing antibiotic susceptibility testing of cultures *M. tuberculosis* in a simple, low-tech manner well-suited to the challenge of drug-resistant TB in the developing world. While further validation of the method is necessary, clinical trials in TB-ravaged countries are currently being initiated.

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