

Evaluation of Fluoromycobacteriophages for Detecting Drug Resistance in *Mycobacterium tuberculosis*[∇]

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We tested a new method for detecting drug-resistant strains of *Mycobacterium tuberculosis* that uses a TM4 mycobacteriophage phAE87::hsp60-EGFP (EGFP-phage) engineered to contain the gene encoding enhanced green fluorescent protein (EGFP). After promising results in preliminary studies, the EGFP-phage was used to detect isoniazid (INH), rifampin (RIF), and streptomycin (STR) resistance in 155 strains of *M. tuberculosis*, and the results were compared to the resazurin microplate technique, with the proportion method serving as the reference standard. The resazurin technique yielded sensitivities of 94% for INH and RIF and 98% for STR and specificities of 97% for INH, 95% for RIF, and 98% for STR. The sensitivity of EGFP-phage was 94% for all three antibiotics, with specificities of 90% for INH, 93% for RIF, and 95% for STR. The EGFP-phage results were available in 2 days for RIF and STR and in 3 days for INH, with an estimated cost of ~2\$ to test the three antibiotics. Using a more stringent criterion for resistance improved the specificity of the EGFP-phage for INH and RIF without affecting the sensitivity. In preliminary studies, the EGFP-phage could also effectively detect resistance to the fluoroquinolones. The EGFP-phage method has the potential to be a valuable rapid and economic screen for detecting drug-resistant tuberculosis if the procedure can be simplified, if it can be adapted to clinical material, and if its sensitivity can be improved.

The early implementation of effective antibiotic treatment for multidrug-resistant tuberculosis and extremely drug-resistant tuberculosis is only possible if drug resistance can be detected quickly. The ideal method for detecting drug resistance in *Mycobacterium tuberculosis* is still unclear, although there have been several recent advances. Relatively rapid phenotypic tests, such as the microscopic observation drug susceptibility (MODS) assay (11), alamarBlue (6), and nitrate reductase (2), have been recently validated by the World Health Organization (9, 15) but generally require at least 1 week of culture in either liquid or solid medium. Tests based on the amplification of nucleic acids, such as the line probe assay (7) or the very promising Xpert MTB/RIF (4), appear to be accurate and very rapid, but their costs limit their usefulness in resource poor settings where most drug-resistant tuberculosis occurs. The same is true for the highly effective BACTEC systems.

Other techniques proposed to detect drug resistance have used mycobacteriophages (10). In one method, *M. tuberculosis* strains are considered resistant if they can support the replication of phage D29 when grown in the presence of rifampin (1). The replicated phage are detected as plaques on rapidly

growing *Mycobacterium smegmatis*. Studies using this method have produced variable rates of sensitivity, and contamination can be a problem (10). Another method uses a phage engineered to carry the luciferase gene (3). Strains of *M. tuberculosis* are grown in the presence of antibiotic and then infected with the luciferase-phage. If the strains are resistant to the antibiotic, they produce the ATP needed for the luciferase enzyme to produce light. This method has shown high sensitivity and specificity but requires manipulation of liquid cultures of *M. tuberculosis* in a relatively costly luminometer.

More recently, a new method has been proposed using phAE87::hsp60-EGFP (EGFP-phage), a TM4 derivative phage engineered to carry the gene for enhanced green fluorescent protein (EGFP) (12). As with the luciferase phage, EGFP-phage is used to infect strains of *M. tuberculosis* growing in the presence of antibiotics, which are then inactivated, spotted onto glass slides, and examined by using a fluorescence microscope for the fluorescent bacilli that indicate drug resistance (Fig. 1). We describe here the first test of the EGFP-phage for detecting drug resistance in natural isolates of *M. tuberculosis* strains.

MATERIALS AND METHODS

Strains. Preliminary studies were performed on *M. smegmatis* mc²155 and *Mycobacterium bovis* BCG, as well as several fluoroquinolone-resistant strains of each that had been previously selected and characterized for *gyrA* mutations in the Laboratorio de Genética Molecular, IVIC. A subsequent pilot study was performed with 20 clinical strains that constitute a test bank of strains donated by Juan Carlos Palomino of the Institute of Tropical Medicine, Antwerp, Belgium, and Dick van Soolingen, RIVM, Bilthoven, Holland. A larger study of 155 strains included the 20 strains previously tested, plus 81 clinical isolates determined to be resistant to at least one of the antibiotics to be tested—INH, RIF,

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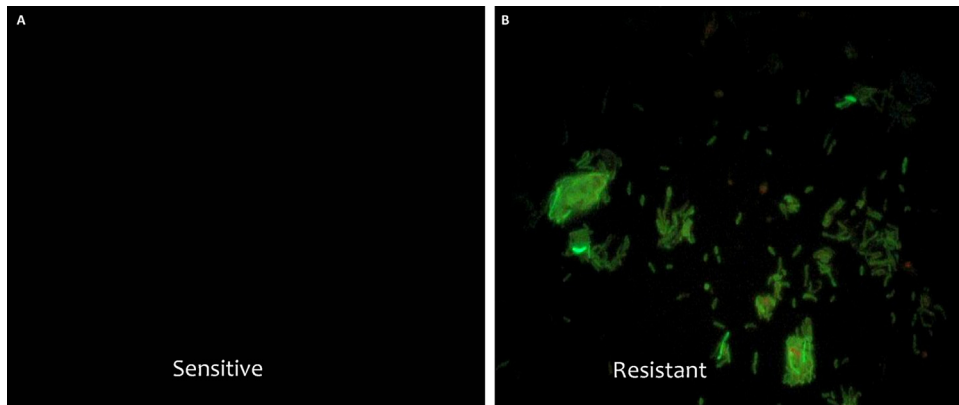


FIG. 1. Two strains of *M. tuberculosis* were incubated separately in 7H9-OAD with 2 µg of RIF/ml for 24 h, infected with the EGFP-phage, killed with paraformaldehyde, and then fixed on microscope slides as described in Materials and Methods. The images, obtained with a fluorescence microscope, show a strain sensitive to RIF (A) and a strain resistant to RIF (B).

or STR—and 49 clinical strains found to be sensitive to all three drugs. These clinical strains were isolated and characterized for drug sensitivity by the resazurin method either in the Laboratorio de Tuberculosis, Instituto de Biomedicina, or the Instituto de Higiene, Caracas, Venezuela, and graciously donated by Sandra Fernández. The present study also included five strains obtained from the American Type Culture Collection (ATCC): H37Rv, H37Ra, and ATCC 355822, resistant to INH; ATCC 35820, resistant to STR; and ATCC 35838, resistant to rifampin.

Phage stocks. The construction of the phAE87:*hsp60*-EGFP phage (EGFP-phage) has been previously described (12). To prepare phage stocks, *M. smegmatis* strain mc²155 was grown to mid-exponential phase in 7H9 with 10% OAD (0.5% oleic acid, 0.85% NaCl, 5% albumin, 2% glucose), 0.5% glycerol, and 0.04% tyloxapol. The bacteria were washed once in phage buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.6], 10 mM MgSO₄, and 8 mM CaCl₂) to eliminate traces of tyloxapol; then 100 µl was added to a tube containing approximately 5,000 to 10,000 EGFP-phage particles, followed by incubation at room temperature for 30 min. Subsequently, 3 ml of 7H9 with 0.7% agar was added to the tube, followed by brief mixing, and poured onto a 100-mm petri dish containing 7H10-OAD which, after the top-agar had solidified, was then incubated at 30°C. After 48 h, 3 ml of phage buffer was pipetted onto plates on which the lytic phage plaques were confluent but distinguishable, and left overnight at 4°C with mild agitation. The buffer was then removed and passed through a 0.2-µm-pore-size syringe filter, and the phage particle titers were determined.

EGFP-phage assay. The protocol for the EGFP-phage assay is outlined in Table 1.

Resazurin assay. Bacterial suspensions with a turbidity of McFarland 1 were diluted 1:20 in 7H9-OAD, and 100 µl was added to the wells of 96-well plates containing 100 µl of 7H9-OAD without antibiotics or with antibiotics in decreasing 2-fold dilutions as follows: INH, 1 to 0.031 µg/ml; RIF, 2 to 0.062 µg/ml; and STR, 8 to 0.25 µg/ml. Each strain was assayed in duplicate. On day 7, 30 µl of an aqueous 0.01% solution of sodium resazurin (Sigma-Aldrich) was added to a control well. If the control well turned pink, indicating bacterial growth, resazurin was then added to the wells containing antibiotics. If the control well did not change color, resazurin was added to a control well on days 9, 11, 13, and 15 until a color change was seen. The cutoff concentrations defining resistance were as follows: INH, 0.25 µg/ml; RIF, 0.25 µg/ml, and STR, 2 µg/ml.

Proportion method. The assay was performed as described earlier (5) but modified to use 7H10-OAD medium, with final results read after 6 weeks. Colonies were scraped from a culture on 7H10-OAD and vortexed in a glass tube with 3-mm glass beads and three drops of sterile distilled water. After sitting for 15 min, 2 ml of sterile distilled water was added, and the tube was shaken and left standing for an additional 15 min. An aliquot was placed into a new tube, the turbidity was adjusted to McFarland 0.5 with sterile water, and then dilutions of 10⁻³, 10⁻⁴, and 10⁻⁵ were plated on 7H10-OAD medium without antibiotics to ascertain the number of CFU present. Liquid cultures in 7H9-OAD were used for some strains and similarly diluted. To determine resistance, 100-µl portions of a 10⁻⁴ dilution of each strain were spread onto plates containing INH (0.2 µg/ml), INH (1 µg/ml), RIF (1 µg/ml), STR (2 µg/ml), or STR (10 µg/ml), as well as two control plates without antibiotics. The plates were kept at room temperature until the inocula were completely absorbed into the agar and then incu-

bated at 37°C and examined weekly for 6 weeks. Resistance was determined when the number of colonies growing on a plate with antibiotics was at least 1% of the colonies growing on plates without antibiotics. The results on plates with INH at 0.2 µg/ml, RIF at 1 µg/ml, and STR at 2 µg/ml were used for comparison

TABLE 1. Protocol for testing *M. tuberculosis* strains for drug resistance using EGFP-phage

| Step | Description |
|---------|---|
| 1..... | <i>M. tuberculosis</i> strains are grown in 7H9-OADC and 0.04% tyloxapol to McFarland 3 |
| 2..... | Aliquots (1 ml) are divided into four tubes |
| 3..... | Centrifugation and then resuspension in 7H9-OADC without Tween or tyloxapol and either (i) no antibiotic, (ii) INH at 0.2 µg/ml, (iii) RIF at 2 µg/ml, or (iv) STR at 4 µg/ml |
| 4..... | Incubation at 37°C for 24 (or 48 h for the INH treatment) |
| 5..... | Centrifugation and resuspension in the same medium to McFarland 3 |
| 6..... | Aliquots (200 µl) from each antibiotic tube are placed into new tubes, with two new tubes from the no-antibiotic control |
| 7..... | A 100-µl portion of a 10 ¹⁰ EGFP-phage stock is added to each tube except for one control tube |
| 8..... | Incubation for 16 to 24 h 37°C |
| 9..... | A 300-µl portion of paraformaldehyde (4% in phosphate-buffered saline [PBS]) is added |
| 10..... | The strains are left at room temperature for 90 min to ensure killing of the bacteria |
| 11..... | Centrifugation and resuspension in 300 µl of 50 mM NH ₄ Cl |
| 12..... | The strains are left at room temperature for 90 min to reduce the background fluorescence |
| 13..... | Centrifugation and resuspension in 400 µl of PBS |
| 14..... | Centrifugation and resuspension in 25 µl of PBS (at this stage the bacteria can be stored at 4°C) |
| 15..... | The samples are spread as 5 µl onto a glass microscope slide |
| 16..... | The samples are covered with a labeled coverslip and sealed with a 1:1:1 mixture of Vaseline-lanolin-paraffin |
| 17..... | The slides are examined by using a Nikon Eclipse TE 2000 fluorescent microscope with 100× objective lens and oil immersion; the slides are interpretable for at least a month |

TABLE 2. Results and performance parameters: phage versus proportion^a

| Antibiotic | No. of strains | | | | | Phage vs proportion | | | |
|------------|----------------|---------|---------|---------|-------|---------------------|---------------|------------|-------------|
| | Prop R | | Prop S | | Total | % Sensitivity | % Specificity | % PPV | % NPV |
| | Phage R | Phage S | Phage R | Phage S | | | | | |
| INH | 46 | 3 | 11 | 95 | 155 | 94 (83–98) | 90 (82–94) | 81 (69–89) | 97 (82–94) |
| RIF | 16 | 1 | 9 | 129 | 155 | 94 (73–99) | 93 (88–97) | 64 (45–80) | 99 (96–100) |
| STR | 58 | 4 | 5 | 88 | 155 | 94 (95–97) | 95 (88–98) | 92 (83–97) | 96 (89–98) |

^a The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the three methods used to detect resistance were compared to each other in Tables 2 to 4. Abbreviations: Prop, proportion; Phage, *EGFP*-phage; R, resistant; S, sensitive. The concentrations used for defining resistance with the proportion method were as follows: INH, 0.2 µg/ml; RIF, 1 µg/ml; and STR, 2 µg/ml. The 95% confidence intervals are indicated in parentheses.

with the other techniques. Confidence intervals for accuracy estimates were calculated with the CEBM online statistics calculator (ktclearinghouse.ca/cebm/practice/ca/calculators/statscale).

RESULTS

In initial studies to standardize the protocol, the *EGFP* phage accurately detected fluoroquinolone resistance in several strains of *M. smegmatis* and *M. bovis* BCG with *gyrA* mutations (data not shown) (14).

Subsequently, the *EGFP*-phage was used in a pilot study to detect resistance to isoniazid (INH), rifampin (RIF), and streptomycin (STR) in a panel of 20 well-characterized strains of *M. tuberculosis* that have been used to test other methods. The strains were also tested with the resazurin microplate assay. Compared to results with the microplate assay, the *EGFP*-phage method had a 100% sensitivity, detecting all resistance to INH, RIF, and STR and specificities of 95% for INH, 85% for RIF, and 90% for STR (data not shown).

The *EGFP*-phage was then used to detect INH, RIF, and STR resistance in 155 strains of *M. tuberculosis*, including the 20 strains previously tested. The isolates were also assayed by using the resazurin microplate assay, with the proportion method used as the reference test.

Compared to the results with the proportion method, the *EGFP*-phage had 94% sensitivity for detecting resistance to the three antibiotics, with specificities of 90% for INH, 93% for RIF, and 95% for STR (Tables 2, 3, and 4). The sensitivity of the resazurin method was also 94% for INH and RIF, but 98% for STR, and the specificities were 97%, for INH, 95% for RIF, and 99% for STR. The only contamination in the study occurred in two strains with the resazurin method, for which results were uninterpretable.

The criterion for resistance with the *EGFP*-phage method was the presence of at least one fluorescent bacillus per high-

power field, but it was reasoned that perhaps the number of strains falsely labeled as resistant might be reduced if the criterion for resistance were more stringent, requiring two or more fluorescent bacilli per field. To test this, the slides were reexamined with a fluorescence microscope a month after the initial reading, and the absolute number of fluorescent bacilli per field was recorded. When resistance was defined as two fluorescent bacilli, the specificity improved for all three drugs tested, and the sensitivity was unchanged for INH and RIF but decreased for STR (Table 5). When the criterion for resistance was three or more fluorescent bacilli per field, the sensitivity dropped for all three antibiotics.

We also examined the results from the strains that were resistant with the proportion method but sensitive with the *EGFP*-phage. Two of the four strains discordantly called sensitive to STR had only 1 or 2% resistant colonies, less than any of the concordantly resistant isolates, but the others had 6 and 11% resistance. Two of the three strains discordantly sensitive to INH with the *EGFP*-phage had only 2 and 4% resistant colonies with the proportion method, but there were two concordantly resistant strains with the same percentage of resistant colonies. The third discordant strain had 20% resistant colonies. The one strain discordantly sensitive to RIF had 5% resistant colonies, and there were four other concordantly resistant strains that had the same or lower percentages of resistant colonies with the proportion method.

With the *EGFP*-phage the results were available for RIF and STR in 48 h and in 72 h for INH, with a total of cost approximately \$2 for testing the three antibiotics (Table 6). The results were available with the resazurin method in an average of 11 days, and an average of 40 days with the proportion method. Costs for these two methods have been estimated elsewhere as approximately \$11 with the resazurin assay and \$2.5 for the proportion method (8).

TABLE 3. Results and performance parameters: phage versus resazurin^a

| Antibiotic | No. of strains | | | | | Phage vs resazurin | | | |
|------------|----------------|---------|---------|---------|-------|--------------------|---------------|------------|------------|
| | Prop R | | Prop S | | Total | % Sensitivity | % Specificity | % PPV | % NPV |
| | Resaz R | Resaz S | Resaz R | Resaz S | | | | | |
| INH | 45 | 12 | 4 | 94 | 155 | 79 (67–88) | 96 (90–98) | 92 (81–97) | 89 (81–93) |
| RIF | 20 | 5 | 3 | 127 | 155 | 80 (60–91) | 98 (93–99) | 87 (68–96) | 96 (91–98) |
| STR | 58 | 5 | 4 | 86 | 153 | 92 (83–97) | 96 (89–98) | 94 (85–98) | 95 (88–98) |

^a The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the three methods used to detect resistance were compared to each other in Tables 2 to 4. Abbreviations: Prop, proportion; Resaz, resazurin; Phage, *EGFP*-phage; R, resistant; S, sensitive. The concentrations used for defining resistance with the proportion method were as follows: INH, 0.2 µg/ml; RIF, 1 µg/ml; and STR, 2 µg/ml. The 95% confidence intervals are indicated in parentheses.

TABLE 4. Results and performance parameters: resazurin versus proportion^a

| Antibiotic | No. of strains | | | | Total | Resazurin vs proportion | | | |
|------------|----------------|---------|---------|---------|-------|-------------------------|---------------|-------------|-------------|
| | Prop R | | Prop S | | | % Sensitivity | % Specificity | % PPV | % NPV |
| | Resaz R | Resaz S | Resaz R | Resaz S | | | | | |
| INH | 46 | 3 | 3 | 103 | 155 | 94 (84–98) | 97 (92–99) | 94 (84–98) | 97 (92–99) |
| RIF | 16 | 1 | 7 | 131 | 155 | 94 (73–99) | 95 (90–98) | 70 (49–84) | 99 (96–100) |
| STR | 61 | 1 | 1 | 90 | 153 | 98 (91–100) | 99 (94–100) | 98 (91–100) | 99 (94–100) |

^a The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the three methods used to detect resistance were compared to each other in Tables 2 to 4. Abbreviations: Prop, proportion; Resaz, resazurin; R, resistant; S, sensitive. The concentrations used for defining resistance with the proportion method were as follows: INH, 0.2 µg/ml; RIF, 1 µg/ml; and STR, 2 µg/ml. The 95% confidence intervals are indicated in parentheses.

DISCUSSION

The EGFP-phage has been tested previously in an experimental setting (12), but this is the first report testing its ability to detect drug resistance in clinical isolates of *M. tuberculosis*. It was found to have a sensitivity of 94% for all three drugs tested and specificities of 90% for INH, 93% for RIF, and 95% for STR compared to results with the proportion method. Parallel studies using the resazurin microplate assay also showed 94% sensitivity for INH and RIF but a higher 98% for STR. The resazurin method also showed higher specificities of 97% for INH, 95% for RIF, and 99% for STR, again using results with the proportion method as the reference.

The principal use of the EGFP-phage method would be as a rapid, inexpensive screen to detect resistant strains requiring full drug sensitivity testing with another method in order to confirm the resistance and determine to which drugs the strain is susceptible. Therefore, very high sensitivity is the priority, and the 94% sensitivity, while equal to that obtained with the resazurin method for INH and RIF, is not optimal. If the problem is that fluorescent bacteria are not always easy to see, the sensitivity should increase with a newer version of the phage that expresses EGFP from a stronger promoter and creates more brightly fluorescent bacilli (W. R. Jacobs, Jr., unpublished data).

TABLE 5. Performance parameters of the EGFP-phage assay depending upon the number of fluorescent bacilli per high-power field used as the criterion for resistance to the antibiotic tested

| Antibiotic tested | Parameter ^a | % Parameter value observed with 1, 2, 3, or 4 fluorescent bacilli as the resistance criterion | | | |
|-------------------|------------------------|---|-----------|-----------|-----------|
| | | 1 bacillus | 2 bacilli | 3 bacilli | 4 bacilli |
| INH | Sensitivity | 94 | 94 | 84 | 73 |
| | Specificity | 90 | 92 | 97 | 100 |
| | PPV | 81 | 85 | 93 | 100 |
| | NPV | 97 | 97 | 93 | 89 |
| RIF | Sensitivity | 94 | 94 | 76 | 65 |
| | Specificity | 94 | 96 | 100 | 100 |
| | PPV | 67 | 76 | 100 | 100 |
| | NPV | 99 | 99 | 97 | 96 |
| STR | Sensitivity | 94 | 90 | 78 | 67 |
| | Specificity | 95 | 97 | 99 | 100 |
| | PPV | 92 | 94 | 98 | 100 |
| | NPV | 96 | 95 | 89 | 85 |

^a PPV, positive predictive value; NPV, negative predictive value.

Resistance is defined with the proportion method as the survival of >1% of bacteria in the presence of antibiotic. It was reasoned that high-power fields might contain many more than 100 bacilli, so that one fluorescent bacillus per field may actually represent <1% of resistant bacteria. When the criterion for resistance was two fluorescent bacilli per high-power field, the specificity improved for INH and RIF without affecting the sensitivity, although the sensitivity dropped from 94 to 90% for STR. Since detection of RIF resistance can be used as an indication of multidrug resistance requiring a change in the antibiotic regimen, this more stringent criterion, if confirmed in subsequent studies, might be appropriate, with the criterion for STR resistance remaining one fluorescent bacillus per field. We did not attempt to determine resistance to ethambutol with the EGFP-phage method, because as an economic and rapid screen, the detection of resistance to INH and especially RIF, would be sufficient to trigger full drug susceptibility testing with a more comprehensive method. In addition, the concentration cutoff for defining ethambutol resistance has been questioned (13).

Most of the strains that were discordantly sensitive with the EGFP-phage had ≤5% resistant colonies with the proportion method, but some had larger proportions, and other strains correctly designated as resistant had ≤5% resistant colonies. So while there is a suggestion that the EGFP-phage method may sometimes have difficulty in correctly detecting resistance present in proportions of 5% or less, this does not appear to be the only source of error. The final results of the modified proportion method were determined after 40 days. If results were read at the standard 21 days, it is possible that some of the discordant strains that showed only one or two percentage resistance with the proportion method might have been called

TABLE 6. Estimated costs for testing 200 strains for resistance to isoniazid, rifampin, and streptomycin

| Item | Cost (US\$) |
|-------------------------------|-------------|
| Antibiotics..... | 40 |
| Medium 7H9 (2 liters)..... | 5 |
| OAD, glycerol, tyloxapol..... | 35 |
| Slides and coverslips..... | 200 |
| Chemicals and buffers..... | 15 |
| Paraformaldehyde..... | 5 |
| Other..... | 35 |
| Eppendorf tubes (1800)..... | 66 |
| Total (for 200 strains)..... | 401 |
| Cost per strain..... | 2.01 |

drug sensitive, which would have improved the calculated sensitivity of the *EGFP*-phage method.

The results here are better than achieved in most studies with the D29 phage replication assay, but 100% sensitivity and specificity have been reported for the luciferase phage (10). The MODS method (9), and the recent Xpert methods have also reported better results (4). Nonetheless, the *EGFP*-phage method has some advantages that make it a promising method deserving further study and development: (i) results for RIF and STR are available in 2 days, and for INH in 3 days, and preliminary tests suggest that it will also detect fluoroquinolone resistance in 48 h; (ii) the liquid cultures are grown in sealed microtubes and then inactivated with paraformaldehyde, so that subsequent processing does not require biosafety facilities; (iii) new, low cost solar powered LED fluorescence microscopes are commercially available; (iv) minimal additional training is required for TB lab personnel to perform the technique; and (v) finally, the assay requires no reagents beyond antibiotics and media, and the phage stocks can be easily and cheaply reproduced. The cost can be as low as \$2 to test for resistance to three drugs, and at least 20 strains can be processed daily (Table 6). If the *EGFP*-phage method can be adapted for clinical material, the sensitivity can be improved to ~98%, and the protocol simplified, it could prove useful as a rapid and economic way to detect multidrug-resistant or extensively drug-resistant strains of *M. tuberculosis* in resource-poor settings with minimal infrastructure.

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