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Evaluation of Reverse Transcription-PCR and a Bacteriophage-Based Assay for Rapid Phenotypic Detection of Rifampin Resistance in Clinical Isolates of *Mycobacterium tuberculosis*

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New rapid phenotypic assays for the detection of rifampin resistance in *Mycobacterium tuberculosis* have recently been described, but most of these require liquid cultures, which reduces the utility of many tests in terms of turnaround times. In the United Kingdom, over 90% of rifampin-resistant isolates are also resistant to isoniazid, so rifampin resistance can be used as a sensitive marker for multidrug-resistant tuberculosis. In this study, two new rapid phenotypic assays were compared to the standard resistance ratio method on 91 clinical isolates of *M. tuberculosis*. One, the phage amplified biologically (PhaB) assay, has been described previously and is based on the inability of susceptible isolates of *M. tuberculosis* to support the replication of bacteriophage D29 in the presence of inhibitory doses of rifampin. The other employed reverse transcription (RT)-PCR to demonstrate a reduction in inducible *dnaK* mRNA levels in susceptible isolates treated with rifampin. After incubation for 18 h with 4 μg of rifampin per ml, the PhaB assay showed concordance with the resistance ratio method for 46 of 46 (100%) susceptible and 31 of 31 (100%) resistant isolates, while RT-PCR showed concordance for 46 of 48 (96%) susceptible and 35 of 36 (97%) resistant isolates. We believe these assays provide a reliable rapid means of susceptibility testing with a total turnaround time of only 48 h, although the PhaB assay is better in terms of its lower technical demand and cost and its applicability to tuberculosis susceptibility testing in developing countries.

The Centers for Disease Control and Prevention recommend that all isolates of *Mycobacterium tuberculosis* be tested for their susceptibility to antibiotics, using the most rapid and reliable means possible, and that susceptibility data be available within 15 to 30 days of receipt of a specimen (19).

Conventional culture-based techniques for susceptibility testing vary in the methodology employed. The proportion method measures the percentage of resistant clones in a population, whereas the resistance ratio method compares the growth of the test organism at three concentrations of a drug with the growths of a number of susceptible wild-type strains, although an element of proportionality is still preserved (2). These methods take several weeks from receipt of a primary specimen to complete, and although proportion-based liquid culture systems have significantly reduced turnaround times, results are still not available for at least 5 days after receipt of an isolate (16). More rapid methodologies providing meaningful data within a few days have been actively sought, and a number have been developed (10, 18, 20, 21). Although commercially available molecular tests offer great benefits in terms of rapidity and reproducibility, there are potential problems. Approximately up to 5% of mutations which confer rifampin resistance are missed by the INNO-LiPA Rif.TB assay (17, 18, 20), and no information concerning the proportion of resistant clones detected in clinical samples or cultures has been presented. Molecular techniques have limited utility for detecting resistance to antibiotics other than rifampin, for which mutations conferring resistance occur at a number of different genetic loci (6). Attention is now focusing on rapid phenotypic methods, which use markers of viability other than an increase in biomass. A number of different strategies have been employed, including the use of vital dyes (12), the particle counting immunoassay (5), and the use of luciferase-reporter phage for the antibiotic susceptibility testing of mycobacteria (15), which has been applied to both first- and second-line agents with some success. However, appraisals of these rapid phenotypic methods sometimes fail to take into account the time taken to establish log-phase liquid cultures prior to testing. As most cultures referred to the Public Health Laboratory Service Mycobacterium Reference Unit (MRU) are subcultures on solid medium, such methods may offer little advantage, in terms of time saved, over conventional methods. Reverse transcriptase (RT)-PCR can be used to demonstrate quantitative changes in mRNA levels (7) and thus offers a relatively rapid method for the demonstration of the viability of organisms. In this paper, we describe an assay based on the detection of changes in heat shock protein mRNA (*dnaK* mRNA) levels in *M. tuberculosis*. This is an extension of the method used to detect viability in *Mycobacterium leprae* by measuring steady-state *dnaK* mRNA levels (13), and it involves the extraction of mRNA by methodology described previously by members of our group (11) after the heat shock of organisms at 45°C for 45 min, which increased *dnaK* mRNA levels 50- to 100-fold in viable mycobacteria (14). We have also compared this with a low-cost rapid phenotypic method described previously by members of our group (22), a method that relies on the ability of rifampin to block the lytic cycle of bacteriophage D29 within susceptible *M. tuberculosis* and hence the production of plaques on a lawn of the rapidly growing indicator organism *Mycobacterium smeg-*

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matis. The results of both assays were compared with those of the resistance ratio method.

MATERIALS AND METHODS

Isolates. Clinical isolates of *M. tuberculosis* from specimens cultured at the MRU or ones that were referred for sensitivity testing from other sites in the United Kingdom were identified by conventional biochemical methodology. For the majority of isolates, identity was also confirmed by DNA hybridization (AccuProbe; GenProbe, Inc., San Diego, Calif.). Isolates were cultured on Lowenstein-Jensen (LJ) egg medium at 37°C and stored at 20°C in the dark, ready for use.

Preparation of samples. A 1-μl loopful, containing approximately 10^6 organisms of a mycobacterial isolate, was transferred from growth on LJ slopes to a 25-ml plastic screw-cap universal container (Bibby-Sterilin, Stone, United Kingdom) containing approximately 1 ml of acid-washed glass beads (1 to 4 mm in diameter) in 1 ml of 7H9 broth (Becton Dickinson, Oxford, United Kingdom) with 10% (vol/vol) oleic acid-albumin-dextrose-catalase enrichment (Difco Laboratories, Detroit, Mich.) and 1 mM $CaCl_2$. After vortexing for 20 s, an additional 4 ml of 7H9 medium was added and the homogenate was allowed to stand for 15 to 20 min to allow larger clumps to settle. One milliliter of homogenate supernatant was then added to aliquots of rifampin stock solution in 25-ml plastic universal containers to yield the final test concentrations. Control samples consisting of 1 ml of organism suspension without rifampin were included. Immediately prior to RNA extraction, organisms were incubated for 45 min at 45°C.

Extraction of RNA. mRNA was extracted from 50 μl of culture (approximately 10^4 CFU) by the method described by Mangan et al. (11). RNA was resuspended in 30 μl of diethyl pyrocarbonate-treated distilled water.

DNase I treatment and reverse transcription. Five microliters of extract was treated with 1 U of DNase I (Life Technologies, Paisley, United Kingdom) at room temperature for 15 min. The reaction was stopped by adding 1 μl of EDTA (final concentration, 2.5 mM), followed by 10 min at 85°C to denature the DNase and RNA prior to reverse transcription. Samples were stored on ice until used. For each new batch of DNase I, control samples were treated with RNase (Life Technologies) for 10 min at 37°C to ensure that any PCR signal was RNA specific and to confirm that no DNA remained after DNase treatment.

Reverse transcription was performed as follows. Ten microliters of a DNase-treated sample was added to 10 μl of master mix containing 100 U of Superscript II RT reverse transcriptase (Life Technologies), deoxyribonucleoside triphosphates at 1.9 mM each (Promega, Madison, Wis.), 10 mM dithiothreitol, 10.8 nM primer HSP70II (primers synthesized by Genosys, Cambridge, United Kingdom), 40 U of rRNasin RNase inhibitor (Promega), and 4.5 μl of first-strand buffer (Life Technologies) in 0.5-ml snap-seal apex tubes (Alphalabs, Eastleigh, Hampshire, United Kingdom) and incubated at 42.7°C for 45 min. Samples were heated to 100°C for 2 min prior to PCR.

PCR. Ten-microliter samples were added to 40 μl of PCR master mix containing 1 U of *Taq* DNA polymerase (Promega), 125 nM concentrations of each primer, 250 μM concentrations of each deoxyribonucleoside triphosphate (Promega), and 2 mM $MgCl_2$. Reaction mixes were overlaid with 40 μl of mineral oil to prevent evaporation. The primers which were designed to amplify a 274-bp fragment, corresponding to nucleotides 1526 to 1800 of *hsp*70 (*dnaK*), were HSP70I (5′-ATTGTGCACGTCACCGCC-3′) and HSP70II (5′-ACCGCGGCA TCAACCTTG-3′).

Resistance ratio method of susceptibility testing. Rifampin susceptibility testing was carried out on all isolates with the modification of the resistance ratio method described by Collins et al. (2).

PhaB assay. The phage amplified biologically (PhaB) assay was performed as described by Wilson et al. (22) after overnight exposure to the antibiotic. Briefly, the PhaB assay depends upon the ability of lytic phage D29 to infect both *M. tuberculosis* and *M. smegmatis* in 7H9 medium containing 1 mM $CaCl_2$. The bacteriophage enters the cells and undergoes a lytic cycle, which can subsequently be demonstrated in a quantitative manner by the production of plaques when the infected *M. tuberculosis* is mixed with a heavy suspension of *M. smegmatis* in solid medium and incubated overnight at 37°C. Antibiotic pretreatment of susceptible *M. tuberculosis* organisms should render these organisms incapable of supporting a lytic cycle, and hence plaques should be produced in the indicator cells. To ensure that only intracellular phage is carried over into the *M. smegmatis* cultures, extracellular viruses are destroyed with the phagicidal agent ferrous ammonium sulfate. Controls with no *M. tuberculosis* cells were included with each batch of isolates. Final plaque counts on untreated plates vary. Typically, 1 ml of sample diluted 1 in 10 in 7H9 medium containing 1 mM $CaCl_2$ produces 100 to 2,000 plaques. Plaque numbers above this are difficult to assess, as lysis becomes semiconfluent.

Antibiotics. Rifampin (Sigma) was made up as a stock solution at 20 mg/ml in dimethylformamide (Sigma) and stored in 30-ml screw-cap glass bottles. Further dilutions were made in sterile diethyl pyrocarbonate-treated water immediately prior to use.

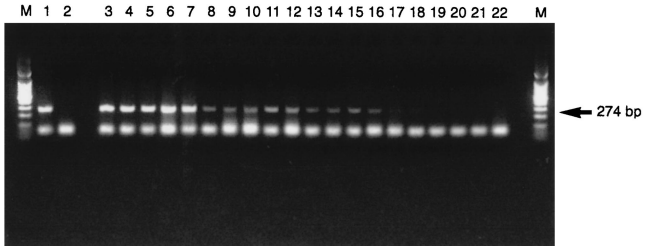

FIG. 1. Agarose (1.5%) gel demonstrating loss of the 274-bp *dnaK* product with increasing concentrations of rifampin. RT-PCR was performed on five rifampin-susceptible clinical isolates of *M. tuberculosis* after overnight incubation in Middlebrook 7H9 broth with no drug (lanes 3 to 7) and with rifampin at 1 μg/ml (lanes 8 to 12), 2 μg/ml (lanes 13 to 17), and 4 μg/ml (lanes 18 to 22) following incubation at 45°C for 45 min. Lane 1, positive DNA control; lane 2, negative PCR control; lanes M, molecular weight markers.

RESULTS

Initial evaluation of RT-PCR. PCR was carried out with a Perkin-Elmer 9600 thermal cycler on genomic DNA extracted from serial dilutions of *Mycobacterium bovis* BCG. The limit of detection was 10^3 to 10^4 genome equivalents. RT-PCR performed on mRNA extracted from 10-fold dilutions of BCG, and compared with colony counts on LJ medium, detected a 274-bp *dnaK* product from as few as 10 organisms after heat shock and 10 to 100 organisms without prior heat shock. The absence of RT-PCR product after treatment with RNase confirmed that the product was derived from RNA.

Inhibition of *dnaK* mRNA by rifampin. RT-PCR was carried out on rifampin-susceptible isolates (determined by the resistance ratio method) of *M. tuberculosis* after overnight incubation with rifampin at 1, 2, and 4 μg/ml (Fig. 1). The loss of product was consistently demonstrated after overnight incubation with rifampin at 4 μg/ml.

To assess the reproducibility of RT-PCR as a screen for rifampin resistance, it was carried out in triplicate on 10 clinical isolates. The persistence of a 274-bp RT-PCR product after overnight incubation with rifampin at 4 μg/ml was used to define resistance. The results were interpreted in a blinded fashion by an observer who correctly scored four resistant and six sensitive strains.

To assess the ability of the assay to detect small subpopulations of rifampin-resistant organisms, RT-PCR was performed on suspensions of rifampin-susceptible and -resistant *M. tuberculosis* cells mixed in various proportions after overnight incubation with and without rifampin at 4 μg/ml. The relative proportions of resistant and susceptible organisms were adjusted in accordance with colony counts on LJ medium with and without rifampin. RT-PCR product was readily demonstrated in populations in which 0.1 to 1% of the organisms were rifampin resistant (Fig. 2).

PhaB assay. A sample was scored as sensitive if the number of plaques on the plate with antibiotic-treated organisms was less than 1% of that on the control plate. When the number was greater than 1%, the sample was scored as resistant. Thus, isolates were scored only if >100 plaques were present on the control plates. Results were deemed noninterpretable if fewer than 100 plaques or confluent lysis was present on the control plate.

To assess the ability of the PhaB assay to detect small percentages of resistant organisms in a drug-susceptible population, the PhaB assay was performed on cultures of susceptible *M. tuberculosis* mixed with 10-fold-higher numbers of resistant organisms. The increase in the plaque counts seen in the antibiotic-treated cultures reflected the 10-fold-higher numbers

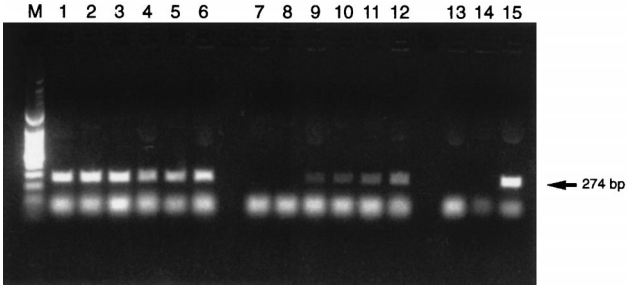

FIG. 2. Agarose (1.5%) gel demonstrating the presence of the 274-bp *dnaK* product in a culture of rifampin-susceptible *M. tuberculosis* organisms mixed with an increasing percentage of rifampin-resistant *M. tuberculosis* organisms. Lanes 1 to 6, cultures with 0, 0.01, 0.1, 1, 10, and 50%, respectively, rifampin-resistant *M. tuberculosis* not exposed to rifampin; lanes 7 to 12, same cultures as lanes 1 to 6, respectively, after overnight exposure to rifampin at 4 μg/ml; lanes 13 and 14, negative PCR controls; lane 15, positive DNA control; lane M; molecular weight markers.

of resistant organisms in the mixed population (Table 1).

Utility of *dnaK* RT-PCR and PhaB assay for detection of rifampin resistance in clinical isolates of *M. tuberculosis*. To assess the utility of the RT-PCR and PhaB assays for rifampin susceptibility testing of clinical isolates referred to the MRU, both assays were performed on 91 clinical isolates of *M. tuberculosis* and the results were compared with those obtained by the resistance ratio method.

Of the 91 isolates tested by RT-PCR, 84 yielded a result on initial testing and seven were repeated after being scored as noninterpretable by a blinded observer. No product was obtained from one isolate on repeat testing. Further examination showed the isolate to be contaminated. Of the 90 isolates yielding an interpretable result, either initially or on retesting, 39 of 40 (98%) resistant and 48 of 50 (96%) susceptible isolates, determined by the resistance ratio method, were correctly assigned (Table 2). Little or no reduction in *dnaK* product occurred in resistant isolates treated with rifampin, compared with susceptible isolates (Fig. 3). On repeat testing, the two isolates incorrectly scored as resistant were both scored as susceptible. The single isolate incorrectly scored as susceptible became contaminated after testing and was not tested further.

Of the 91 isolates tested by the PhaB assay, 77 yielded a result on initial testing and 14 were tested again due to insufficient plaque numbers in the "no-antibiotic" control. On repeat testing, it was discovered that four cultures were contaminated and three subcultures were no longer viable. Of the remaining 84 isolates, 31 of 31 (100%) resistant and 46 of 46 (100%) susceptible isolates, determined by the resistance ratio method, were correctly assigned (Table 2).

TABLE 1. Detection of resistant organisms by the PhaB assay in mixed cultures of susceptible and resistant organisms

% Resistant organisms in culture[a]	Mean plaque count		% Plaques obtained with antibiotic
	Without rifampin	With rifampin (4 μg/ml)	
0	2,200	0	0
0.01	2,159	1	0.05
0.1	2,154	6	0.3
1	2,006	20	1
50	647	199	31
100	355	208	59

[a] Calculated from colony counts performed for resistant and susceptible strains on LJ medium.

TABLE 2. Concordance of RT-PCR and PhaB assay with results obtained by resistance ratio method for testing susceptibility of *M. tuberculosis* isolates to rifampin

Method and result[a]	No. of isolates with resistance ratio method result of[a]:		% Concordance
	Susceptible	Resistant	
PhaB assay			
Susceptible	47	0	
Resistant	0	37	100
RT-PCR			
Susceptible	48	1	
Resistant	2	39	97

[a] After overnight incubation with rifampin at 4 μg/ml.

DISCUSSION

In this paper we have compared two phenotypic assays, both of which yield results for rifampin susceptibility within 48 h of receipt of a culture on solid media. Other phenotypic assays have been developed for use with log-phase liquid broth cultures, which can take 2 to 15 days to establish (5, 12, 15) from solid cultures.

We have shown here that RT-PCR is capable of detecting reductions in *dnaK* mRNA levels after the exposure of *M. tuberculosis* to rifampin, and this provides a reliable marker of drug susceptibility in clinical isolates of *M. tuberculosis*. We also confirmed that this assay is capable of detecting small percentages (0.1 to 1%) of resistant organisms. As the natural occurrence of rifampin-resistant clones in strains without prior drug challenge is on the order of 10^{-7} to 10^{-8} (3, 4), the demonstration of rifampin resistance by this assay is likely to be of clinical significance. Other workers have used RNA as a marker of viability for *M. tuberculosis* drug susceptibility testing. Cangelosi et al. used radiolabeled nucleic acid probes to demonstrate differences in short-lived precursor RNA levels in *M. tuberculosis* treated with rifampin and ciprofloxacin (1). Jou et al. employed a single-tube nested RT-PCR to detect 85B mRNA and demonstrated a loss of RT-PCR product in cultures incubated for 1 week in isoniazid (9). More recently, Hellyer et al. demonstrated that the exposure of *M. tuberculosis* ATCC 27294 to rifampin for 24 h reduced 85B mRNA levels to less than 0.01% of those present in untreated controls (8).

The advantage of using *dnaK* as a target for assessing the

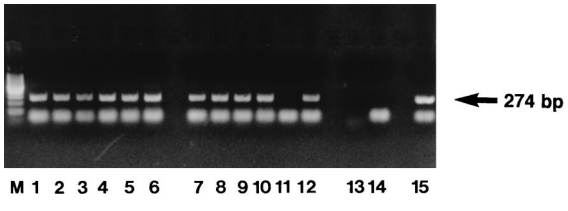

FIG. 3. Agarose (1.5%) gel of products of RT-PCR of six clinical isolates of *M. tuberculosis* without (lanes 1 to 6) and with (lanes 7 to 12) overnight exposure to rifampin. Lane M, molecular weight markers; lanes 1 and 7, sample 41; lanes 2 and 8, sample 42; lanes 3 and 9, sample 43; lanes 4 and 10, sample 44; lanes 5 and 11, sample 91; lanes 6 and 12, sample 45; lane 13, negative PCR control; lane 14, RNase-treated sample; lane 15, positive control. The samples were scored as resistant to rifampin except for sample 91, which was scored as susceptible.

viability of *M. tuberculosis* is that mRNA levels are increased more than 50-fold after the exposure of living organisms to a heat shock stimulus (45°C for 45 min) (14), which produces greater differences in template concentration between untreated cultures and those exposed to drug. This may improve discrimination between drug-resistant and -susceptible organisms.

The assay detects low numbers of organisms; thus, it may be possible to demonstrate rifampin resistance in clinical specimens directly. However, the primers used in this study were not specific for *M. tuberculosis*, so further evaluation with a species-specific primer set would be required.

The other methodology described in this paper, the PhaB assay, has been described previously (22). In the present study, we evaluated more isolates (91 compared with 46); included a larger proportion of rifampin-resistant isolates (45% compared with 20%); and employed a shorter exposure time to rifampin (24 h compared with 48 h), which reduced the turnaround time for the assay from 3 days to 2 days. We also showed that the PhaB assay detects small populations of rifampin-resistant organisms and thus distinguishes between drug-naïve isolates and populations of organisms in which the proportion of resistant clones may have reached clinically significant levels.

One weakness of the PhaB assay in its present format is the primary failure rate when plaque counts on control plates are below 100. Thus, 7 of 84 (8%) of the viable cultures failed to produce adequate plaque counts on initial testing, which is consistent with previous results (unpublished data). The reason for this is unclear, although as each isolate gave a valid result on retesting, it is likely that the inoculum was inadequate for the initial test. Work is under way to address this problem by simplifying the detection format in an automated system.

We have assessed the utility of two new phenotypic methodologies for use in a routine diagnostic setting and feel confident that both the assays described have a role in the diagnosis of rifampin resistance in *M. tuberculosis*. However, the PhaB assay offers a marked advantage over *dnaK* RT-PCR in terms of cost and technical demand, and it is likely to have greater utility in reducing turnaround times for drug susceptibility testing in developing countries.

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