Comparison of Three Molecular Assays for Rapid Detection of Rifampin Resistance in *Mycobacterium tuberculosis*

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Multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) is an emerging problem of great importance to public health, with higher mortality rates than drug-sensitive TB, particularly in immunocompromised patients. MDR-TB patients require treatment with more-toxic second-line drugs and remain infectious for longer than patients infected with drug-sensitive strains, incurring higher costs due to prolonged hospitalization. It is estimated that 90% of United Kingdom rifampin-resistant isolates are also resistant to isoniazid, making rifampin resistance a useful surrogate marker for multidrug resistance and indicating that second- and third-line drugs to which these isolates are susceptible are urgently required. Resistance in approximately 95% of rifampin-resistant isolates is due to mutations in a 69-bp region of the *rpoB* gene, making this a good target for molecular genotypic diagnostic methods. Two molecular assays, INNO-LiPA Rif.TB (Innogenetics, Zwijndrecht, Belgium) and MisMatch Detect II (Ambion, Austin, Tex.), were performed on primary specimens and cultures to predict rifampin resistance, and these methods were compared with the resistance ratio method. A third method, the phenotypic PhaB assay, was also evaluated in comparison to cultures in parallel with the genotypic assays. In an initial evaluation 16 of 16, 15 of 16, and 16 of 16 rifampin-resistant cultures (100, 93.8, and 100%, respectively), were correctly identified by line probe assay (LiPA), mismatch assay, and PhaB assay, respectively. Subsequently 38 sputa and bronchealveolar lavage specimens and 21 isolates were received from clinicians for molecular analysis. For the 38 primary specimens the LiPA and mismatch assay correlated with culture and subsequent identification and susceptibility tests in 36 and 38 specimens (94.7 and 100%), respectively. For the 21 isolates submitted by clinicians, both assays correlated 100% with routine testing.

Historically tuberculosis (TB) has been and today it remains the single greatest cause of mortality due to an infectious agent, and with the increasing prevalence of TB’s resistance to the drugs of choice (4, 30) the problem posed by TB to public health should not be underestimated. This was reflected in 1993 when the World Health Organization declared TB to be a global emergency (22). Estimates that one-third of the world’s population is infected with *Mycobacterium tuberculosis*, the causative organism, leading to 3 million deaths annually, are frequently quoted (8, 10, 22).

In the United Kingdom, notification of TB became mandatory in 1913. Changes in social conditions contributed to a decline in the incidence of TB, and the advent of *Mycobacterium bovis* BCG vaccination and effective chemotherapeutic agents in the 1950s was accompanied by a 10-fold decrease in notifications between 1948 and 1987 (18). However, this decline ceased in 1987 and from then until 1993 notifications increased by 15% in England and Wales and by 34% in London (18). These increases have been attributed in part to immigration from countries with high incidences of infection (19), although the real threat to the success of national tuberculosis control programs globally, and the single most important risk factor for the development of TB, is coinfection with the human immunodeficiency virus (9). The problem is compounded by the rising incidence of drug resistance and particularly the emergence of multidrug-resistant TB (MDR-TB) (13). The World Health Organization initiated the Global Program in Drug Resistance in 1994 in order to estimate the true global rate of drug resistance in TB (4, 30). Primary MDR-TB was found in every country surveyed except Kenya. In the United Kingdom a surveillance system, MYCOBNET, was created in 1993 to collate drug resistance data from all the reference laboratories in the country. In England and Wales initial MDR-TB rates from 1993 to 1995 increased from 0.6 to 1.5%, with the combined clinical prevalence rate for MDR-TB increasing from 0.6 to 1.7% (2).

The primary aim of prompt diagnosis and treatment of pulmonary TB is to cure the individual, rendering him or her noninfectious and so interrupting the chain of transmission. Quadruple therapy with isoniazid, rifampin, pyrazinamide, and ethambutol is designed to achieve this and to prevent the emergence of MDR-TB (9, 13). In the United Kingdom it is estimated that 90% of rifampin-resistant isolates are also resistant to isoniazid (unpublished data). Rifampin resistance therefore serves as a useful surrogate marker for the detection of multidrug resistance. Furthermore, rifampin resistance means that short-course therapy is no longer an option and that second- and third-line drug susceptibilities are required in order to make an informed choice for alternative therapy (9, 13).

The genetic basis for rifampin resistance in the majority of rifampin-resistant isolates of the *M. tuberculosis* complex is mutation in the *rpoB* gene, which codes for the β subunit of the RNA polymerase (14, 26–28). Approximately 95% of rifampin-resistant isolates have a mutation in the 69-bp region corre-
sponding to codons 511 to 533 of the \textit{rpoB} gene. Current genotypic methods of testing for rifampin resistance rely on detection of mutations in this region. The mechanism of resistance in the remaining 5% of resistant isolates remains under-
determined with the exception of further mutations at codons 381 (25), 481 (21), 505 (17), 508 (17), and 509 (21) of the \textit{rpoB} gene. Molecular assays that have been used to screen the \textit{rpoB} gene for rifampin resistance mutations include DNA sequencing (14), heteroduplex analysis (31), PCR single-stranded con-
formational polymorphism (PCR-SSCP) (27, 28), line probe assay (LiPA) (6, 7), and mismatch analysis (21).

Current methods of drug susceptibility testing with primary specimens can take 6 to 8 weeks, although once the specimens have been cultured this time is reduced to 7 to 10 days (12). In this study two genotypic methods, a LiPA (7) (INNO-LiPA 
\textit{Rif.TB}; Innogenetics, Zwijndrecht, Belgium) and an RNA-
RNA mismatch assay (21) (MisMatch Detect II; Ambion, Aus-
tin, Tex.), using cultures of \textit{M. tuberculosis} and atypical myco-
bacteria were compared to standard culture methods of identification and susceptibility testing. A third assay, the PhaB assay (32), was applied to rifampin resistance detection in cultures of \textit{M. tuberculosis}.

The second part of the study was a blinded analysis of con-
secutive sputum and bronchealveolar lavage specimens (BALs) submitted to the laboratory for molecular testing and isolation of mycobacteria as part of our special Fastrack diag-
nostic service over a 12-month period. Samples were accepted for analysis only when patients had significant risk factors for MDR-TB or if there was a clinical suspicion of resistance. Results were compared prospectively to standard primary cul-
ture results and subsequent biochemical identification and drug susceptibility test results.

MATERIALS AND METHODS

Preliminary evaluation of methods. For the initial evaluation of the methods 49 isolates were selected from a bank of stored cultures. These included 16 isolates of \textit{M. tuberculosis} which were resistant to rifampin by resistance ratio drug susceptibility testing, 15 isolates which were sensitive to rifampin, 15 non-
tuberculous mycobacteria (NTM), and 3 other members of the \textit{M. tuberculosis} complex (\textit{M. bovis} BCG, and \textit{M. africanum}). All isolates were tested by the LiPA and the mismatch assay. Only \textit{M. tuberculosis} isolates were tested by the PhaB assay. Susceptibility testing and biochemical identification were repeated by standard methods. To avoid observer bias, results were interpreted by a third party with no knowledge of the species identity or drug susceptibility of the cultured isolates.

Evaluation of assays in real-time diagnostic setting. The LiPA and mismatch assay were applied prospectively to primary specimens and cultures submitted to the laboratory as part of a national molecular testing service. A total of 59 submissions met the minimum scientific criteria (i.e., quality and quantity) for testing. Of these, 38 were smear-positive spu
ta or BALs and 21 were cultures on Löwenstein-Jensen (LJ) slopes or in BACTEC fluid (\textit{n} = 1). Results of the molecular assays were returned to the requesting clinician within 72 h of receipt of the specimen. The molecular results were not available to personnel involved in the interpretation of the biochemical identification and drug susceptibility tests. The PhaB assay was not included in this part of the study to avoid com-
promising diagnosis by splitting the sample among too many tests.

Processing of primary specimens. Sputum and BAL smears were prepared without prior concentration and were stained with auramine-phenol (12) and Ziehl-Neelsen (12) stains. Decontamination was performed by the NaOH-
NALC method (5). One milliliter of decontaminated specimen was transferred to a sterile screw-cap microcentrifuge tube for DNA extraction. The remaining fluid was used to inoculate an MB/BacT rapid culture vial (Organon Teknika 
Corp., Durham, N.C.) and pyruvate- and glycerol-containing LJ slopes.

Preparation of DNA extracts. DNA was extracted from mycobacteria by a simple and rapid method using chloroform to assist in disrupting cells and to precipitate proteins. All steps, including addition of DNA extracts to PCR mixtures, were performed in a class 1 biological safety cabinet under strict 
control to avoid cross-contamination of samples.

In order to ensure that the DNA extract added to the PCR was at an optimal pH for the PCR, samples were washed in 50 mM Tris-HCl (pH 8.0) before further processing: spu
ta, BALs, and the BACTEC fluid were centrifuged at 13,000 × g for 30 min to pellet any proteins present in the sample. The supernatant was removed and discarded into phenolic 
disinfectant. The pellet was resuspended in 1 ml of 50 mM Tris-HCl (pH 8.0). Isolates on LJ slopes were washed by transferring a small amount of growth to 1 ml of 50 mM Tris-HCl (pH 8.0) in a sterile screw-cap microcentrifuge tube by using a sterile swab. At this point all specimens were in Tris-HCl. The samples were centrifuged at 13,000 × g for 30 min to pellet any proteins present in the sample. The supernatant was removed and discarded into phenolic 
disinfectant. Pelleted samples from spu
ta, BALs, and the BACTEC fluid were resuspended in chloroform (50 μl) and sterile double-distilled water (50 μl). Pelleted samples from solid cultures were resuspended in chloroform (100 μl) and sterile double-distilled water (100 μl). Samples were heated at 80°C for 20 
min to disrupt the cells and release the DNA. Samples were allowed to cool to room temperature before centrifugation at 13,000 × g for 5 min in a scaled 
centrifuge. Taking care not to disturb the proteinaceous interphase, we trans-
ferred the upper (aqueous) phase to a clean sterile microcentrifuge tube and stored it at −20°C. Ten-microliter aliquots of these DNA extracts were used in each of the PCRs.

Resistance ratio method for drug susceptibility testing. MIC testing by plate 
microlithifications, and proportional testing using a radiometric system. Drug sus-
pceptibility testing by resistance ratio, microlithification, and proportional meth-
ods was performed as described by Heifets and Good (12), Talles and Yates (29), and Siddiqui (24).

PCR. The PCR mix was the same for each primer pair used. The 40-μl reaction mixture contained 1 U of BIOTAQ DNA polymerase (Bioline, London, United Kingdom). The supplied reaction buffer was used at the recommended working 
strength, with 1.5 mM MgCl₂, 200 μM of each dATP, dCTP, and 
dGTP, and 0.1 mM (each) dTTP and dUTP. Each reaction 
mixture contained 20 pmol of each primer. The primers and cycling parameters were as fol-

The LiPA is available commercially as a kit, INNO-LiPA Rif.TB (Innogenet-
ics). The hybridization assay was performed as described in the user manual 
supplied. For primary specimens a nested PCR was performed with 10 
μl of DNA extract in a 40-μl PCR mixture containing the outer LiPA primers; 1 μl of the first-round product was transferred to a 40-μl second-round reaction mixture 
containing the inner primers. The primers and cycling parameters were as fol-

The PhaB assay was performed as described by Wilson et al. (32).

Preparation of test DNA. The supplied reaction buffer was used at the recommended working 
strength, with 1.5 mM MgCl₂, 200 μM of each dATP, dCTP, and 
dGTP, and 0.1 mM (each) dTTP and dUTP. Each reaction 
mixture contained 20 pmol of each primer. The primers and cycling parameters were as fol-

The mismatch assay is based on the ability of double-
stranded DNA to withstand digestion with RNase A. Target DNA is amplified by 

The assay is available commercially as MisMatch Detect II (Ambion) and can 
be used for the detection of mutations in any suitable target sequence. It has 
been applied to detection of rifampin resistance mutations in cultures of \textit{M. tuberculosis} (21) but has not been previously applied to primary specimens. The assay was described as performed by Nash et al. (21) with the exception that the PCR amplification conditions were as described above. For the primary 
specimens a nested PCR was performed with the outer primers rp61451 (5'-GCA 
GACGCCTTGGAATCACT-3') and rp62225S (5'-TAGTCACCTCAGACCG 
AGG-3'). The cycling parameters were as follows: 95°C for 30 s, 55°C for 40 s, 
and 72°C for 30 s. Only Sp6 RNA polymerase. The complementary transcripts from the test and 
reference PCR products are allowed to hybridize, and the resulting hybrids are 
treated with RNase. Any mutations in the test transcript will not pair with the reference 
transcript, and so the hybrid will be cleaved at that point. Undigested 
transcripts and cleavage products can be detected by analysis using agarose gel 
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TABLE 1. DNA sequencing results

<table>
<thead>
<tr>
<th>Isolate</th>
<th>DNA sequence</th>
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<tr>
<td>wt</td>
<td>cGctctcagtttatggaccagaacaacccgctgtctgagattacgcacaaacgtcgtatctccgcactc</td>
</tr>
<tr>
<td>RM6</td>
<td>cGctctcagtttatggaccagaacaacccgctgtctgagattacgcacaaacgtcgtatctccgcactc</td>
</tr>
<tr>
<td>RM10</td>
<td>cGctctcagtttatggaccagaacaacccgctgtctgagattacgcacaaacgtcgtatctccgcactc</td>
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*From codon 511 to 533. An upper case letter denotes a base change.

Testing of Fastrack specimens. All Fastrack isolates were correctly scored by the LiPA and mismatch assays applied prospectively—6 of 6 resistant isolates, 12 of 12 sensitive isolates, and 3 of 3 NTM isolates. From the primary specimens submitted, 10 patients with rifampin-resistant isolates, 24 with sensitive isolates, and 4 with NTM isolates were subsequently detected based on culture and drug susceptibility testing by the resistance ratio method. The LiPA correctly scored 8 of 10 (80%) of the resistant specimens, 24 of 24 (100%) of the sensitive specimens, and 4 of 4 (100%) of the NTM specimens. The mismatch assay correctly scored 10 of 10 (100%) of the resistant specimens, 24 of 24 (100%) of the sensitive specimens, and 4 of 4 (100%) of the NTM specimens. The overall correlations of the LiPA and mismatch assays, when performed on primary specimens, with subsequent culture-based identification and drug susceptibility assays were 94.7 and 100%, respectively.

DISCUSSION

This study was initiated as a result of the growing demand from clinicians for rapid molecular diagnostics for patients for whom clinical details and history suggested the presence of drug-resistant TB, i.e., previous TB, recent immigration from or travel to an area with high prevalence of drug-resistant TB, failure to respond to therapy, or contact with a known MDR-TB patient. Overall, the performances of the LiPA and mismatch assay were similar, with both correlating to 97.8% of culture results in the initial evaluation. The in-house phenotypic method, the PhaB assay, when used on a subset of cultures for the rapid detection of rifampin resistance, compared favorably with the two genotypic methods, correlating with culture in 100% of specimens. The PhaB assay was originally developed for use in developing countries and is particularly suited to that environment as a result of its low cost and its relative simplicity of use, features which are not shared by the genotypic assays.

In the initial evaluation of the assays, two isolates (RM6 and RM10) possessing a CTG-to-CCG mutation at codon 511 were identified. Although these isolates contained the mismatch assay failed to detect the mutation, although the assay as originally described by Nash et al. (21) was capable of identifying this mutation. However, it is known that G-U mismatches are relatively resistant to cleavage with RNase A (20) and it seems likely that RNase digestion conditions are more critical for identification of mutations of this type. These two isolates were the only ones sequenced that possessed a T-to-C mutation.

Although the substitution of proline for leucine at codon 511 has been shown to confer resistance in a number of studies (3, 27), the two isolates in this study that possessed such a substitution gave variable results when tested by different phenotypic methods of drug susceptibility testing. Furthermore, Taniguchi et al. (25) demonstrated sensitivity to rifampin in one of three isolates with a leucine-to-proline substitution at codon 533. It can be postulated that sensitivity to rifampin in an isolate with

aggar plate. The bacteriophage in turn infect, replicate within, and lyse the M. smegmatis cells, and the lysis is detected as plaques in a lawn of bacterial growth. The number of plaques is directly related to the number of protected mycobacte riophage, which is in turn related to the number of viable bacilli present after treatment with the drug. Interpretation is based on the proportional method, by comparing the numbers of plaques on plates where the M. tuberculosis bacilli have been incubated with and without drugs.

DNA sequencing. DNA sequencing was performed with ABI PRISM dye terminator technology with AmpliTaq DNA polymerase FS on an ABI 373 DNA sequencer. DNA was extracted from cultures and amplified in a PCR containing the outer primer from the LiPA. The PCR product was purified on spin columns (Wizard PCR Prep; Promega, Southampton, United Kingdom) and sequenced with the inner LiPA primers in the forward and reverse orientations. DNA sequencing results were only used to confirm the identity of mutations and were not used as part of the diagnostic service.

RESULTS

Preliminary evaluation of assays. Using the resistance ratio method as the “gold standard,” 14 of 15 rifampin-sensitive isolates (93.3%) were scored as sensitive by the LiPA and 15 of 15 sensitive isolates (100%) were scored as sensitive by the PhaB and mismatch assays. A total of 16 of 16 resistant isolates (100%) were scored as resistant (presence of mutation) by the LiPA and the PhaB assay. A total of 15 of 16 resistant isolates (93.8%) were scored as resistant by the mismatch assay.

Two isolates, RM6 and RM10, were sensitive and resistant to rifampin, respectively by both resistance ratio and PhaB assays. Resistance ratio results were confirmed at a second reference laboratory. Both isolates demonstrated S1 band deletions when tested by the LiPA. Drug susceptibility testing by the proportional method using the BACTEC system found RM10 and RM6 to be sensitive and borderline resistant to rifampin, respectively, at a breakpoint of 0.5 mg/l.

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Although the substitution of proline for leucine at codon 511 has been shown to confer resistance in a number of studies (3, 27), the two isolates in this study that possessed such a substitution gave variable results when tested by different phenotypic methods of drug susceptibility testing. Furthermore, Taniguchi et al. (25) demonstrated sensitivity to rifampin in one of three isolates with a leucine-to-proline substitution at codon 533. It can be postulated that sensitivity to rifampin in an isolate with
a known resistance mutation is an artifact of subculture on artificial media. However, subsequent to the completion of this study we received a smear-positive sputum specimen for detection of *M. tuberculosis* and screening for rifampin resistance mutations. An S1 band deletion was obtained by the LiPA, but the cultured isolate proved to be sensitive to all first-line antituberculous drugs by resistance ratio and proportion drug susceptibility testing in the BACTEC system. It is logical that a mutation would not be selected for in a population if it did not confer some form of selective advantage, and it is therefore reasonable to assume that a leucine-to-proline amino acid substitution at codon 511 does indeed confer some level of resistance to rifampin. The clinical significance of this mutation is not clear and reinforces the importance of a measured approach to rapid methods of diagnosis in which, as in any area of medicine, synthesis of several pieces of evidence is used to manage patients. It is essential that rapid tests always be verified by standard culture-based methods of diagnosis.

The results obtained in this study compare favorably with those obtained by other methods used to detect mutations. Most screening methods use an amplification step, typically PCR, followed by an analysis step. The complexity of the post-PCR procedure can vary dramatically. PCR-SSCP has been performed successfully on cultures (26, 27) but with less success on primary specimens (28). In addition the results of PCR-SSCP can sometimes be hard to interpret (11). Distinctive results can be seen with the use of dideoxy fingerprinting (11), a modification of SSCP, which produces a unique pattern for each mutation. The LiPA provides very clear results, especially for the most common mutations, relying on probe binding and color detection. Similarly the mismatch assay provides clear results although, like SSCP, the actual mutation present is not identified. DNA sequencing is the “gold standard” (27) for mutation detection, because it provides a definitive identification of any mutation present. However, although DNA sequencing is simple for laboratories already performing it routinely, the costs of capital equipment and maintenance do not make it a cost-effective option for most clinical laboratories.

The assays used in this study are relatively simple post-PCR manipulations. The LiPA is the simplest to perform and interpret, requiring only a basic knowledge of molecular biology to perform successfully. In contrast the mismatch assay utilizes a transcription step and subsequent manipulation of RNA, although with proper precautions to avoid RNase contamination, this should not cause a problem.

The cost of an assay is an important factor in its applicability to a diagnostic setting. The LiPA kit costs $720 and provides 20 test strips, while MisMatch Detect II costs $555 and provides enough reagents for 120 tests. Other consumable costs are involved with both assays. Outer primers and the primer for incorporating the transcriptional promoter sites must be purchased for the mismatch assay. Although the LiPA kit includes sufficient inner primer for 30 PCRs, outer primers are required for a nested PCR when the starting material is a primary specimen. In addition insufficient inner primer was supplied for an adequate number of negative control reactions, necessitating the purchase of more inner primers, further increasing the cost per test of the LiPA. It is essential in any PCR that enough negative controls be performed to control for false positives (15), particularly in a nested PCR, to control for carryover contamination during the nesting procedure.

Although many studies specifically do not include samples from patients who are on therapy, the nature of this study meant that such samples would be received. An illustration of this is a particular patient from whom we initially received a culture for identification and susceptibility testing. The patient was not responding to standard first-line therapy, and so a request was made for screening for rifampin resistance mutations. A result was returned within 24 h, saying that the isolate was likely to be sensitive to rifampin. Sensitivity to rifampin was confirmed 2 weeks later by culture when isoniazid resistance was also identified. Therapy was modified accordingly, but when the patient had still failed to respond to therapy 5 weeks later, a sputum sample was sent for molecular testing and rapid culture. On this occasion a common rifampin resistance mutation (S531 to L) was identified 6 weeks before culture identified resistance to rifampin, isoniazid, and streptomycin. Subsequently, it became clear that the patient had not been compliant with therapy.

Of the samples submitted for molecular testing, NTM were present in 7 of 59 specimens (11.9%). Of the remaining samples which were subsequently identified as *M. tuberculosis*, rifampin resistance was identified in 16 of 52 specimens (30.8%) by culture-based methods. Overall, 40% of the samples submitted contained *M. tuberculosis* strains which were resistant to rifampin or were NTM strains. Although the NTM most commonly presenting as pulmonary disease will partially respond to therapy with the rifampin and ethambutol components of quadruple therapy for TB (1), such therapy will still be suboptimal. In addition a sputum smear-positive patient with an NTM infection can also be taken out of negative-pressure isolation.

Clearly there is a role for the identification of mycobacteria and detection of rifampin resistance by molecular methods. However, it is essential that the limits of the assays be recognized (16, 23) in order to avoid misdiagnosis through false-positive and -negative results. With limited resources and budgets for purchasing expensive molecular assays, it is important that laboratories focus on those samples from patients with significant risk factors for rifampin-resistant *M. tuberculosis*. We have found that encouraging clinicians to send high-quality specimens, accompanied by sufficient clinical information, has a key role to play in the effective use of these assays for diagnosis.

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