Fluorometric Assay for Testing Rifampin Susceptibility of *Mycobacterium tuberculosis* Complex[†]

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The emergence and transmission of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) have raised concern about diagnostic delay associated with culture-based drug susceptibility testing methods. The association between rifampin resistance and MDR-TB or XDR-TB makes it an important genetic marker for genotypic drug susceptibility testing. In this article, we describe the analysis of the physical properties of the rifampin resistance-determining region (RRDR) in the *rpoB* gene by high-resolution thermal melt analysis as a method for detecting rifampin resistance in *Mycobacterium tuberculosis* complex. The RRDR from the *M. tuberculosis* complex was amplified by PCR from DNA templates extracted from sputum cultures of *M. tuberculosis* or the laboratory strain (H37Rv) in the presence of a fluorescent DNA binding dye. Subsequent mixing of the amplification products from the respective sputum cultures and the laboratory strain and thermocycling allowed the formation of DNA duplexes. The thermal denaturation properties of these DNA duplexes were determined by measuring the derivative of the intensity of fluorescence at different temperatures. Analysis of DNA extracted from 153 sputum cultures showed a sensitivity of 98% and a specificity of 100% for the detection of rifampin resistance compared to the “gold standard” culture-based phenotyping method. No statistical difference was detected in the performance of the method when applied to crude DNA from 134 boiled cultures. This method, named “FAST-Rif” (“fluorometric assay for susceptibility testing of rifampin”), allowed the rapid, reliable, and easy detection of genotypic rifampin resistance as a marker for MDR-TB and XDR-TB.

Drug resistance remains a serious threat to tuberculosis (TB) control worldwide (21). This is especially true for multidrug-resistant TB (MDR-TB), which is defined as disease caused by *Mycobacterium tuberculosis* that is resistant in vitro to both isoniazid and rifampin, with or without resistance to other anti-TB drugs. A recent survey estimated that 424,203 MDR-TB cases were diagnosed worldwide among new and retreatment cases in 2004 (23). In 2004, a study of samples submitted to selected national reference laboratories worldwide estimated that in the 4 years between 2000 and 2004, 20% of the samples tested represented MDR-TB cases and that 10% of these were extensively drug-resistant TB (XDR-TB) cases (defined at that time as resistant to three of six classes of second-line drugs) (3). In October 2006, the World Health Organization redefined XDR-TB as being MDR-TB with additional resistance to any fluoroquinolone (e.g., ciprofloxacin, ofloxacin, or moxifloxacin) and to at least one of three injectable second-line anti-TB drugs used in treatment (capreomycin, kanamycin, or amikacin) (22).

In order to combat the threat of drug resistance, it is essential that new rapid diagnostics are developed to complement a

well-functioning TB control program. Recent advances in phenotypic drug susceptibility testing include the use of mycobacterial growth indicators (6, 9) and phage-based assays (1). Although these methods are able to report phenotypic resistance in 2 to 10 days, the culture of viable bacilli poses a health risk to laboratory personnel and thereby requires high levels of biosafety. To overcome these limitations and to improve the speed of detection of drug resistance, numerous PCR-based methods have been described (reviewed in reference 11). However, the number of different nonsynonymous single nucleotide polymorphisms (nsSNPs) conferring resistance remains a major challenge to the successful development of genotypic drug susceptibility testing methods. Pragmatically, this has been partially circumvented by developing assays which analyze the most prominent nsSNPs, with some reduction in sensitivity and specificity due to this. However, many of these methods are hampered by the need for downstream processing to enable the detection of nsSNPs within the PCR-amplified domain (e.g., hybridization to immobilized oligonucleotides [7], microarray [4], dot blot hybridization [17], denaturing high-performance liquid chromatography [15], and DNA sequencing [2, 8]). The complexity of these methods and the need for multiple steps to perform them greatly increase the risk of cross-contamination and thereby misdiagnosis. An assay which is rapid, sensitive, and specific and does not require downstream processing, thereby minimizing cross-contamination, would be ideal.

A study by Williams et al. (20) showed that heteroduplexes could be used to determine rifampin susceptibility by analyzing

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conformational changes created by nsSNPs in the DNA fragments. We proposed that the analysis of thermal denaturation profiles of heteroduplexes could be used to enhance the detection of nsSNPs conferring resistance in *M. tuberculosis*. In theory, the thermal denaturation profile of a specific DNA fragment is dependent on the nucleotide sequence of that fragment (13). Thus, any change in the nucleotide sequence would alter the thermal denaturation profile, which in turn could be detected by measuring the efficiency of binding of a fluorescent dye to the DNA fragment at different temperatures (10). However, nucleotide transversions (A:T and G:C) remain difficult to detect, since they have very little influence on the overall thermal denaturation profile. To circumvent this limitation, we proposed that the efficiency of detecting transversions and transitions could be enhanced by analyzing the thermal denaturation profiles of DNA duplexes formed by annealing DNA fragments with and without nucleotide change (heteroduplex and homoduplex, respectively).

In this study, we tested these concepts by analyzing the unique thermal denaturation properties of the rifampin resistance-determining region (RRDR) of the *rpoB* gene to develop a method for the detection of rifampin resistance. Monoresistance to rifampin is rare and is mostly accompanied by isoniazid resistance. Therefore, the rifampin resistance profile could be used as a marker for suspected MDR-TB and XDR-TB cases.

MATERIALS AND METHODS

Preparation of pure DNA templates. *M. tuberculosis* was cultured from sputa (obtained from TB patients) on Löwenstein-Jensen medium, and genomic DNA was extracted as previously described (18).

Preparation of crude DNA templates. Decontaminated sputum specimens were cultured at 37°C in Bactec 12B medium (Becton Dickinson) for 7 days in the Bactec 460 system, and the bacteria were pelleted by centrifugation, resuspended in 100 µl Bactec 12B medium, and boiled to generate a crude-DNA template (19).

Drug susceptibility testing. Drug susceptibility testing was done by the National Health Laboratory Service, using the indirect proportion method on Middlebrook medium containing critical concentrations of 0.2 µg/ml isoniazid and 30 µg/ml rifampin. Following DNA sequencing and high-resolution thermal melt analysis, specimens with discrepant phenotypic results were subjected to rifampin drug susceptibility testing in mycobacterial growth indicator tubes (Becton Dickinson) according to the manufacturer's instructions.

DNA sequencing of the RRDR of the *rpoB* gene. The DNA sequence of the RRDR of the *rpoB* gene of each sputum culture (pure and crude DNA) was determined as previously described (16). The genotypes and phenotypes of the respective isolates were determined prior to high-resolution thermal melt analysis, and these results were blinded to the high-resolution thermal melt operator.

PCR amplification of the RRDR. DNA templates (pure or crude) extracted from different sputum cultures, the laboratory strain (H37Rv, having a rifampin-susceptible genotype), different members of the *M. tuberculosis* complex (*M. canettii*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, and *M. bovis* [Mycobacterial Reference Laboratory, Institut Pasteur, France]), or different species of nontuberculous mycobacteria (*M. alvei*, *M. anupense*, *M. avium*, *M. chelonae*, *M. elephantis*, *M. fortuitum*, *M. goodii*, *M. intracellulare*, *M. interjectum*, *M. kansasii*, *M. kamamotomense*, *M. marinum*, *M. montefiorensense*, *M. monacense*, *M. morikawaense*, *M. paraffinicum*, *M. peregrinum*, *M. porcinum*, *M. rhodesiae*, and *M. terrae* [speciated by 16S rRNA DNA sequencing {5}]) were subjected to PCR amplification (Fig. 1, step 1) in a reaction mixture containing 25 ng DNA template, 5 µl Q-Buffer, 2.5 µl 10× buffer, 2 µl 25 mM MgCl₂, 4 µl 10 mM deoxynucleoside triphosphates, 1 µl of each primer (50 pmol/µl) (*rpoB* Forward, 5'-CGC CGC GAT CAA GGA GTT C-3'; *rpoB* Reverse short, 5'-GCC CGG CAC GCT CAT GT-3'), 1 µl (1/100 dilution) Syto 9 fluorescent dye (Molecular Probes), and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) and made up to 25 µl with distilled water. Amplification was initiated by incubation at 95°C for 15 min, followed by 35 to 45 cycles at 94°C for 45 s, 64°C for 45 s, and 72°C for 45 s. After the last cycle, the samples were

FIG. 1. Flow diagram of the high-resolution thermal melt genotypic drug susceptibility testing methodology. In step 1, DNA extracted from the laboratory strain (H37Rv) or the respective sputum cultures was subjected to PCR amplification of the RRDR of the *M. tuberculosis* complex *rpoB* gene. In step 2, RRDR DNA duplexes were formed between the amplified RRDR of the laboratory strain and the amplified RRDR of the respective sputum cultures by mixing of the PCR products and subsequent thermocycling. In step 3, DNA duplexes were subjected to high-resolution thermal melt analysis (HRM). Genotypic drug susceptibility was scored according to the shape of the dF/dT plot and the presence of derivative peaks in defined temperature bins.

incubated at 72°C for 10 min. Amplification was confirmed by high-resolution melt analysis (see below). To minimize laboratory cross-contamination, the preparation of the PCR mixes, the addition of the DNA, and the PCR amplification were conducted in physically separated rooms. Negative controls (water) were included to detect reagent contamination.

DNA duplex formation. After amplification, a 12.5-µl aliquot of the PCR product containing the amplified RRDR from each sputum culture was mixed with a 12.5-µl aliquot of the amplified RRDR from the laboratory strain H37Rv (Fig. 1, step 2). Each mixture was subjected to a second round of cycling without adding additional PCR components (95°C for 1 min, followed by 10 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 5 min). This procedure generated DNA duplexes between the respective RRDR amplification products from the sputum culture and the standard laboratory strain.

High-resolution thermal melt analysis. The DNA duplexes were subjected to high-resolution thermal melt analysis in a Rotorgene 6000 real-time analyzer (Corbett, Australia) (Fig. 1, step 3). The thermal denaturation profile was measured over the temperature range from 80°C to 95°C, and fluorometric readings were taken every 0.1°C. Rotorgene software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peak(s) represents the T_m value of the DNA

FIG. 2. dF/dT values of the DNA duplexes. (A) Isolates were scored as susceptible to rifampin based on the presence of a single derivative peak within a defined temperature bin (homoduplex). (B) Isolates were scored as resistant to rifampin based on the presence of two derivative peaks in defined temperature bins (homo- and heteroduplex).

duplexes. Rifampin drug susceptibility was assigned by the software according to the presence of a derivative peak(s) located within a defined temperature bin(s) (width, 1.5°C). A derivative plot with a single derivative peak (homoduplex) was classified as drug susceptible, while a derivative plot with two derivative peaks (homo- and heteroduplexes) was classified as drug resistant.

Statistical analysis. The statistical software program Statistica 7.1 was used to calculate the sensitivity and specificity of PCR amplification or genotypic drug susceptibility testing at a confidence interval (CI) of 95%.

RESULTS

To determine the specificity of the primers used for the PCR amplification of the *M. tuberculosis* complex RRDR, DNA templates from 7 different members of the *M. tuberculosis* complex and 20 different species of nontuberculosis mycobacteria were amplified. The specificity of *M. tuberculosis* complex RRDR amplification was 100% compared to the 16S rRNA DNA sequence data.

To test whether the thermal denaturation properties of the RRDR could be used to detect nsSNPs conferring rifampin resistance, DNA duplexes (formed between the RRDRs amplified from DNA extracted from phenotypically and genotypically well-characterized sputum cultures [$n = 34$] and the laboratory strain [H37Rv], respectively) were subjected to

high-resolution thermal melt analysis in the presence of a saturating concentration of the fluorescent dye. Figure 2 shows the dF/dT values of DNA duplexes. Rifampin-susceptible isolates were characterized by the presence of a single derivative peak, representing homoduplexes (Fig. 2A), while rifampin-resistant isolates were characterized by the presence of two distinct derivative peaks at defined temperatures, representing homo- and heteroduplexes, respectively (Fig. 2B). This demonstrated that the high-resolution thermal melt method could be used to identify nsSNPs conferring rifampin resistance.

To determine the performance of this method, purified DNA from 80 phenotypically rifampin-susceptible and 73 phenotypically rifampin-resistant sputum cultures was analyzed (the operator was blinded to the genotypic and phenotypic rifampin resistance data). The method showed a sensitivity of 98% (95% CI, 94% to 101%) and a specificity of 100% (95% CI, 100% to 100%) for scoring the presence of nsSNPs encoding rifampin resistance in relation to the “gold standard” phenotypic drug susceptibility testing method (see the supplemental material). The positive predictive value was 100%, and the negative predictive value was 97%.

To determine whether the high-resolution thermal denatur-

ation genotyping technique could be used to analyze crude DNA templates, 44 phenotypically rifampin-susceptible and 90 phenotypically rifampin-resistant boiled sputum cultures were analyzed. Performance analysis showed the technique had a sensitivity of 94% (95% CI, 90% to 99%) and a specificity of 96% (95% CI, 90% to 101%) (see the supplemental material). The positive predictive value was 98%, and the negative predictive value was 88%.

Using the thermal denaturation genotyping technique, a total of 14 different nsSNPs were detected at six codons in the RRDR and an insertion between codons 514 and 515 (data not shown).

DISCUSSION

In this article, we report the development of a novel PCR-based fluorometric assay for testing of susceptibility to rifampin, called FAST-Rif. This method allowed for the rapid detection of 15 different mutational events in the RRDR of the *rpoB* gene conferring rifampin resistance in *M. tuberculosis* complex. The advantage of this method over previously described methods (2, 4, 7, 8, 15, 17) was the simplicity of the method, the broad spectrum of nsSNPs detected by each analysis, and the high sensitivity and specificity achieved. However, the method did not provide information about which nsSNP conferred resistance. This should not be seen as a limitation, since the primary objective for routine diagnostics would only be to determine the presence or absence of an nsSNP conferring resistance, thereby enabling the correct treatment to be administered to the patient. The sensitivity of this technique was not confounded by the presence of synonymous single nucleotide polymorphisms, since these have not been identified in the RRDR (12). We acknowledge that our method was not able to identify nsSNPs conferring rifampin resistance which fell outside of the RRDR. This implies that approximately 5% of cases with rifampin resistance will be not be detected by the described method. Similarly, most of the previously described genotypic drug susceptibility testing methods (2, 4, 7, 8, 15, 17) would not be able to detect nsSNPs outside of the RRDR.

In this study, the FAST-Rif method was based on two independent PCRs, which ensured the optimal noncompetitive amplification of both the clinical and reference templates. Attempts to convert the method to a single-tube format were hampered by the competitive nature of the PCR. We envisage that this method could be adapted to a single-tube format if the two PCRs were separated by a septum. After PCR amplification, the two PCRs could be mixed by inverting the tube, thereby avoiding the need to open the reaction tube. This would have further advantages, since it would simplify the method while avoiding the risk of cross-contamination following aerosolization of amplicons. Furthermore, the single-tube (South African provisional patent 2007/06915) format would allow for high throughput and automation.

We acknowledge that the quality and amount of the input DNA remain a bottleneck for the applicability of all PCR-based genotypic drug susceptibility testing methods. However, in this study we showed that the FAST-Rif method could efficiently amplify "crude DNA", thereby enabling a diagnosis to be made within a matter of 3 to 4 h following short-term

culture. This diagnostic interval could be shortened further if the RRDR could be efficiently and routinely amplified from *M. tuberculosis* DNA purified from sputum.

Application of this methodology in the routine screening of clinical isolates would enable the rapid diagnosis of rifampin resistance (14), thereby increasing the suspicion index for both MDR-TB and XDR-TB. This information would permit the attending clinician to request drug susceptibility testing for additional anti-TB drugs, thereby ensuring the appropriate management of the TB case and preventing the acquisition of other drug resistance phenotypes. Furthermore, the rapid diagnosis of rifampin resistance would assist in limiting the transmission of drug-resistant TB to close contacts. Given the affordability of the described method (cost not exceeding that of a standard PCR), as well as the ease of use, it should be possible to implement this methodology to improve case detection in most settings, thereby enhancing the overall control of the drug-resistant-TB epidemic. The versatility of this method implies that it could be adapted to detect nsSNPs conferring resistance to other anti-TB drugs.

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REFERENCES

1. Albert, H., A. Trollip, T. Seaman, and R. J. Mole. 2004. Simple, phage-based (FASTPlaque) technology to determine rifampicin resistance of *Mycobacterium tuberculosis* directly from sputum. *Int. J. Tuberc. Lung Dis.* **8**:1114–1119.
2. Bakonyte, D., A. Baranauskaite, J. Cicenaitis, A. Sosnovskaja, and P. Stakenas. 2005. Mutations in the *rpoB* gene of rifampicin-resistant *Mycobacterium tuberculosis* clinical isolates from Lithuania. *Int. J. Tuberc. Lung Dis.* **9**:936–938.
3. Centers for Disease Control and Prevention. 2006. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs—worldwide, 2000–2004. *MMWR Morb. Mortal. Wkly. Rep.* **55**:301–305.
4. Gryadunov, D., V. Mikhailovich, S. Lapa, N. Roudinskii, M. Donnikov, S. Pan'kov, O. Markova, A. Kuz'min, L. Chernousova, O. Skotnikova, A. Moroz, A. Zasedatelev, and A. Mirzabekov. 2005. Evaluation of hybridisation on oligonucleotide microarrays for analysis of drug-resistant *Mycobacterium tuberculosis*. *Clin. Microbiol. Infect.* **11**:531–539.
5. Harmsen, D., S. Dostal, A. Roth, S. Niemann, J. Rothganger, M. Sammeth, J. Albert, M. Frosch, and E. Richter. 2003. RIDOM: comprehensive and public sequence database for identification of *Mycobacterium* species. *BMC Infect. Dis.* **3**:26.
6. Hasan, R., and S. Irfan. 2007. MODS assay for the diagnosis of TB. *N. Engl. J. Med.* **356**:188.
7. Hillemann, D., S. Rusch-Gerdes, and E. Richter. 2007. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *J. Clin. Microbiol.* **45**:2635–2640.
8. Kapur, V., L. L. Li, S. Iordanescu, M. R. Hamrick, A. Wanger, B. N. Kreiswirth, and J. M. Musser. 1994. Characterization by automated DNA sequencing of mutations in the gene (*rpoB*) encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *J. Clin. Microbiol.* **32**:1095–1098.
9. Mengatto, L., Y. Chiani, and M. S. Imaz. 2006. Evaluation of rapid alternative methods for drug susceptibility testing in clinical isolates of *Mycobacterium tuberculosis*. *Mem. Inst. Oswaldo Cruz* **101**:535–542.
10. Monis, P. T., S. Giglio, and C. P. Saint. 2005. Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Anal. Biochem.* **340**:24–34.
11. Palomino, J. C. 2006. Newer diagnostics for tuberculosis and multi-drug resistant tuberculosis. *Curr. Opin. Pulm. Med.* **12**:172–178.
12. Ramaswamy, S., and J. M. Musser. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuberc. Lung Dis.* **79**:3–29.
13. Reed, G. H., J. O. Kent, and C. T. Wittwer. 2007. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. *Pharmacogenomics* **8**:597–608.

14. Rossau, R., H. Traore, H. De Beenhouwer, W. Mijs, G. Jannes, P. De Rijk, and F. Portaels. 1997. Evaluation of the INNO-LiPA Rif. TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. *Antimicrob. Agents Chemother.* **41**:2093–2098.
15. Shi, R., K. Otomo, H. Yamada, T. Tatsumi, and I. Sugawara. 2006. Temperature-mediated heteroduplex analysis for the detection of drug-resistant gene mutations in clinical isolates of *Mycobacterium tuberculosis* by denaturing HPLC, SURVEYOR nuclease. *Microbes Infect.* **8**:128–135.
16. Van Der Zanden, A. G., E. M. Te Koppele-Vije, B. N. Vijaya, D. van Soolingen, and L. M. Schouls. 2003. Use of DNA extracts from Ziehl-Neelsen-stained slides for molecular detection of rifampin resistance and spoligotyping of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **41**:1101–1108.
17. Victor, T. C., A. M. Jordaan, A. van Rie, G. D. van der Spuy, M. Richardson, P. D. van Helden, and R. Warren. 1999. Detection of mutations in drug resistance genes of *Mycobacterium tuberculosis* by a dot-blot hybridization strategy. *Tuber. Lung Dis.* **79**:343–348.
18. Warren, R., M. de Kock, E. Engelke, R. Myburgh, N. Gey van Pittius, T. Victor, and P. van Helden. 2006. Safe *Mycobacterium tuberculosis* DNA extraction method that does not compromise integrity. *J. Clin. Microbiol.* **44**:254–256.
19. Warren, R. M., T. C. Victor, E. M. Streicher, M. Richardson, N. Beyers, N. C. Gey van Pittius, and P. D. van Helden. 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. *Am. J. Respir. Crit. Care Med.* **169**:610–614.
20. Williams, D. L., L. Spring, T. P. Gillis, M. Salfinger, and D. H. Persing. 1998. Evaluation of a polymerase chain reaction-based universal heteroduplex generator assay for direct detection of rifampin susceptibility of *Mycobacterium tuberculosis* from sputum specimens. *Clin. Infect. Dis.* **26**:446–450.
21. World Health Organization and International Union against Tuberculosis and Lung Disease Global Project. 2004. Anti-tuberculosis drug resistance in the world. World Health Organization, Geneva, Switzerland. http://www.who.int/tb/publications/who_htm_tb_2004_343/en/.
22. World Health Organization. 2006. WHO Global Task Force outlines measures to combat XDR-TB worldwide. World Health Organization, Geneva, Switzerland. <http://www.who.int/mediacentre/news/notes/2006/np29/en/index.html>.
23. Zignol, M., M. S. Hosseini, A. Wright, C. L. Weezenbeek, P. Nunn, C. J. Watt, B. G. Williams, and C. Dye. 2006. Global incidence of multidrug-resistant tuberculosis. *J. Infect. Dis.* **194**:479–485.