Temperature-Mediated Heteroduplex Analysis for Detection of *pncA* Mutations Associated with Pyrazinamide Resistance and Differentiation between *Mycobacterium tuberculosis* and *Mycobacterium bovis* by Denaturing High-Performance Liquid Chromatography

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The goal of this study was to apply temperature-mediated heteroduplex analysis using denaturing high-performance liquid chromatography to identify pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis* isolates and simultaneously differentiate between *M. tuberculosis* and *Mycobacterium bovis*. Features that contributed to an optimal assay included the use of two different reference probes for the *pncA* gene targets from wild-type *M. tuberculosis* and wild-type *M. bovis*, optimization of the column temperature, increasing the starting concentration of the elution buffer, and reducing the rate of elution buffer increase (slope). A total of 69 strains were studied, including 48 wild-type *M. tuberculosis* strains (13 were PZA-resistant strains) and 21 *M. bovis* strains (8 were BCG strains). In all isolates tested, wild-type *M. tuberculosis* generated a single-peak pattern when mixed with the *M. tuberculosis* probe and a double-peak pattern with the *M. bovis* probe. In contrast, all *M. bovis* isolates generated a double-peak pattern when mixed with the *M. tuberculosis* probe and a single-peak pattern with the *M. bovis* probe. PZA-resistant mutant *M. tuberculosis* isolates generated characteristic patterns that were easily distinguishable from both wild-type *M. tuberculosis* and *M. bovis* isolates. Chromatographic patterns generated by the two reference probes allowed the rapid detection of PZA resistance with the simultaneous ability to distinguish between *M. tuberculosis* and *M. bovis*. This approach may allow the detection of drug resistance-associated mutations, with potential application to clinical and epidemiological aspects of tuberculosis control.

Pyrazinamide (PZA) is a first-line drug for the treatment of tuberculosis (3). PZA appears to act on semidormant tubercle bacilli that are unaffected by any other antituberculosis drug (11). In combination with isoniazid, rifampin, and ethambutol, it allows shortening of the treatment period from 18 to 6 months (2, 25). Whereas resistance to PZA is rare in *Mycobacterium tuberculosis*, almost all strains of *Mycobacterium bovis* are naturally resistant (13, 28). PZA is a prodrug that requires activation to its toxic form, pyrazinoic acid, a process mediated by pyrazinamidase (Pzase), an enzyme produced by mycobacterial species (29). The correlation between PZA resistance and Pzase activity is supported by the demonstration of loss of this activity in resistant isolates (18, 32).

The genetic basis for PZA resistance involves mutation within the *pncA* gene, which encodes Pzase activity (20, 28). Although cases of PZA-resistant *M. tuberculosis* isolates with no *pncA* mutations have been reported, mutations of *pncA* and its putative promoter remain the major mechanism of PZA resistance (15, 20). Over 40 different mutations in either the *pncA* structural gene or its putative promoter associated with

PZA resistance in *M. tuberculosis* have been described. The changes are either mutations that involve substitution of nucleotides or mutations in the form of nucleotide insertions or deletions (15, 20, 27). In contrast, the natural resistance to PZA demonstrated by *M. bovis* strains is uniformly due to a unique single-point mutation (C₁₆₉G) in *pncA*. This mutation involves the replacement of histidine (CAC) with aspartic acid (GAC), leading to the production of inactive enzyme (26, 28).

Susceptibility testing to detect PZA resistance has recently received increased attention for a number of reasons, including the important role of PZA in shortening the time course for treatment of tuberculosis and the recent recognition of PZA-monoresistant strains of *M. tuberculosis* (9), the increasing frequency of tuberculosis infections following intravesical instillation of the naturally PZA-resistant *M. bovis* BCG strain for the treatment of superficial bladder cancer (1, 17, 19), and the increasing incidence of zoonotic tuberculosis in developing countries due to naturally PZA-resistant *M. bovis* (6, 16, 24).

Conventional mycobacterial susceptibility testing for PZA is dependent on growth of the organism in the presence of the drug. This technique is both time-consuming and potentially unreliable due to the poor growth of *M. tuberculosis* in the highly acidic medium required for PZA activity (7, 12). Automated testing systems, such as the BACTEC 460TB and BACTEC MGIT 960 systems, are more sensitive than conven-

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FIG. 1. Alignment of *pncA* genes of wild-type *M. tuberculosis* and *M. bovis* and their putative promoters showing the positions of mutations in the 13 different mutant strains used in the study; mutant 1, G₂₃₃A; mutant 2, C₂₉₇G; mutant 3, del G₇₁; mutant 4, A₄₁₀G; mutant 5, T₁₁C; mutant 6, T₋₇C; mutant 7, A₂₀C; mutant 8, A₁₃₉G; mutant 9, T₃₉₈A; mutant 10, T₅₁₅C; mutant 11, A₁₅₂C; mutant 12, C₁₈₅G; and mutant 13, C₄₅₈A. *, unique mutation of *M. bovis* (C₁₆₉G) that conveys natural PZA resistance. The arrow at the top indicates the start of the open reading frame.

tional testing but require from 8 to 12 days to determine antibacterial susceptibility and have the potential for cross-contamination (12, 14, 31).

Genotypic assays for the detection of drug resistance have been applied to both cultured isolates and direct patient specimens. These include amplification techniques, DNA sequence analysis, PCR-single-strand conformation polymorphism electrophoresis, and structure-specific cleavage and DNA probe detection assays, all of which are capable of detecting mutations associated with drug resistance (8, 22, 30).

Temperature-mediated heteroduplex analysis (TMHA) using denaturing high-performance liquid chromatography (DHPLC) was originally applied to the detection of specific gene polymorphisms (21). The technology was recently applied to the detection of mutations associated with antituberculosis drug resistance (5). The technique utilized differential retention of homoduplex and heteroduplex DNAs under partial denaturing conditions for the identification of mutations in *rpoB*, *katG*, *rspL*, *embB*, and *pncA* that are responsible for rifampin, isoniazid, streptomycin, ethambutol, and PZA resistance, respectively. Additionally, a separate genetic element (*oxyR*) was utilized to differentiate between *M. tuberculosis* and *M. bovis*. Although the study demonstrated the feasibility of this approach for detecting resistance to multiple antimicrobial agents, detection of mutations in *pncA* was found to be problematic. The difficulty of detecting *pncA* mutations was attributed to the diverse natures of the mutations and their distribution throughout the gene and its putative promoter. It was

proposed that the potential for highly stable DNA helices due to increased GC content within specific regions of the *pncA* gene represented a major technical challenge for TMHA methodology (5).

To overcome these difficulties, the analysis conditions of the TMHA assay were reengineered, and a second probe was added. In combination, these changes allowed the rapid identification of *pncA* mutations associated with PZA resistance and the ability to distinguish between the two closely related species of the complex, *M. bovis* and *M. tuberculosis*, using the same genetic target.

MATERIALS AND METHODS

Mycobacterial strains. Sixty-nine isolates of the *M. tuberculosis* complex were studied: 48 *M. tuberculosis* strains, 13 of which were PZA resistant, and 21 *M. bovis* strains, 8 of which were BCG strains. The PZA-resistant *M. tuberculosis* isolates were obtained from either the Tuberculosis Diagnostic Laboratory of the Centers for Disease Control and Prevention (CDC) (20) or the Tuberculosis Diagnostic Section of the Michigan Public Health Laboratory. The *pncA* genes from the 13 PZA-resistant *M. tuberculosis* strains had been sequenced and found to contain different mutations distributed throughout the *pncA* open reading frame, as well as the promoter region (Fig. 1). The study isolates included six reference *M. bovis* BCG strains (ATCC 35743, ATCC 35744, ATCC 35739, ATCC 35731, ATCC 35738, and ATCC 35748) from the CDC collection. Fifty clinical isolates were obtained from either Creighton University Medical Center (5 *M. tuberculosis* and 5 *M. bovis* isolates), CDC (4 *M. bovis* isolates), or the University of Nebraska Medical Center (UNMC) (4 *M. bovis*, 2 *M. bovis* BCG, and 30 *M. tuberculosis* isolates). Clinical isolates were identified as either *M. tuberculosis* or *M. bovis* as previously described (6, 32) using the standard biochemical reactions, including nitrate reduction, niacin accumulation, and Pzase

TABLE 1. Modified gradient buffer concentrations for mutation detection within GC-rich *pncA* gene in comparison with universal gradient for mutation detection on WAVE system

Step	Modified gradient for <i>pncA</i> gene mutation detection			Universal gradient for mutation detection		
	Time (min)	Buffer (%)		Time (min)	Buffer (%)	
		A ^a	B ^b		A	B ^c
Loading	0.0	40	60	0.0	65	35
Start gradient	0.5	39	61	0.1	60	40
Stop gradient	6.2	29	71	16.1	28	72
Start clean	6.3	0 ^d	0 ^d	16.2	0 ^d	0 ^d
Stop clean	6.8	0 ^d	0 ^d	16.7	0 ^d	0 ^d
Start equilibrate	6.9	40	60	16.8	65	35
Stop equilibrate	7.8	40	60	18.0	65	35

^a Buffer A, ion-pairing reagent (triethylammonium acetate).

^b Buffer B, elution buffer (0.1 M triethylammonium acetate in 25% acetonitrile) with a slope of 1.2% increase per min.

^c Buffer B, with a slope of 2% increase per min.

^d Active cleaning step with 100% buffer D (75% acetonitrile).

activity. PZA susceptibility was previously determined for all isolates, with resistance defined by an MIC of >25 µg/ml using the proportion method with Middlebrook 7H10 medium (4). Two reference strains were used as probes in the TMHA study, *M. tuberculosis* H37Rv, obtained from UNMC, and *M. bovis* ATCC 19210, obtained from the CDC. Amplicons for use as probes in the assay were generated from these reference strains by using the primers described below. To determine the analytical specificity and cross-reactivity of our assay, six additional reference strains of nontuberculous *Mycobacterium* species were included: *Mycobacterium avium* (ATCC 25291), *Mycobacterium intracellulare* (ATCC 13950), *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium chelonae* (ATCC 35751), *Mycobacterium kansasii* (ATCC 35775), and *Mycobacterium goodii* (ATCC 14470).

Genomic-DNA isolation. Genomic DNA was extracted from cultured isolates by the glass bead agitation method as previously described (23). The crude DNA extract was purified using the QIAamp Blood Kit (Qiagen Inc., Valencia, Calif.) according to protocols provided by the manufacturer.

Primer selection and PCR conditions. Specific primers were designed using Oligo version 6.4 software (Molecular Biology Insight, Inc., Cascade, Colo.) to generate a 638-bp amplicon that included the entire *pncA* gene and its putative promoter. The sequence of the forward primer, AW-A3 (5'-GTCATGGACCC TATATCTGTGGCTGCCGCTCG-3'), began at bp -77 upstream of the open reading frame, and that of the reverse primer, AW-A6 (5'-TCAGGAGCTGCA AACCAACTCGACGCTGG-3'), began at the stop codon (bp 561). The PCR assay was performed using 5 µl of template DNA (10 ng/µl) in a total reaction volume of 50 µl, including PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl); 0.1 mM (each) dATP, dGTP, dTTP, and dCTP; 1.5 mM MgCl₂; 0.3 µM (each) primer; and 1.5 U of PlatinumTaq High-Fidelity DNA polymerase (Gibco BRL-Life Technologies, Gaithersburg, Md.). Amplification was performed on a Stratagene (La Jolla, Calif.) Robocycler model 96 thermocycler starting with an initial denaturation step at 95°C for 10 min, followed by 35 cycles, each cycle consisting of a denaturation step at 95°C for 1 min, an annealing step at 64°C for 1 min, and an extension step at 72°C for 1 min. An additional extension step at 72°C for 7 min was performed after the last cycle. The amplicons were stored at 4°C until they were used.

Cloning of PCR products and sequence analysis. PCR products from selected PZA-resistant *M. tuberculosis* isolates were cloned directly following amplification using the standard protocol of the Original TA Cloning kit (Invitrogen, San Diego, Calif.). Purified plasmids from selected colonies were screened for the correct insert by digestion with the endonuclease *EcoRI* (New England Biolabs, Beverly, Mass.) and analyzed by gel electrophoresis for the presence of an ~600-bp product. Selected plasmids were sequenced at the Epply Molecular Biology Core Laboratory (UNMC, Omaha) using the universal M13 forward and reverse sequencing primers. The sequences were analyzed for the presence of mutations of interest by alignment against the wild-type *M. tuberculosis* sequence using MacVector sequence analysis software version 6.5 (Oxford Molecular Group, Inc., Campbell, Calif.).

TMHA assay. The TMHA assay was performed using the commercial WAVE-DHPLC System (Transgenomics Inc. Omaha, Neb.). Since the hydrophobic matrix (polystyrene-divinylbenzene copolymer beads) of the WAVE-

DNASep cartridge is electrostatically neutral and does not readily react with DNA, an ion-pairing reagent, triethylammonium acetate (buffer A), was used to adsorb DNA to the cartridge according to the manufacturer's protocol. An elution buffer composed of 0.1 M triethylammonium acetate in 25% acetonitrile (buffer B) was used to elute DNA based on size and/or sequence composition. Once eluted, the DNA was detected spectrophotometrically by UV absorption at 260 nm. The DNA molecules were analyzed for integrity by using nondenaturing conditions at a column temperature of 50°C, and they were analyzed for mutation detection by using partially denaturing conditions at a column temperature range of 52 to 70°C (21).

PCR product integrity analysis. The PCR products of all isolates were analyzed for purity, specificity, and DNA concentration using the universal DNA-sizing gradient concentration program and a column temperature of 50°C with DHPLC. The phiX174 DNA ladder was used as the sizing marker. The sizing capability of the WAVE system allowed analysis of purity, and only those amplicons shown to generate a single uniform peak of the correct size were used for subsequent analysis.

DNA hybridization. DNAs from the reference strains, *M. tuberculosis* H37Rv (ATCC 25618) and *M. bovis* ATCC 19210, were used for individual hybridization with each of the test isolates. In a total volume of 50 µl, equimolar ratios of test and reference DNA molecules were mixed together in the presence of polymerization inactivation buffer (5.0 mM EDTA, 60.0 mM NaCl, and 10.0 mM Tris [pH 8.0]). The mixture was heated to 95°C for 4 min and then left at room temperature for gradual cooling to 35°C over 45 min. For heteroduplex analysis, both homoduplex and heteroduplex molecules were generated by hybridization of the PCR product for each of the tested isolates with each of the reference DNA probes.

Heteroduplex analysis. Following hybridization, mixtures of test isolates and reference probes were analyzed for *pncA* mutations using the partially denatured mode of the DHPLC. A variety of gradient concentrations were examined with different starting concentrations of buffer B at different rates of increase (slope), and a range of column temperatures from 64.8 to 66.8°C was evaluated. A modified gradient concentration program (Table 1) and a column temperature of 65.8°C were chosen for all subsequent mutation detection studies. A set of three mixtures of wild-type reference DNAs (both *M. tuberculosis* and *M. bovis*) and reference probes was included with each run of the test isolates. Each of the test isolates was analyzed at least three times on three successive days using three different PCR products from each template to test the reproducibility of the chromatographic patterns. The chromatographic patterns of test isolates were compared with those of reference isolates, and interpretations were made according to the proposed protocol (Table 2). Accordingly, any test isolate which generated a single-peak pattern with the *M. tuberculosis* reference probe and a double-peak pattern with the *M. bovis* reference probe was identified as wild-type *M. tuberculosis*, whereas any test isolate which generated a double-peak pattern with the *M. tuberculosis* reference probe and a single-peak pattern with the *M. bovis* reference probe was identified as *M. bovis* or BCG. Isolates that produced a double-peak pattern with both reference probes were identified as mutant strains of *M. tuberculosis* (PZA resistant). A double-peak pattern was defined as a negative deflection following a peak that created a visible trough between adjacent peaks. For each of the double-peaked chromatographic patterns, the distance between the peaks was recorded.

RESULTS

The specificity, purity, and concentration of PCR products from PZA-resistant mutant *M. tuberculosis*, wild-type *M. tuberculosis*, wild-type *M. bovis*, and *M. bovis* BCG were determined

TABLE 2. Proposed protocol for identification of test isolates using two different reference probes for hybridization in TMHA-DHPLC assay

Proposed identification of test isolate	TMHA-DHPLC peak pattern for reference probe:	
	<i>M. tuberculosis</i>	<i>M. bovis</i>
<i>M. tuberculosis</i> (wild type; PZA susceptible)	Single	Double
<i>M. bovis</i> (wild type; PZA resistant)	Double	Single
<i>M. tuberculosis</i> (mutant; PZA resistant)	Double	Double

FIG. 2. TMHA of *pncA* gene PCR products from reference control and test wild-type isolates using *M. tuberculosis* reference probe (A) and *M. bovis* reference probe (B). The chromatographic patterns *a* and *b* in each panel depict the wild-type reference control isolates of *M. tuberculosis* and *M. bovis*, respectively, with the reference probes. Chromatographic patterns 1, 3, and 5 are representative wild-type *M. tuberculosis* test isolates, and patterns 2, 4, and 6 are representative *M. bovis* test isolates.

using the nondenaturing mode of the DHPLC system at a column temperature of 50°C. All tested isolates generated uniform products with identical relative retention times and approximate sizes of 600 bp compared to the PhiX 174 DNA ladder. The analytical specificity of the assay was demonstrated through testing of DNAs from six different reference species of nontuberculous mycobacteria which generated either variable small peaks consistent with nonspecific products or no product (data not shown).

Following optimization of the system, duplexes formed between PCR products of the tested isolates and each of the two reference probes were analyzed using the partially denatured mode of the system at the optimal buffer concentration gradient (Table 1) and column temperature (65.8°C).

Chromatographic patterns produced by the wild-type PZA-susceptible isolates of *M. tuberculosis* demonstrated single-peak patterns when mixed with the *M. tuberculosis* reference probe and double-peak patterns when mixed with the *M. bovis* reference probe, as predicted (Fig. 2A). In contrast, *M. bovis* isolates produced double-peak patterns when mixed with the

M. tuberculosis reference probe and single-peak patterns when mixed with the *M. bovis* reference probe (Fig. 2B).

TMHA of the PZA-resistant *pncA* mutant *M. tuberculosis* strains generated the predicted chromatographic patterns with two peaks or more in 11 of the 13 isolates tested with both reference probes (Fig. 3). Two of the mutant isolates (mutant 3 and mutant 9) produced nonstandard but reproducible chromatographic patterns when they were mixed with the *M. tuberculosis* reference probe (Fig. 3). Further investigation showed that these chromatographic patterns contained distinct features that allowed their consistent recognition. In comparison with the single sharp peak generated by the wild-type PZA-susceptible *M. tuberculosis* isolates when mixed with the *M. tuberculosis* reference probe, mutant 3 produced a broad peak with a shoulder on one side while mutant 9 produced a double-shouldered peak (Fig. 4A). When mixed with the *M. bovis* reference probe, both mutants 3 and 9 generated the predicted double-peak patterns characteristic of all other mutant isolates. However, in comparison with chromatographic patterns generated by wild-type isolates, the mutant isolates demon-

FIG. 3. TMHA of *pncA* gene PCR products from reference control and test mutant isolates using *M. tuberculosis* reference probe (panels A) and *M. bovis* reference probe (panels B). Chromatographic patterns *a* and *b* in each panel depict the wild-type reference control isolates of *M. tuberculosis* and *M. bovis*, respectively, with the reference probes. Chromatographic patterns 1 to 13 depict the 13 test mutant isolates with each of the reference probes. All mutant isolates demonstrated the predicted double-peak patterns with both probes, with the exception of mutant 3 and mutant 9 (circled).

FIG. 4. (A) TMHA of *pncA* gene PCR products of mutant isolates 3 and 9 with the *M. tuberculosis* reference probe. The chromatographs show the difference in shape between the patterns obtained by mutant isolates 3 (*Mut.3*) and 9 (*Mut.9*) and that of wild-type *M. tuberculosis* (*WT.*). (B) TMHA of *pncA* gene PCR products of mutant isolates 3 and 9 with the *M. bovis* reference probe. The differences in retention time between the double-peak patterns of mutant isolates 3 and 9 and that of wild-type *M. tuberculosis* are illustrated.

strated earlier elution of the first peak (heteroduplex DNA) than of the second peak (homoduplex DNA). This resulted in greater separation between the double peaks generated by the mutant isolates than between those generated by the wild-type isolates (Fig. 4B). When all of these observations were combined in the analysis, a protocol was developed that allowed the identification of all mutant isolates as distinct from wild-type *M. tuberculosis* isolates. Furthermore, since the chromatographic patterns for all *M. bovis* isolates were distinct, it was possible to distinguish them from either mutant or wild-type *M. tuberculosis* isolates.

DISCUSSION

TMHA using DHPLC technology was originally applied to the rapid detection of DNA polymorphisms associated with genetic diseases and drug resistance (5, 21). The existence of specific *pncA* mutations associated with PZA resistance in the *M. tuberculosis* complex provided a means to both detect PZA resistance and distinguish *M. tuberculosis* from *M. bovis*. A naturally occurring polymorphism within *pncA* of *M. bovis* (C₁₆₉G) has been used to differentiate the species from *M. tuberculosis* (26). The polymorphism within *M. bovis* strains is unique and different from all of the known acquired mutations of *pncA* of PZA-resistant *M. tuberculosis*. Therefore, a second probe was generated from the *M. bovis pncA* gene for use in combination with the wild-type *M. tuberculosis* probe. Differentiation between wild-type *M. tuberculosis* and *M. bovis* BCG

strains and identification of PZA-resistant mutant strains of *M. tuberculosis* were achieved using a protocol to interpret chromatographic patterns produced by TMHA of the test isolates after mixing them with the two reference probes.

In order to identify the optimal assay conditions, an extended range of column temperatures and various gradient concentrations were studied. This resulted in a modification of the universal gradient concentration recommended by the manufacturer for mutation detection. The modification process included shortening the run time from 18 to <10 min by starting the gradient at a higher elution buffer concentration (61 rather than 40). This change was made based on the predicted retention time of analyzed duplexes according to size. In addition, the slope of elution buffer during the run was reduced from 2 to 1.2% per min. The modification process also included evaluation of a range of column temperatures, starting from the 64.8°C recommended by the system software and ranging up to 66.8°C in 0.1°C increments. The optimal column temperature was determined to be 65.8°C, since all higher and lower temperatures failed to induce the production of the predicted chromatographic patterns. These modifications improved the correlation among the predicted chromatographic patterns based on the theoretical helical structure of heteroduplexes of GC-rich sequences and the observed patterns. The essential outcome of these changes was that the previously cryptic mutations within the GC-rich sequence of *pncA* could be revealed.

The observed chromatographic patterns following TMHA of

the wild-type isolates of *M. tuberculosis* and *M. bovis* (Fig. 2) were consistent with the predicted patterns on which the study was based and allowed the differentiation of the two closely related members of the *M. tuberculosis* complex.

Given the diversity of *pncA* mutations that convey PZA resistance, it was important to test mutations from within all regions of the coding sequence, as well as the promoter element. To test the clinical applicability of our assay, 13 different PZA-resistant mutant strains of *M. tuberculosis* were evaluated. Eleven of these mutant isolates generated the predicted chromatographic pattern when mixed with both reference probes, i.e., a double-peak pattern with clear demonstration of an intervening trough between the peaks. Two mutant *M. tuberculosis* isolates (mutant 3 and mutant 9) did not produce the standard double-peak pattern when mixed with the *M. tuberculosis* reference probe. These patterns were found to be highly reproducible when mutant isolates 3 and 9 were tested repeatedly. Review of the sequence showed that mutant isolates 3 and 9 had mutations in two different regions of *pncA* with high GC content. This was consistent with the original suggestion by Cooksey et al. that the difficulty in detecting *pncA* mutations was due to the presence of GC-rich sequences adjacent to the mutated nucleotides (5). The influence of the GC-rich region on the chromatographic pattern generated by mutations within such sequences was subsequently confirmed by analyzing two additional mutant isolates within GC-rich regions (C₄₀₁T and G₅₁₁A). Under the same optimized conditions, these mutants produced patterns similar to those of mutant isolate 9 (data not shown). We concluded that single-point mutations within or near GC-rich regions of *pncA* were unable to disrupt the helical structure of the heteroduplex DNA under the given conditions, rendering them indistinguishable from the homoduplex DNA. It was subsequently demonstrated that mutations within GC-rich regions could be uncovered through an optimal combination of both column temperature and gradient buffer concentration. Although the entire *M. tuberculosis* genome is GC rich (65% overall), certain regions have higher GC contents than others, which is expected to affect the melting points of the DNA molecules within those regions. Depending on where the boundaries are drawn for the calculation, the melting temperature is considerably higher; for example, the region surrounding the mutation of isolate 3 has a GC content of 82%.

Production of chromatographic peaks using TMHA-DHPLC (WAVE) technology is a function of temperature and the interaction between the DNA duplex and the cartridge matrix under given buffer gradients. It has been reported that the DNASep cartridge, under nondenaturing conditions, resolves the DNA fragment independently of sequence composition (10). However, in our laboratory, shouldered peaks have been observed with certain GC-rich sequences, even under nondenaturing conditions (D. R. Bastola, P. C. Iwen, and S. H. Hinrichs, unpublished data). We demonstrated that specific sequences with predicted secondary structure generated by these GC-rich sequences were responsible for these shouldered peaks. At higher temperatures and under the optimal gradient concentration used in the present study, the chromatographic patterns generated from mutant isolate mixtures that contain both homoduplex and heteroduplex populations were expected to contain double peaks, or at least shouldered

peaks, that were distinguishable from those of wild-type isolates that contain only homoduplex populations.

Another important difference between the chromatographs produced by mutant isolates 3 and 9 and those produced by wild-type *M. tuberculosis* isolates was apparent when both were analyzed with the *M. bovis* reference probe. Mutants 3 and 9 produced chromatographic patterns with two peaks that were separated by a greater distance than that of wild-type isolates (Fig. 4B). This increase in peak separation was also seen in all other mutant isolates when they were mixed with the *M. bovis* probe. The generation of widely separated peaks was a function of an earlier elution time for the heteroduplex formed by the mutant DNA in comparison with the heteroduplex formed by the wild-type *M. tuberculosis* DNA. One explanation for this observation is that the mutant heteroduplexes have greater secondary structure than the wild-type heteroduplexes. This is due to the presence of two base pair mismatches in the mutant heteroduplex, one in the mutant DNA and one in the *M. bovis* reference probe, compared to the wild-type heteroduplexes, which have only a single base pair mismatch that is present in the *M. bovis* reference probe. The greater secondary structure in the mutant-isolate heteroduplexes is believed to result in its elution earlier than the wild-type heteroduplexes.

When the observed patterns from both reference probes were considered together, mutants 3 and 9 could be distinguished from wild-type *M. tuberculosis* isolates, a characterization that could not be made if only one probe was utilized in the analysis.

Under optimal conditions, mixed DNA populations (homoduplex and heteroduplex) produce two separate double-peak patterns. The first double peak represents the two different heteroduplex DNA populations, and the second double peak represents the two different homoduplex populations. Mixed DNA populations can produce two, three, or four peaks, depending on the type and position of the studied isolate mutation. The reaction conditions used for this study were optimized to recover all types of mutations. In isolate 7, the run temperature, gradient concentration, and gradient slope resulted in three peaks, while in other isolates, the same conditions differentiate only the larger double peaks of homoduplex and heteroduplex combinations.

Although rare, processing of a mixed culture of wild-type *M. tuberculosis* and *M. bovis* may generate a double-peak pattern with both reference probes. This pattern could be confused with that of a PZA-resistant mutant *M. tuberculosis* isolate. However, differentiation between the two cases is still possible based on the distance between the double peaks generated by the unknown isolate when mixed with the *M. bovis* reference probe (Fig. 4B). The distance between the two peaks of the wild-type *M. tuberculosis* strain (in the mixed culture) with the *M. bovis* reference probe is shorter than that of a PZA-resistant mutant *M. tuberculosis* isolate.

Inclusion of interpretive software to automate the interpretation of chromatographic patterns, especially those that need careful analysis, is under development and is expected to greatly assist the use of this new technology within the clinical setting.

Demonstration of the specificity of the present assay was also important, since cross-contamination with nontuberculous *Mycobacterium* species is a well-known problem in other stan-

dard culture-based automated assays (14, 31). Specificity was achieved through the use of specific primers that selectively amplify the *pncA* target only from the *M. tuberculosis* complex and not from nontuberculous mycobacteria (data not shown). The simultaneous screening for PZA resistance and identification of *M. tuberculosis* complex members was generally accomplished within 24 h of obtaining an isolate. Since PCR can be applied to direct patient specimens such as bronchial wash fluid (30), even faster analysis is feasible and should be further investigated.

Detection of clinically important drug-resistant subpopulations among predominantly susceptible bacilli represents a challenge for most genotypic assays used for drug resistance screening. We are investigating the sensitivity of our assay for the detection of PZA-resistant subpopulations in heterogeneous primary clinical isolates.

The ability to detect mutations within GC-rich sequences, essential for identification of PZA resistance, and the simultaneous ability to distinguish between the closely related *Mycobacterium* species *M. tuberculosis* and *M. bovis* significantly expand the utility of TMHA-DHPLC methodology for clinical applications.

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