Detection by Denaturing Gradient Gel Electrophoresis of \textit{pncA} Mutations Associated with Pyrazinamide Resistance in \textit{Mycobacterium tuberculosis} Isolates from the United States-Mexico Border Region

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Pyrazinamide (PZA) is a front-line drug used in the treatment of tuberculosis (TB). In a typical short-course (6-month) therapy, PZA is administered with rifampin (RIF), isoniazid (INH), and ethambutol (EMB) for the first 2 months of treatment, followed by 4 months of treatment with INH and RIF (1). During the initial acute phase, PZA and RIF are responsible for much of the killing of persisting \textit{M. tuberculosis} bacilli (48). PZase inactivation is the primary mechanism by which PZA kills tubercle bacilli (23, 48). The mode of action of PZA is complex and not fully understood. It requires activation to pyrazinoic acid by a pyrazinamidase/nicotinamidase (PZase) encoded by the \textit{pncA} gene (48). The activated acid form is excreted and then reabsorbed in a protonated form. It is thought that the acidification of the \textit{M. tuberculosis} cells by the protonated form represents the primary mechanism by which PZA kills tubercle bacilli (48, 49). PZase inactivation is the primary mechanism for developing resistance to PZA (31, 35, 47, 48). Any mutation in \textit{pncA} that inactivates the encoded enzyme appears to be sufficient to confer resistance. Consequently, PZA resistance mutations are highly diverse and are found throughout \textit{pncA}. Cumulative reports indicate that between 72 and 98% of PZA-resistant isolates harbor \textit{pncA} mutations (33, 46, 49). More recent reports, however, tend to support the upper end of this range (48). Earlier reports may have been subject to inaccurate susceptibility testing using culture methods. Susceptibility testing media must have a pH of 6 for PZA to be active against susceptible cells, and this pH is near the limit for growth of tubercle bacilli. Problems with susceptibility testing are magnified in developing countries with limited resources and a high burden of TB. While newer formulations of media yield more consistent results (32), alternative assays to detect PZA resistance are needed.

Molecular techniques (44) are receiving increased scrutiny as alternatives to traditional culture methods of drug susceptibility testing since they can directly detect drug resistance as DNA mutations (31, 47). Denaturing gradient gel electrophoresis (DGGE) is a simple yet powerful technique that is capable of detecting many mutations over large stretches of DNA (10, 44). With DGGE, mutations within an amplicon are recognized as alterations in melting temperature as the fragment migrates through a gradient of denaturants. GC-rich portions at the 5′ end of the PCR primers serve as the highest melting domains and as clamps that prevent DNA fragments from denaturing completely. Mutational alterations in a fragment result in bands that migrate differently from the wild type. For organisms like \textit{M. tuberculosis}, having a single gene copy, heteroduplexing of the DNA to a reference DNA (usually wild type) generates additional homoduplex and heteroduplex bands that facilitate mutation detection. DGGE can...
detect point mutations, insertions, and deletions and is more sensitive than DNA sequencing in identifying mutations in complex DNA samples (11, 40). DGGE has previously been sensitive than DNA sequencing in identifying mutations in rpoB mutations associated with RIF resistance in *M. tuberculosis* (20, 33), including in primary clinical samples from infected patients (33).

In this study, DGGE was used for the first time to detect PZA resistance mutations in *pncA*. Overlapping PCR products were used to scan the *pncA* open reading frame and flanking noncoding regions in a large set of *M. tuberculosis* isolates from the U.S.-Mexico border states of Texas and Tamaulipas, respectively. By using DGGE, DNA alterations were detected in 99% of PZA-resistant isolates, while only 1% of PZA-susceptible isolates harbored DNA alterations. Coupled with comparable data for detecting RIF resistance mutations in *rpoB* (20, 33), these results suggest that DGGE is a sensitive, fast, and inexpensive method for detecting drug resistance mutations in *M. tuberculosis*.

**MATERIALS AND METHODS**

**Bacterial isolates and susceptibility testing.** For this study, 181 individual patient isolates were identified and cultured on Lowenstein-Jensen medium, and mycobacterial DNA was extracted as previously described (20, 30). In this data set, 118 (65%) *M. tuberculosis* strains were isolated from patients residing in the U.S. state of Texas, while 63 (35%) strains were isolated from patients residing in the neighboring Mexican state of Tamaulipas (30). Strain genotypes were determined by IS610 restriction fragment length polymorphism analysis (42) and spoligotyping (5) as previously described (30). Isolates were chosen for this study because they were isolated from patients residing in the neighboring states of Texas and Tamaulipas and were identified as susceptible isolates for PZA susceptibility testing as described elsewhere (20) (Table 1). Samples were loaded onto denaturing gradient gels, 40 to 90% urea/formamide/8% acrylamide (19:1) with TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8) (primer sets A to D), or 50 to 90% urea/formamide/8% acrylamide with TAE (primer set E). After electrophoresis, gels were stained with ethidium bromide in TAE for 16 to 20 h at 100 V in an OptiAnalyzer 8000 electrophoresis tank (CBS Scientific, Solana Beach, CA) containing TAE buffer (21, 43, 45). After electrophoresis, DNA samples that harbored mutations were visualized as a single band. DNA samples that harbored mutated bands were sequenced using two homoduplexes and two heteroduplexes that denatured at distinctive concentrations of denaturant.

**DNA sequencing of *pncA*.** Initial sequencing of *pncA* alleles (see Fig. 2) was performed as described previously (46). Subsequent sequencing was performed as described elsewhere (20) (Table 1). DNA sequencing was performed by Lark Technologies (Houston, TX), an FDA-approved sequencing facility. DNA changes relative to *pncA* alleles from H37Rv (Rv2043c) were noted. Previous reports of DNA sequencing in *pncA* (3, 4, 6, 8, 9, 14–19, 22, 24–29, 31, 34–36, 38, 39, 46, 47) were used to identify unique alterations for this report.

**RESULTS**

**DGGE detection of *pncA* mutations.** Four primer sets, A through D, were initially designed to scan for mutations in *pncA* (Fig. 1). An additional primer set, E, was later added to detect a particular subset of *pncA* mutations (see below). Collectively, these primer sets amplified the entire *pncA* open reading frame and approximately 75 bp of noncoding flanking sequence. These primer pairs were tested using six independent *M. tuberculosis* PZA-resistant isolates from which *pncA* mutations had been identified by DNA sequencing. All mutations were correctly detected within the appropriate PCR fragment (Fig. 2). The primer sets were then used to screen for nucleotide polymorphisms in PZA-resistant and -susceptible isolates. These independent strains were isolated from the U.S.-Mexico border states of Texas and Tamaulipas, respectively (30). All isolates were scanned for DNA polymorphisms in regions A through D. Positive isolates had detectable alterations in at least one PCR fragment. Since the scanned regions

### Table 1. Sequences of primers used in this study

<table>
<thead>
<tr>
<th>PCR fragment</th>
<th>Forward primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reverse primer&lt;sup&gt;b&lt;/sup&gt;</th>
<th>bp (codons)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5'-gcegGCGTCGTTAGGCAACACTG-3'</td>
<td>5'-gcegGCGTCGTTAGGCAACACTG-3'</td>
<td>50–182 (27–53)</td>
</tr>
<tr>
<td>B</td>
<td>5'-gcegGCGTCGTTAGGCAACACTG-3'</td>
<td>5'-gcegGCGTCGTTAGGCAACACTG-3'</td>
<td>131–284 (52–86)</td>
</tr>
<tr>
<td>C</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>212–403 (80–126)</td>
</tr>
<tr>
<td>D</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>340–588 (121–187)</td>
</tr>
<tr>
<td>E</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>5'-ACAGGTCATCAGCCGTTGCAATATC-3'</td>
<td>3–186 (9–53)</td>
</tr>
<tr>
<td>SEQ</td>
<td>5'-CGGCGTCATGACCTATATC-3'</td>
<td>5'-CGGCGTCATGACCTATATC-3'</td>
<td>81–594</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primers set amplify the PCR fragments for DGGE; SEQ, primers used to amplify pncA for sequencing.

<sup>b</sup> Clamp sequences are in lowercase, and pncA-specific sequences are in uppercase.

<sup>c</sup> The 5' nucleotides of forward and reverse primers relative to the *pncA* open reading frame; 50, base pairs upstream of pncA initiation codon. The codons scanned by each PCR product represent the range of whole codons between the 3' ends of primers and include a gap of 2 to 5 bp.
overlapped, some mutations were detected in multiple fragments (Tables 2 and 3).

Once analysis was completed, the DGGE assay was able to detect \textit{pncA} alterations in 82 of 83 PZA-resistant isolates. On the other hand, only 1 of 98 PZA-susceptible isolates harbored a DGGE-detectable DNA alteration. A portion of these alterations are shown in Fig. 3 and 4. Only denaturing gradient gels in which alterations were detected are shown. Each DNA alteration generated a distinct denaturation pattern of bands (corresponding to the homoduplex wild type, homoduplex mutant, and two heteroduplex fragments) that was characteristic of the DNA alteration. Isolates with identical denaturation patterns in the same PCR product (e.g., Fig. 4, lanes 23 and 26) were confirmed by DNA sequencing to harbor the same mutation.

\textbf{Sequencing of \textit{pncA} mutations.} \textit{The pncA} gene was sequenced from 41 isolates. Isolates were chosen from three categories: PZA-susceptible isolates with DGGE-detectable mutations, PZA-resistant isolates without DGGE-detectable mutations, and PZA-resistant isolates with DGGE-detectable mutations. Mutations selected for sequencing were distributed throughout \textit{pncA} (Fig. 1A) and do not represent a random distribution of mutations within the gene (Table 3). DNA alterations were identified in one PZA-susceptible isolate and in 31 PZA-resistant isolates (Tables 2 and 3). The PZA-susceptible isolate harbored a synonymous polymorphism (TCC→TCT) at codon 65, which encodes a Ser residue. Another synonymous polymorphism at codon 75 (GGT→GGC), both encoding Gly residues, was observed in a PZA-resistant isolate that also harbored a D49A mutation. Interestingly, the latter alteration was not detected within fragment A even though it was within the region scanned by the PCR product. Other mutations in this same region were also not detected within the fragment (see below). One isolate contained an IS6110 insertion after codon 114. The insertion was apparent from agarose gels of the PCR-amplified products from this isolate, and it also resulted in an aberrant migration pattern on the denaturing gels (Fig. 4, lane 16). Two isolates harbored small insertions. One contained a 2-bp insertion after the first nucleotide of codon five and resulted in a frameshift mutation. The other was a 6-bp insertion after the second nucleotide of codon 160 that would result in the insertion of two amino acids (Pro and Glu) within the protein. Interestingly, another isolate contained a T160TPE mutation. The remaining isolates harbored single nucleotide changes. Three mutations resulted in nonsense mutations at codons 41, 91, and 103. Two strains with differing genetic backgrounds contained the same mutation (GCC→GTC), which encoded an A134V alteration, and the denaturation patterns from these isolates were identical (Fig. 4, lanes 23 and 26). One DNA sample yielded both wild-type and mutant sequence at codon 103; the mutant sequence encodes a Tyr-to-His alteration. As reported previously, DGGE
M. bovis gene of M. bovis (Rv2043c) open reading frame.

Three of eighty-three isolates were monoresistant to PZA, and since this is a property of M. bovis, these isolates were investigated in more detail. The pncA gene of M. bovis (Mb2069c) encodes an Asp residue at codon 57, and it appears that this residue prevents the M. bovis PZase from activating PZA. M. tuberculosis pncA normally encodes a His residue at codon 57, and the encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug. An H57D mutation has also been identified encoded PZase can activate PZA and render the cells susceptible to the drug.

## Table 2. Sequenced pncA mutations

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Codon</th>
<th>Base change</th>
<th>Amino acid change</th>
<th>DGGE fragment(s)</th>
<th>Fig: lane</th>
</tr>
</thead>
<tbody>
<tr>
<td>2778</td>
<td>4</td>
<td>TTG→TCG</td>
<td>Leu→Ser</td>
<td>A</td>
<td>3:1</td>
</tr>
<tr>
<td>4067</td>
<td>5</td>
<td>ATC→ATTG TC.</td>
<td>Ile→Met+fs</td>
<td>A, E</td>
<td>2:2, 3:2</td>
</tr>
<tr>
<td>5242</td>
<td>12</td>
<td>GAC→GCC</td>
<td>Asp→Ala</td>
<td>A, E</td>
<td>3:3</td>
</tr>
<tr>
<td>2381</td>
<td>17</td>
<td>GGC→AGC</td>
<td>Gly→Ser</td>
<td>A, E</td>
<td>2:1, 3:4</td>
</tr>
<tr>
<td>3579</td>
<td>27</td>
<td>CTG→CCG</td>
<td>Leu→Pro</td>
<td>(A), E</td>
<td>3:5</td>
</tr>
<tr>
<td>2609</td>
<td>35</td>
<td>CTG→CCG</td>
<td>Leu→Pro</td>
<td>(A), E</td>
<td>3:6</td>
</tr>
<tr>
<td>2886</td>
<td>41</td>
<td>TAC→TAG</td>
<td>Tyr→Stop</td>
<td>(A), E</td>
<td>3:7</td>
</tr>
<tr>
<td>3772</td>
<td>47</td>
<td>ACC→GCC</td>
<td>Thr→Ala</td>
<td>(A), E</td>
<td>3:8</td>
</tr>
<tr>
<td>1908</td>
<td>49</td>
<td>GCC→GAC</td>
<td>Asp→Ala</td>
<td>(A), E</td>
<td>3:9</td>
</tr>
<tr>
<td>1417</td>
<td>54</td>
<td>CGG→CTG</td>
<td>Gly→Gly</td>
<td>B</td>
<td>4:5</td>
</tr>
<tr>
<td>5745</td>
<td>57</td>
<td>ACC→GAC</td>
<td>His→Asp</td>
<td>B</td>
<td>4:3</td>
</tr>
<tr>
<td>4508</td>
<td>65</td>
<td>TCC→TCT</td>
<td>Ser→Ser</td>
<td>B</td>
<td>4:2</td>
</tr>
<tr>
<td>4862</td>
<td>67</td>
<td>TCG→CCG</td>
<td>Ser→Pro</td>
<td>B</td>
<td>4:7</td>
</tr>
<tr>
<td>2753</td>
<td>71</td>
<td>CAT→TAG</td>
<td>His→Tyr</td>
<td>B</td>
<td>4:1</td>
</tr>
<tr>
<td>3377</td>
<td>71</td>
<td>CAT→GAT</td>
<td>His→Asp</td>
<td>B</td>
<td>4:4</td>
</tr>
<tr>
<td>5482</td>
<td>76</td>
<td>ACT→ACT</td>
<td>Thr→Pro</td>
<td>B</td>
<td>4:8</td>
</tr>
<tr>
<td>2091</td>
<td>91</td>
<td>GAG→TAG</td>
<td>Glu→stop</td>
<td>C</td>
<td>4:15</td>
</tr>
<tr>
<td>4086</td>
<td>93</td>
<td>GTG→CTG</td>
<td>Val→Leu</td>
<td>C, D</td>
<td>2:4, 4:12</td>
</tr>
<tr>
<td>4074</td>
<td>96</td>
<td>AAG→AAG</td>
<td>Lys→Thr</td>
<td>C</td>
<td>4:13</td>
</tr>
<tr>
<td>820</td>
<td>97</td>
<td>GTG→AGT</td>
<td>Gly→Ser</td>
<td>C</td>
<td>4:14</td>
</tr>
<tr>
<td>2319</td>
<td>103</td>
<td>TAG→TAG</td>
<td>Tyr→stop</td>
<td>C, D</td>
<td>2:3, 4:11</td>
</tr>
<tr>
<td>3455</td>
<td>103</td>
<td>TAG→aAC</td>
<td>Tyr→His</td>
<td>C</td>
<td>4:10</td>
</tr>
<tr>
<td>5171</td>
<td>114</td>
<td>T14′ IS6110</td>
<td>fs</td>
<td>C</td>
<td>4:16</td>
</tr>
<tr>
<td>570</td>
<td>125</td>
<td>GTC→TTC</td>
<td>Val→Phe</td>
<td>C, D</td>
<td>4:17</td>
</tr>
<tr>
<td>1902</td>
<td>134</td>
<td>GCC→GTC</td>
<td>Ala→Val</td>
<td>D</td>
<td>4:23</td>
</tr>
<tr>
<td>5330</td>
<td>134</td>
<td>GCC→GTC</td>
<td>Ala→Val</td>
<td>D</td>
<td>4:26</td>
</tr>
<tr>
<td>1419</td>
<td>139</td>
<td>GTG→GCC</td>
<td>Val→Ala</td>
<td>D</td>
<td>4:21</td>
</tr>
<tr>
<td>1969</td>
<td>154</td>
<td>ΔGG→GGG</td>
<td>Arg→Gly</td>
<td>D</td>
<td>4:24</td>
</tr>
<tr>
<td>4694</td>
<td>160–161</td>
<td>ACA GCC→ACA CCT GAA GCC</td>
<td>Thr Ala→Thr Pro Glu Ala</td>
<td>D, E</td>
<td>2:5, 4:20</td>
</tr>
<tr>
<td>5387</td>
<td>160</td>
<td>ACA→CCA</td>
<td>Thr→Pro</td>
<td>D</td>
<td>4:22</td>
</tr>
<tr>
<td>4250</td>
<td>175</td>
<td>ATG→GTG</td>
<td>Met→Val</td>
<td>D</td>
<td>4:25</td>
</tr>
<tr>
<td>4547</td>
<td>180</td>
<td>GTG→TTC</td>
<td>Val→Phe</td>
<td>D</td>
<td>2:6, 4:19</td>
</tr>
<tr>
<td>4720</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

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*a* All isolates are resistant to pyrazinamide except 4508; isolate 5745 is M. bovis.

*b* Codons of pncA (Rv2043c) open reading frame.

*c* Base change of altered codon with altered nucleotide(s) underlined; ′, insertion; lowercase letter, minor nucleotide mixed with wild type as determined by DNA sequence.

*d* Amino acid alteration of mutation is indicated; fs, frameshift; stop, nonsense mutation; ′, insertion.

*e* DNA alteration was identified within DGGE fragments A to E as indicated; (A), mutation is within fragment A but was not detected.

*f* Fig: lane, figure and lane in which the denaturation profile of the mutation is shown.

*g* DGGE profile is not shown.
the gene containing a high GC content. While most of pncA
shorter than fragment A and contains a different combination
designed. The fragment amplified by primer set E is slightly
phoresis. To address this problem, an additional primer set was
the heteroduplexes from melting properly during electro-
main. We hypothesized that these mutations were not detected
detected mutations were associated with this high melting do-
a melting temperature peak close to 90°C (Fig. 1B). The un-
has a melting temperature around 81 to 82°C, this region had
were identified by sequencing. The mutations resided in a
sequencing their
by retesting these isolates for PZA susceptibility, by DNA
confirmed as resistant to PZA, and mutations within
lacked any alteration within
previously, and PZA-resistant isolates harboring this amino
acid alteration lack PZase activity (26). The two amino acid
changes at codon 71 have not been reported previously, but
another alteration at this codon, H71R, has been reported in

Resolving problem isolates. Problems were encountered in
the initial analysis of 18 isolates whose PZA susceptibility did
not match their DGGE pattern. These problems were resolved by
retesting these isolates for PZA susceptibility, by DNA
sequencing their pncA alleles, and by further DGGE analysis.
Initially, 11 isolates were identified as PZA resistant but did
not harbor DGGE-detectable mutations. Susceptibility was re-
vised from resistant to susceptible for six of these isolates, and
DNA sequencing confirmed that they lacked alterations in
pncA. A single isolate was confirmed as resistant to PZA but
lacked any alteration within pncA. Finally, four isolates were
confirmed as resistant to PZA, and mutations within pncA
were identified by sequencing. The mutations resided in a
region of pncA amplified by primer set A and spanned codons
27 to 47 (Fig. 1, lanes 5 to 8 and 10). Only the nonsense
mutation at codon 41 resulted in a partial denaturation pat-
tern. An additional isolate harbored a defect at codon 49 that
was also not detected (lane 9). This isolate also contained a
synonymous alteration at codon 75 that was detected within
fragment B. Inspection of the pncA gene revealed a domain of
the gene containing a high GC content. While most of pncA
has a melting temperature around 81 to 82°C, this region had
a melting temperature peak close to 90°C (Fig. 1B). The un-
detected mutations were associated with this high melting do-
main. We hypothesized that these mutations were not detected
because the high melting temperature of this region prevented
the heteroduplexes from melting properly during electro-
phoresis. To address this problem, an additional primer set was
designed. The fragment amplified by primer set E is slightly
shorter than fragment A and contains a different combination
of clamps to fine tune denaturation (Table 1). The electro-
phoretic conditions were also modified to detect denaturation
products (see Materials and Methods). All of the mutations
not detected in fragment A were detected in fragment E (Fig.
1, lanes 5 to 10). Once this was achieved, every sequenced pncA
mutation was detected within one of the DGGE amplicons
(Fig. 3 and 4). Only one PZA-resistant isolate, described
above, lacked a mutation in pncA as assayed by DGGE and
DNA sequencing.

Seven PZA-susceptible isolates were identified with DGGE
mutations during the initial screening. One isolate remained
PZA susceptible upon retesting and harbored the synonymous
S65S polymorphism within pncA, as described above. PZA
susceptibility results were revised from susceptible to resistant
for three isolates, and DNA sequencing indicated that they
harbored alterations in pncA indicative of drug resistance.
These alterations included amino acid changes L4S, H71Y, and
H71D (Table 2). The L4S mutation has been reported
previously, and PZA-resistant isolates harboring this amino
acid alteration lack PZase activity (26). The two amino acid
changes at codon 71 have not been reported previously, but
another alteration at this codon, H71R, has been reported in
PZA-resistant isolates lacking PZase activity (26–28). Intu-
atively, isolates with these amino acid alterations have been
defined as PZA resistant. Three other isolates remained PZA susceptible upon retesting. DNA sequencing indicated that these isolates lacked alterations in \( \text{pncA} \), and repeat DGGE assays confirmed the absence of polymorphisms.

**DISCUSSION**

In this study, DGGE was used to screen \( M. \) *tuberculosis* isolates from the U.S.-Mexico border region for nucleotide polymorphisms in \( \text{pncA} \) that impart resistance to the anti-TB drug PZA. The five DGGE PCR products were used to scan for polymorphisms in approximately 600 bp of the \( M. \) *tuberculosis* genome that encompass the \( \text{pncA} \) gene. Using DGGE, we detected DNA alterations in 98.8% (82 of 83) of PZA-resistant isolates and in only 1% (one of 98) of PZA-susceptible isolates. These results by DGGE rival the theoretical maximum attained by DNA sequencing (31, 44, 47) and suggest that DGGE can be a viable, more economic alternative to sequencing in detecting \( \text{pncA} \) mutations. DGGE can be performed on clinical samples, such as sputum (33), and can be completed rapidly with high throughput methodology (21). DGGE also detected apparent reporting errors in PZA susceptibility testing by culture methods. Based on the initial results reported here, we can compare the diagnostic criteria of the DGGE genotypic assay with those of the culture phenotypic assay of PZA susceptibility. Initial culture testing had a sensitivity of 96.4% (80 of 83) in predicting PZA resistance and a specificity of 93.9% (92 of 98) in predicting PZA susceptibility. The sensitivity of the DGGE assay was 98.8% (82 of 83), and the specificity was 95% (94 of 98). Since the values for the DGGE PZA assay exceed those of the culture assay, this suggests that the DGGE genotypic assay may be a suitable alternative to the phenotypic culture assay for predicting PZA susceptibility. Blinded studies will be necessary, however, to confirm these predictions.

Although \( \text{pncA} \) was not sequenced from all 181 isolates, the concordance between DNA sequencing and DGGE was 100% for the 41 isolates from which \( \text{pncA} \) was sequenced. Of the 32 isolates with DGGE-detected DNA alterations, all 32 yielded \( \text{pncA} \) alterations upon sequencing. Furthermore, all of the codon changes in these isolates were localized to the correct region of \( \text{pncA} \) probed by the PCR fragments. In addition, all nine isolates without DGGE-detectable alterations were shown to lack \( \text{pncA} \) alterations by DNA sequencing. Similar concordance was observed between DGGE and DNA sequencing for RIF resistance mutations associated with \( \text{rpoB} \) (20). Altogether, we identified 32 distinct alterations in \( \text{pncA} \), 11 of which have not been reported previously (3, 4, 6, 8, 9, 14–19, 22, 24–29, 31, 34–36, 38, 39, 46, 47). DNA alterations were distributed throughout \( \text{pncA} \) (Table 3), and representatives detected within the four major DGGE PCR products (A to D) were sequenced to establish a distribution of DNA alterations throughout the gene (Fig. 1). This strategy proved useful when testing whether the four primer sets were sufficient for detecting all \( \text{pncA} \) mutations in these isolates.

Initial analysis identified 18 isolates whose DGGE profiles did not match their PZA susceptibilities, including 11 PZA-resistant isolates without DGGE patterns and 7 PZA-susceptible isolates with DGGE patterns. The PZA susceptibility profiles of half of these isolates were reassigned upon retesting, and DNA sequencing confirmed that these changes were justified. Six isolates without DNA alterations in \( \text{pncA} \) were classified as PZA susceptible upon retesting, and three isolates with \( \text{pncA} \) polymorphisms were revised to PZA resistant. The altered residues of the latter isolates were characteristic of PZA resistance. One isolate remained PZA resistant, but defects in \( \text{pncA} \) could not be detected by DGGE or by sequencing. A small number of PZA-resistant isolates do not harbor mutations in \( \text{pncA} \), and these isolates also usually retain PZase activity (31, 47, 48). PZase activity was not tested, and so this correlation remains to be confirmed for this isolate. Similarly, one PZA-susceptible isolate harbored a S67S synonymous polymorphism.

Problems with the initial DGGE assays were apparent for seven isolates. This included three PZA-susceptible isolates that were incorrectly assigned mutations based on the initial DGGE screen but lacked \( \text{pncA} \) alterations by sequencing or by repeat DGGE assays. In addition, four PZA-resistant isolates harbored \( \text{pncA} \) mutations that were not detected within the initial four DGGE PCR products. All four DNA alterations were located in a region scanned by fragment A; a fifth mutation was also identified in this region that was not detected. These mutations were clustered in a region of \( \text{pncA} \) that had an unusually high melting temperature due to a high GC content. The melting temperature of this region peaked around 90°C, which was about 10°C higher than that of the rest of the gene (Fig. 1B). Indeed, the GC clamps attached to the ends of the

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**TABLE 3. Summary of \( \text{pncA} \) mutation detection by DGGE and DNA sequencing**

<table>
<thead>
<tr>
<th>PZA phenotype</th>
<th>Assay</th>
<th>Mutation detection by DGGE PCR fragment(s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DGG + sequencing</td>
<td>A + E</td>
</tr>
<tr>
<td>Resistant (83 [98.8%])</td>
<td>DGG + sequencing</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>DGG only</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>28</td>
</tr>
<tr>
<td>Susceptible (98 [1.0%])</td>
<td>DGG + sequencing</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>DGG only</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>97</td>
</tr>
</tbody>
</table>

* Mutations were observed by DGGE using PCR fragments A to E as indicated; A + E, mutations detected within fragments A or E or both as described in the text and in Table 2; multiple, mutations detected in more than one fragment; deletion, \( \text{pncA} \) was not amplified from isolate.

a DGGE + sequencing, mutation confirmed by DGGE and DNA sequencing of \( \text{pncA} \). DGGE only, mutation detected by DGGE only.

b Does not include synonymous G75G polymorphism; H57D polymorphism of \( M. \) *bovis* is detected within this fragment.

c Total number of resistant isolates scanned is shown in parentheses; percentage of isolates with detectable DNA alterations is shown in brackets.
amplicons have a peak melting temperature of 95°C, which is only slightly higher than this naturally occurring \textit{M. tuberculosis} domain. The GC clamps are attached to prevent complete separation of the DNA strands during electrophoresis. These results suggested that this region was not melting properly in the denaturing gradient gels, and therefore the mutations were not detected. Similar problems are expected for any molecular technique that probes for DNA alterations in \textit{pncA}, probably including sequencing. For instance, Mohamed et al. (25) reported problems detecting a 1-bp deletion within codon 24 using denaturing high-performance liquid chromatography. Branch migration inhibition (18) and single-stranded conformation polymorphism (6, 34) have also been used to detect \textit{pncA} mutations. These three techniques work on the same basic principles as DGGE in that they detect DNA mismatches that represent mutations. In theory, any molecular technique that relies on denaturation, hybridization, or DNA conformation (44) may face problems in detecting \textit{pncA} alterations in this region. Once problems associated with this high-melting region were understood, an additional DGGE primer set was used to successfully detect mutations in this region. Mutations from five PZA-resistant isolates were localized to this region from a total of 83 PZA-resistant isolates scanned. Based on these results, it is estimated that 6% of PZA-resistant isolates harbor mutations in this region.

Mutations that inactivate the PZase encoded by \textit{pncA} are distributed throughout the gene, and no major hot spots have been observed. Alterations, such as T47A and H57D, are commonly observed among groups of PZA-resistant isolates, but these mutations do not represent a significant proportion of the total resistant isolates. The \textit{pncA} gene can be divided into eight regions that are scanned by the five DGGE PCR products, including four regions in which these fragments overlap. Based on the denaturation patterns that are characteristic of each mutation and on the location of a mutation to one of the aforementioned subdomains, it is possible to predict mutations by DGGE (e.g., Fig. 4, lanes 23 and 26). This property could be used in several ways. First, it is possible to detect the same mutation in different isolates based on their identical denaturation pattern. Second, it can be used to identify synonymous DNA alterations that are not associated with drug resistance. These are infrequent in \textit{pncA}, although a few have been observed (3, 4, 6, 8, 9, 14–19, 22, 24–29, 31, 34–36, 38, 39, 46, 47). By recognizing these polymorphisms by their denaturation fingerprint, it should be possible to improve the specificity of a DGGE clinical assay to predict PZA susceptibility. Third, it could be used to track outbreaks and transmission of PZA-resistant strains. Fourth, polymorphisms can be used to type strains by multilocus sequence typing (41) or comparable techniques. Multilocus sequence typing can be performed using DGGE because of its high throughput and low cost (13). Finally, as shown here, DGGE fingerprints can also be used to help distinguish \textit{M. bovis} isolates from other members of the \textit{M. tuberculosis} complex (14).

The results obtained here are similar to those obtained when DGGE was used to probe \textit{rpoB} for mutations associated with RIF resistance (20, 33). Using two DGGE PCR products, we were able to detect mutations in 99% of RIF-resistant isolates, which were also collected from the U.S.-Mexico border region (20). By contrast, only 2% of RIF-susceptible isolates produced detectable polymorphisms. RIF resistance mutations in \textit{rpoB} are highly localized to an 81-bp stretch of \textit{rpoB} between codons 507 and 533, but mutations outside this region exist as well. The dispersed nature of mutations in both genes makes it expensive to identify mutations by DNA sequencing (2, 7, 44). From the analyses of \textit{rpoB} and \textit{pncA} polymorphisms from our data sets, we have estimated that mutation detection by DNA sequencing is 15- to 50-fold more expensive than detection by DGGE. The lower range includes material costs only, while the upper range includes equipment, data analysis, and labor costs. Expense, sensitivity, and mutation detection present problems for other molecular assays since multiple probes (such as oligonucleotides) must be used. Although multiple primer sets are used for DGGE, they are capable of scanning large (50 to 500 bp) stretches of DNA and are capable of detecting all alterations in the amplicon.

Finally, DGGE assays revealed several instances of apparent errors in drug susceptibility testing. These included both false-positive and false-negative errors. These observations point out the inherent problems with drug susceptibility testing. This problem is more apparent with PZA since the pH of the culture assay must be around 6, which is near the limits for growth of mycobacteria. In our analysis of PZA and RIF resistance by DGGE, the number of apparent culture testing errors was comparable to the number of DGGE errors for both false-positive and false-negative scoring of susceptibility. For both RIF and PZA, it will be interesting to perform blinded studies comparing our DGGE assays to culture methods to predict drug susceptibility.

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REFERENCES

DGGE DETECTION OF pncA MUTATIONS IN PZA-RESISTANT TB


