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Peptide Nucleic Acid Probe Detection of Mutations in *Mycobacterium tuberculosis* Genes Associated with Drug Resistance

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ABSTRACT

The emergence of drug-resistant strains of *Mycobacterium tuberculosis* is a serious public health problem. Many of the specific gene mutations that cause drug resistance in *M. tuberculosis* are point mutations. We are developing a PCR-peptide nucleic acid (PNA)-based ELISA as a diagnostic method to recognize point mutations in genes associated with isoniazid and rifampin resistance in *M. tuberculosis*. Specific point mutation-containing sequences and wild-type sequences of cloned mycobacterial genes were PCR-amplified, denatured, and hybridized with PNA probes bound to microplate wells. Using 15-base PNA probes, we established the hybridization temperatures (50°C–55°C) and other experimental conditions suitable for detecting clinically relevant point mutations in the *katG* and *rpoB* genes. Hybridization of PCR-amplified sequences that contained these point mutations with complementary mutation-specific PNAs resulted in significant increases in ELISA response compared with hybridization using wild-type-specific PNAs. Conversely, PCR-amplified wild-type sequences hybridized much more efficiently with wild-type PNAs than with the mutation-specific PNAs. Using the *M. tuberculosis* cloned genes and PCR-PNA-ELISA format developed here, *M. tuberculosis* sequences containing point mutations associated with drug resistance can be identified in less than 24 h.

INTRODUCTION

Pulmonary tuberculosis is one of the most important, life-threatening bacterial diseases in the world. The World Health Organization (WHO) has estimated that *Mycobacterium tuberculosis* infections are responsible for about 8 million new disease cases and 2 million deaths each year (16). In recent years, tuberculosis control efforts have been

severely threatened by the emergence of potentially dangerous antibiotic-drug-resistant strains of *M. tuberculosis*. A recent WHO study documented that rates of deadly multiple-drug-resistant *M. tuberculosis* exceeded 30% in some eastern European and African countries (17).

Many of the specific gene mutations that cause drug resistance in *M. tuberculosis* have been defined by nucleotide sequence analyses (10). These studies have shown that the drug-resistant phenotype of *M. tuberculosis* usually results from point mutations in known chromosomal genes. The *M. tuberculosis* genes *katG*, which codes for a catalase-peroxidase, and *rpoB*, which encodes the RNA polymerase β subunit, are associated with resistance to isoniazid and rifampin, respectively (10). Although *katG* and *rpoB* alterations have been defined in many isolates, the considerable number of point mutations in these genes that may confer resistance (greater than 40 for *katG* and at least 20 for *rpoB*) has complicated the design and development of appropriate diagnostics. New methods for the rapid detection of drug-resistant *M. tuberculosis* strains are clearly needed because classical bacteriological methods are tedious and time consuming.

Peptide nucleic acid (PNA) probes are useful for detecting mutant nucleic acids because they have high thermal stability, strong binding capacity, and high binding specificity (4,7). It has been demonstrated that PNA probes can be used in fluorescence hybridization assays for the identification and differentiation of tuberculous and nontuberculous mycobacteria in liquid cultures (3,6,8,13). PNA probes can be used to detect single point mutations (5,15). The purpose of this study was to demonstrate that a relatively rapid and cost-effective PCR-PNA-ELISA procedure could be used to detect clinically relevant point mutations in the *M. tuberculosis katG* and *rpoB* genes.

MATERIALS AND METHODS

Templates for PCR

A standard TOPO[®] Cloning procedure (Invitrogen, Carlsbad, CA, USA)

was used to clone the *M. tuberculosis* genes into plasmid vectors. Briefly, the *katG* gene and “core region” (10) of the *rpoB* gene were PCR-amplified from chromosomal DNA extracted from *M. tuberculosis* strain H37Rv (no. 27294/TMC 102; ATCC, Manassas, VA, USA). The products were directly cloned into the pCRII-TOPO vector, according to the manufacturer’s instructions (Invitrogen).

PCR Primers and Products

The design of the DNA oligomeric primers used for PCR amplification of sequences from cloned *M. tuberculosis* genes was based on the published genome sequence of *M. tuberculosis* (2) and published data on mutations in *M. tuberculosis* genes that are associated with drug resistance (10,12). Table 1 shows the four sets of primer pairs used, their sequences, and the names and sizes of their products. PCR product *wtkatG275* was amplified from the cloned *katG* gene and includes codon 275 (ACC) in its sense strand. PCR product *mutkatG275*, which was amplified from the same gene, contains codon 275 with a single altered nucleotide (C; ACC→CCC) inserted into its sense strand. This was achieved by amplification using forward primer *FkatG275-mut(for)*, which contains *katG* codon 275 with the altered nucleotide inserted. PCR product *wtrpoB516* was amplified from the cloned *rpoB* gene and includes codon 516 (GAC) in its sense strand. PCR product *mutrpoB516* was amplified from the same gene and contains codon 516 with a single altered nucleotide (T; GAC→GTC) introduced into its sense strand by the use of the forward primer *FrpoB516mut(for)*. This primer contains *rpoB* codon 516 with the altered nucleotide inserted. PCR primers were prepared with an automated DNA synthesizer (model 394; Applied Biosystems, Foster City, CA, USA) using cyanoethyl phosphoramidite nucleosides.

PCR

PCR was performed as previously described (1), except that the number of cycles was 40, and the annealing temperature was 55°C for wild-type prod-

Table 1. PCR Primer Sequences Used

Name	Sequence (5'→3')	PCR Product (Size)
<i>FkatG275</i> wt(for) <i>katG275</i> wt(rev)	ATGGCCATGAACGACGTCG CGGTCGAAACTAGCTGTGAG	<i>wtkatG275</i> (663 bp)
<i>FkatG275</i> mut(for) <i>katG275</i> mut(rev)	CACTTTCGGTAA <u>CC</u> * <u>CCC</u> ATGG TATTGCCAAGCGCCAGCAG	<i>mutkatG275</i> (250 bp)
<i>FrhoB516</i> wt(for) <i>rhoB516</i> wt(rev)	TTCAT <u>GGA</u> CCAGAACAACCCGC CCATGAGGGGCACGGTTGGCG	<i>wtrpoB516</i> (507 bp)
<i>FrhoB516</i> mut(for) <i>rhoB516</i> mut(rev)	TTCAT <u>GGT</u> *CCAGAACAACCCGC CCATGAGGGGCACGGTTGGCG	<i>mutrhoB516</i> (507 bp)

F, 5' end-labeled fluorescein; for, forward; rev, reverse; wt, wild-type; mut, mutant; 275, codon 275; 516, codon 516; and primer *rhoB516*mut(rev)-primer *rhoB516*wt(rev). Altered codon *katG275*, wild-type codon *rhoB516*, and altered codon *rhoB516* are underlined. An asterisk identifies the altered nucleotide inserted. Further details are given in the Materials and Methods section.

Table 2. PNA Sequences Used

Name	Sequence
<i>wtkatG275</i>	biotin-OO-CCATGGTCTTACCG
<i>mutkatG275</i>	biotin-OO-CCATGGG <u>G</u> *CTTACCG
<i>wtrpoB516</i>	biotin-OO-GTTCTG <u>GT</u> CCATGAA
<i>mutrhoB516</i>	biotin-OO-GTTCTG <u>GA</u> *CCATGAA

The biotin label is at N-terminal (5') end, and base sequences are N(5')→C(3') terminus.
wt, wild-type; mut, mutant; 275, codon 275; 516, codon 516; and O, spacer unit.
Complementary wild-type and altered codons *katG275* and *rhoB516* are underlined.
An asterisk identifies the altered base inserted. Further details are given in the Materials and Methods section.

ucts and 50°C for mutant products. We observed a single band (expected size) for each of the PCR products used in this study by agarose gel electrophoresis (data not shown).

PNAs

Four 15-base PNA oligonucleotide probes were designed and used for the detection of the four PCR products amplified from the cloned *M. tuberculosis* genes. Table 2 shows the names of these PNAs and their sequences. For each of the PNAs, two O spacer units (O = 8-amino-3,6-dioxaoctanoic acid) were inserted between the biotin and the N-terminal (5') end of the PNA. PNA *wtkatG275* is complementary to a 15-base segment that includes codon 275

(ACC) in the sense strand of the *katG* gene and to the same segment in PCR product *wtkatG275*. PNA *mutkatG275* is complementary to the same 15-base segment in the sense strand of the *katG* gene (and of PCR product *wtkatG275*), except for the G* [Table 2; single base mismatch in the complementary sequence to *katG* gene codon 275 containing the point mutation (ACC→CCC)]. This PNA has the same base sequence as PNA *wtkatG275*, except for the altered base G*. PNA *wtrpoB516* is complementary to a 15-base segment that includes codon 516 (CAC) in the sense strand of the *rhoB* gene and to the same segment in the PCR product *wtrpoB516*. PNA *mutrhoB516* is complementary to the same 15-base segment in the sense strand of the *rhoB* gene (and

of PCR product *wtrpoB516*), except for the single base mismatch A* in the complementary sequence to the *rhoB* gene codon 516 containing the point mutation (Table 2; GAC→GTC). This PNA has the same base sequence as PNA *wtrpoB516*, except for the altered base A*. The PNAs were synthesized on a Perceptive Biosystems Expedite™ 8900 instrument (Applied Biosystems), using the manufacturer's preprogrammed synthesis cycles.

ELISA

Streptavidin (Sigma, St. Louis, MO, USA) was bound to microplate wells by incubation (12 µg/well) in 1× coating solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) at 40°C for 1 h. The wells were washed with PBS, 0.05% Tween® 20. All additional washes were performed with this buffer. Before performing ELISA, we added fluorescein-labeled PCR-amplified product (approximately 450 ng/well) to biotin-labeled PNA (50 pmol/well in 50 mM Na₂HPO₄, 1 mM EDTA, pH 8.5), and this mixture was heated at 95°C for 10 min. The mixture was cooled in an incubator at hybridization temperature for 10 min, added to a streptavidin-coated well, incubated at hybridization temperature for 1 h, and the attachment of biotin-labeled PNA to streptavidin plus hybridization of PCR product and PNA occurred. The wells were washed and blocked with 3% BSA in 50 mM

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Na_2HPO_4 , 1 mM EDTA, pH 8.5, at 40°C for 30 min. After washing, 150 mU of anti-fluorescein-alkaline phosphatase Fab fragments (Roche Diagnostics, Penzberg, Germany) were added to each well, followed by incubation at 40°C for 30 min. After washing, alkaline phosphatase substrate (100 $\mu\text{g}/\text{well}$ of *p*-nitrophenylphosphate in 1 M diethanolamine buffer; Kirkegaard & Perry Laboratories) was added, and the wells were incubated at room temperature for 20 min. We stopped the reaction by adding an equal volume of 5% EDTA solution and measured the absorbance at 405 nm.

RESULTS AND DISCUSSION

The goal of this study was to establish a PCR-PNA-ELISA for the detection of clinically relevant point mutations in PCR products amplified from two cloned *M. tuberculosis* genes (*katG* and *rpoB*) that are associated with antibiotic drug resistance. Figure 1 shows a schematic of the PCR-PNA-ELISA format that was used. During the development of the PCR-PNA-ELISA protocol, the optimal concentrations of critical reagents used in the ELISA (e.g., PCR products, PNAs, streptavidin, and antibody conjugate) were determined and are given in the Materials and Methods section. Previously, we have shown that substitution of a proline for a threonine at *katG* codon 275 results in a dramatic reduction in catalase/peroxidase activity and, consequently, increases resistance to isoniazid (11). To determine whether the PCR-PNA-ELISA technique could be used to detect this codon 275 mutation, PCR product *wtkatG275* that contains this codon was prepared. A 15-base PNA probe (PNA *wtkatG275*) complementary to a 15-base segment of PCR product *wtkatG275* that contained codon 275 was designed and synthesized. We prepared another 15-mer PNA probe (PNA *mutkatG275*) that has the same base sequence as PNA *wtkatG275*, except for a single base substitution (T→G) in the triplet that is complementary to codon 275 (Table 2).

Aliquots of PCR product *wtkatG275* and PNA *wtkatG275* were mixed and heated to 95°C and were then incubated

at 5° temperature intervals from 40°C to 60°C. Figure 2A shows that hybridization of the wild-type PNA with the wild-type PCR product was dependent on temperature. Maximum ELISA response occurred at 40°C, and no response was observed at 60°C. When PNA *mutkatG275* was hybridized with the wild-type PCR product, results similar to the wild-type PNA were obtained at 40°C and 60°C (Figure 2A). However, little or no response was observed at 50°C and 55°C. Comparison of the data shows that at 50°C the wild-type PNA probe hybridized with the wild-type PCR product much more efficiently than the mutant PNA probe. These results indicate that the wild-type and mutant *katG275* PNA probes used with the PCR-PNA-ELISA format and hybridization at 50°C can distinguish between the wild-type and the mutant *katG275* PCR products.

The point mutation (ACC→CCC) in

katG gene codon 275 is clinically relevant and is associated with drug resistance (10). Figure 2B shows the results of hybridizing both the wild-type and mutant *katG* PNAs with PCR product *mutkatG275*, which contains the codon 275 mutation. As observed when using the wild-type *katG* PCR product, the optimum ELISA response for point mutation detection occurred at 50°C. The results shown in Figure 2, A and B, indicate that, with the PCR-PNA-ELISA procedure, the mutant and wild-type *katG275* PNA probes can be used to readily distinguish between a PCR product that contains a wild-type *katG* codon 275 (ACC) and a product that contains a mutant codon 275 with a single nucleotide change (CCC) under optimum hybridization temperature conditions.

To demonstrate that this point mutation detection procedure could be applied to another *M. tuberculosis* gene associated with drug resistance, similar

Figure 1. Schematic diagram that illustrates the PCR-PNA-ELISA format developed for detecting mutations in *M. tuberculosis* genes associated with drug resistance. PCR with fluorescein-labeled/nonlabeled primer pairs is carried out to amplify specific sequences of mutant or wild-type DNA from *M. tuberculosis* genes that are associated with resistance to antibiotic drugs. Biotinylated PNA probes that hybridize with the specific PCR products are synthesized. The PNAs are incubated with the denatured, fluorescein-labeled, PCR-amplified gene sequences at predetermined optimum temperatures and then bound to streptavidin-precoated ELISA microplate wells. After PNA/PCR product hybridization and PNA/streptavidin binding, color (405 nm) is developed following the addition of an anti-fluorescein, alkaline phosphatase conjugate and substrate system. Additional details are given in the Materials and Methods section.

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PCR-PNA-ELISA experiments were performed with wild-type and mutant PCR products amplified from the *rpoB* gene. For the *M. tuberculosis rpoB* gene, an asparagine-to-valine substitution at codon 516 has been associated with the development of resistance to rifampin in clinical isolates (10). For these experiments, the cloned core region of *rpoB* was used as a PCR template to amplify the PCR products *wtrpoB516* and *mutrpoB516*. The nucleotide sequences of these products were identical, except for a clinically relevant, single nucleotide change in codon 516 (GAC→GTC) (10). The probe pair PNA *wtrpoB516* and PNA *mutrpoB516* were synthesized and dif-

fer by only a single base substitution (T→A) in the triplet that is complementary to codon 516 (Table 2).

Figure 2, C and D, shows the results of two experiments involving hybridization of wild-type and mutant PNA probes with wild-type and mutant PCR products amplified from the *rpoB* gene. The data resemble those shown for the *katG* experiments. In Figure 2C, the ELISA responses indicate that the wild-type PCR product hybridized efficiently at 50°C with the wild-type-specific PNA but not with the mutant-specific PNA. For the data shown in Figure 2D, 55°C was optimal for PNA probe detection of the mutant PCR product. However, detection at 50°C was still

significantly different. These data indicate that a temperature between 50°C and 55°C is optimal for the PCR-PNA-ELISA detection of wild-type and mutant *M. tuberculosis katG* and *rpoB* gene sequences.

In conclusion, using the PCR-PNA-ELISA format with the two *M. tuberculosis* cloned genes described in this report, the point mutations in PCR-amplified *M. tuberculosis* sequences of genes associated with drug resistance could be identified in less than 24 h. We are currently examining the PCR-PNA-ELISA detection of point mutations in other codons of the two *M. tuberculosis* genes. Recently, we established that this format could also be used to detect mu-

Figure 2. Detection of point mutations in sequences of *M. tuberculosis katG* and *rpoB* genes using a PCR-PNA-ELISA format. The purpose of the experiments shown in this figure was to establish the optimum PNA probe-PCR product hybridization temperatures for microplate detection of point mutations in PCR products amplified from two cloned *M. tuberculosis* genes that are associated with drug resistance. The biotinylated PNA probes and heat-denatured PCR products were incubated together at various temperatures (abscissas) and bound to streptavidin-coated ELISA microplate wells after hybridization. The ordinates indicate A_{405} measured 20 min following the addition of substrate to the ELISA reaction. (A) Upper curve (▲), denatured *wtkatG275* PCR product hybridized with *wtkatG275* PNA and lower curve (■), denatured *wtkatG275* PCR product hybridized with *mutkatG275* PNA. (B) Upper curve (■), denatured *mutkatG275* PCR product hybridized with *mutkatG275* PNA, and lower curve (▲), denatured *mutkatG275* PCR product hybridized with *wtkatG275* PNA. (C) Upper curve (▲), denatured *wtrpoB516* PCR product hybridized with *wtrpoB516* PNA and lower curve (■), denatured *wtrpoB516* PCR product hybridized with *mutrpoB516* PNA. (D) Upper curve (■), denatured *mutrpoB516* PCR product hybridized with *mutrpoB516* PNA and lower curve (▲), denatured *mutrpoB516* PCR product hybridized with *wtrpoB516* PNA. All experimental points represent the averages of triplicate sample data. Vertical bars show the standard deviation measured at each point. The error bars for the data points at or close to the maximum absorption reading (4.0) of the microplate reader could not be determined. The strategies for amplification of the PCR products and other details of the PCR-PNA-ELISA format are described in the Materials and Methods section. Table 2 indicates the DNA base sequences of the PNAs.

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tations in the clinically relevant and drug resistance-associated *katG*108 and *rpoB* 531 codons (data not shown). There was less specificity when certain other PNA probes/point mutations were investigated, and this could be due to the location of the altered base within the PNA sequence (Anker Lundermose, personal communication), influence from adjacent sequences, or the formation of secondary or tertiary structures within the PCR products. The feasibility study shown here suggests that the PNA-based technique should be considered along with other diagnostic procedures such as molecular beacons (9) and microarrays (14) for the rapid detection of mutations in *M. tuberculosis* genes associated with drug resistance. Although further refinement of the PNA procedure will be required for its application to clinical diagnoses, we believe that, with suitable optimization and standardization, a rapid and relatively inexpensive PCR-PNA-ELISA format could be used to identify wild-type *M. tuberculosis* se-

quences and to distinguish them from mutant sequences amplified from patient *M. tuberculosis* isolates.

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