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Capillary Electrophoresis – Single Strand Conformation Polymorphism for the Detection of Multiple Mutations Leading to Tuberculosis Drug Resistance

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Abstract

Drug resistant tuberculosis (TB) is a major health problem in both developed and developing countries. Mutations in the *Mycobacterium (M.) tuberculosis* bacterial genome, such as those to the *rpoB* gene and *mabA-inhA* promoter region, have been linked to TB drug resistance in against rifampicin and isoniazid, respectively. The rapid, accurate, and inexpensive identification of these and other mutations leading to TB drug resistance is an essential tool for improving human health. Capillary electrophoresis (CE) single strand conformation polymorphism (SSCP) can be a highly sensitive technique for the detection of genetic mutation that has not been previously explored for drug resistance mutations in *M. tuberculosis*. This work explores the potential of CE-SSCP through the optimization of variables such as polymer separation matrix concentration, capillary wall coating, electric field strength, and temperature on resolution of mutation detection. The successful detection of an *rpoB* gene mutation and two *mabA-inhA* promoter region mutations while simultaneously differentiating a TB-causing mycobacteria from a non-TB bacteria was accomplished using the optimum conditions of 4.5% (w/v) PDMA in a PDMA coated capillary at 20°C using a separation voltage of 278 V/cm. This multiplexed analysis that can be completed in a few hours demonstrates the potential of CE-SSCP to be an inexpensive and rapid analysis method.

Keywords

capillary electrophoresis; *mabA-inhA*; mutation detection; *rpoB*; SSCP; tuberculosis

1. Introduction

Multi-drug resistant tuberculosis (MDR-TB) is still a major emerging health problem in developed and developing countries (WHO, 2007). In World Health Organization (WHO) 2010 global statistics, there were 8.8 million new cases of tuberculosis, including 1.4 million deaths (WHO, 2010). In addition for 2010, there were 440,000 new cases of MDR-TB including 150,000 deaths with 58 countries having confirmed cases of extensively drug

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resistant TB. There are several contributing factors for the large number of tuberculosis (TB) cases such as longer diagnosis time, reduced TB control and treatment resources, homelessness, overcrowding in institutional settings, HIV infection, and immigration from high TB prevalence areas (WHO, 2007; Bhattacharya *et al*, 2003; Dye *et al*, 1999). However, WHO reported that possibly millions of TB cases are not yet reported because of slow and expensive diagnostic methods. TB detection in clinics often relies on culture-based methods, therefore leading to slower diagnosis (Bhattacharya *et al*, 2003).

MDR-TB is defined as TB that is resistant to treatment by rifampicin (RIF) and isoniazid (INH). RIF is the most commonly used drug and acts on bacterial RNA polymerase to inhibit essential protein transcription (WHO, 2010; McClure *et al*, 1978). Typically, RIF resistant TB is developed because of mutations present in the *rpoB* gene, which encodes RNA polymerase, in *Mycobacterium (M.) tuberculosis*. The RIF resistance determining region (RRDR), from codons 507 to 533 in the *rpoB* gene, accounts for 95% of the mutations (McCammon *et al*, 2205). These mutations are due primarily to substitution in codons 531 (41%), 526 (40%), and 516 (4%) (Yue *et al*, 2003).

INH prevents TB by interrupting the mycolic acid (cell wall) synthesis once it is converted into its active form (Karakousis *et al*, 2008). INH resistance is caused by mutations in different genes such as *mabA-inhA* promoter gene, *inhA*, and *katG* (Afanas'ev *et al*, 2007). Mutations in the *mabA-inhA* gene promoter region lead to overexpression of activated INH targets (β -ketoacyl reductase and ACP) and therefore INH resistance (Afanas'ev *et al*, 2007). Mutations in the promoter region at -15 and -8 are most common.

An improvement in the understanding of the molecular basis of TB drug resistance has furnished new genotypic methods for the faster detection of drug resistance. Genotypic methods can be used to identify both the strain resistance and the mutation that caused the resistance in less than 24 hrs. Automated DNA sequencing, line probe assays, DNA microarrays, and single strand conformation polymorphism (SSCP) are some of the currently developed genotypic methods for the detection of TB, as described briefly below.

Automated DNA sequencing has been used to identify isoniazid, ciprofloxacin, and streptomycin resistance (Takiff *et al*, 1994; Cooksey *et al*, 1996; Nusrath Unissa *et al*, 2008). Although automated DNA sequencing can achieve a sensitivity of 100% when performed with multiple repeats, it is expensive and time consuming, especially when there is a need to check more than one gene fragment, as in the case of multiple drug resistance genes. Line probe assays involve the hybridization of the denatured biotinylated PCR amplicons to a set of nucleotide probes attached on a nitrocellulose strip. Although line probe assays are commercially available, they have been reported to have lower sensitivity when used directly on clinical samples (Morgan *et al*, 2005). Microarrays work on the principle of hybridization of fluorescently labeled PCR amplicons to the microarray, nucleotides that are immobilized in polyacrylamide gel pads (MAGIChip), or on glass substrates (GeneChip, Affymetrix Inc., SantaCruz, CA) (Mikhailovich *et al*, 2001; Troesch *et al*, 1999). Mutations in the amplicons are identified depending on the intensity of the fluorescence signal. Gingeras *et al*. and Troesch *et al*. have evaluated DNA microarrays to identify *rpoB* mutations and observed that microarray results are in agreement with DNA sequencing (Troesch *et al*, 1999; Gingeras *et al*, 1998). However, hybridization based assays have been frequently reported to have difficulty differentiating and identifying single base mutations, which are common in development of drug resistance in these genes (Gao and Yeung, 2000; Hacia, 1999). In addition, this method requires optimized conditions for the identification of mutations (Carrilho, 2000).

SSCP is a simple, sensitive, and inexpensive, sequence mutation detection methodology (Orita *et al*, 1989). SSCP can achieve >90% sensitivity and >80% specificity with the optimization of several experimental conditions, such as separation buffer composition, separation matrix, temperature, voltage, etc (Ren and Ueland, 1999; Ren, 2000; Heller, 2000; Hestekin *et al*, 2011). SSCP exploits the property of mobility shifts for different single stranded DNA tertiary structures (conformations) that are formed with variations in sequence. The variations in single stranded DNA conformations can be because of insertion, deletion and substitution mutations.

Slab gel electrophoresis (SGE) is the traditional form of electrophoresis which has been used frequently for TB mutation detection. SGE-SSCP has been implemented for the detection of resistance to RIF, INH, streptomycin, and ciprofloxacin (Takiff *et al*, 1994; Cooksey *et al*, 1996; Telenti *et al*, 1993; Telenti *et al*, 1997; Heym *et al*, 1994). Telenti *et al*. tested RIF resistant isolates and identified 17 different RIF resistant mutations (Telenti *et al*, 1993). In another study, Telenti *et al*. were able to identify the mutations in *rpoB*, *mabA-inhA* regulatory region, *katG*, *inhA*, and *katG-ahpC* genes (Telenti *et al*, 1997). They determined that PCR-SSCP had a sensitivity of >87% for detection of *inhA* mutations. Although SGE-SSCP is a great analytical tool in research labs for biomolecular separations, it is a slow and laborious method that has trouble achieving the clinically needed sensitivity and specificity. In addition, problems with Joule heat dissipation in slab gel systems require the use of either thin gels or low electric strengths.

Capillary electrophoresis (CE)-SSCP is a promising alternative method to SGE-SSCP for DNA analysis. CE has high separation efficiency, short analysis times, and small sample and reagent requirements. CE utilizes a high surface area to volume ratio allowing for faster heat dissipation. This allows the use of higher electric fields resulting in faster separations. Typically an entangled polymer is used as a separation matrix to identify the mutations. To our knowledge there is no previous research on identification of mutations leading to TB drug resistance using CE-SSCP.

For this study we have chosen to explore the potential of CE-SSCP for detection of a complex sample of *rpoB* gene and *mabA-inhA* promoter region mutations in a TB-causing bacterial DNA sequence along with two non-TB causing bacterial DNA samples. By multiplexing the analysis of multiple drug resistance regions and identification of TB-causing bacteria from non-TB causing bacteria, the cost and analysis time of CE-SSCP could be greatly reduced. In order to obtain this challenging separation, we first explored the importance of system variables including the interaction of separation and coating polymer, temperature, and electric field strength.

2. Materials and Methods

2.1 DNA Sample Preparation

Bacterial samples of 5 µg of *Mycobacterium (M.) sp. BCG* (ATCC 19015D-5), *M. gordonae* (ATCC 35760D-5) and *M. smegmatis* (ATCC 23037D-5) were obtained from ATCC in the form of dried DNA which was then diluted with water to a concentration of 7 ng/µl. *M. sp. BCG* represented wildtype (wt) TB bacteria, while *M. gordonae* and *M. smegmatis* represented non-TB bacteria. These samples were the templates for the amplification of *rpoB* and *mabA-inhA* fragments using PCR as described below. Mutant (mut) DNA for the *rpoB* gene and *mabA-inhA* promoter region were based on previously reported mutations and ordered from BioBasic Inc. (Ontario, Canada) (Telenti *et al*, 1993; Guo *et al*, 2006). The wildtype or unmutated sequence and notation of the mutations is outlined in Table 1. 20 µgs of DNA were diluted using 10 mM Tris-HCl (pH 7.8) to obtain a

DNA stock solution at a concentration of 1 µg/µl. They were then further diluted with water before analysis to obtain a final concentration of 5 ng/µl.

2.2 PCR amplifications

The PCR reagents (5* Go Taq Flexi Buffer, 25 mM MgCl₂, 10 mM dNTP and Taq Polymerase) were obtained from Promega (Madison, WI, USA) and the PCR primers were obtained from Invitrogen (Carlsbad, CA, USA). For *rpoB* and *mabA-inhA*, the forward (fwd) and reverse (rev) primers were labeled with FAM and HEX respectively. These primers amplify a 157 bp fragment in the *rpoB* gene and a 248 bp fragment in the *mabA-inhA* promoter region which contain the regions where mutations commonly occur (Telenti *et al*, 1993; Telenti *et al*, 1997). The amplification was performed using 20 µl of PCR mixture, containing 1.4 µl of 5* Go Taq Flexi Buffer, 2 mM MgCl₂, 2% DMSO, 0.2 mM dNTP, 2.5 U Taq polymerase, 0.5 µM of fwd and rev primers. After preparation, the mixture was shaken gently and centrifuged at 145,000 rpm for ~15-30 s to remove bubbles. Samples were amplified using an ATC 401 Thermocycler (Ramsey, Minnesota, USA). For *rpoB*, the thermocycle protocol consisted of 94°C for 5 min followed by 20 cycles each of 94°C for 1 min, 52.9°C for 1 min, and 72°C for 1 min, followed by 1 cycle of final extension step at 72°C for 8 min, while the *mabA-inhA* thermocycle protocol consisted of 94°C for 6 min followed by 20 cycles each of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by 1 cycle of final extension step at 72°C for 10 min.

2.3 SSCP sample preparation

Amplified wt and mut fragments were purified using the Qiaquick-spin PCR purification kit (Qiagen Inc., Valencia, CA) following the manufacturer's instructions. Quantification of purified DNA was determined with a NanoDrop 1000 (Thermo scientific, Wilmington, DE, USA). The purified samples were diluted to 12-15 nM using 10 mM Tris-HCl (pH 8.0) and were stored at -20°C. Samples were loaded into a 96-well plate as follows: 3 µL for wt alone, 3 µL each of wt and mut samples for mutation detection, 3 µL of each TB and non TB bacterium samples for testing complex sample. After loading the samples were denatured for 3 min at 95°C and snap-cooled on ice for 3 min.

2.4 Polymer Synthesis

Polydimethylacrylamide (PDMA) was synthesized using the method of Albarghouthi *et al*, but with 3 hrs of nitrogen bubbling time before initiation with V-50 (Albarghouthi *et al*, 2002). The polymer was dialyzed using membranes with a molecular mass cutoff of 100 kDa (Spectrum, Gardena, CA, USA) in de-ionized water for 10 water changes followed by lyophilization. Dry polymers were dissolved in 1x TBE buffer + 10% glycerol to obtain polymer concentrations of 3, 3.5, 4 and 4.5% (w/v) and stored at room temperature. Poly-N-hydroxyethylacrylamide (PHEA) was used as a coating polymer and synthesized using a protocol previously described by Albarghouthi *et al*. (Albarghouthi *et al*, 2002).

2.5 CE conditions

CE was performed on an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) in fused-silica capillary arrays of 36 cm in length and 50 µm inner diameter. The glass capillaries were dynamically coated with either 0.1% (w/v) PHEA polymer in water using the procedure described by Albarghouthi *et al*. (Albarghouthi *et al*, 2002) or dynamically coated with PDMA after cleaning with NaOH. The polymer matrix (PDMA) was loaded from 0.5 to 3 min depending on concentration (3-4.5%), samples were injected at 333 V/cm for 15 sec with pre-electrophoresis for 3 min at 416 V/cm. Samples were run at 20-30°C with separation voltages of 5-15 kV (139-416 V/cm) as specified in discussion of results and figure legends.

3. Calculations

Resolution is one way commonly used to measure the quality of mutation detection (Andersen *et al*, 2003). Resolution is proportional to the separation between peaks and inversely proportional to peak width. Therefore, a wider spacing between the wt and mut peaks or sharp, less broad peaks produce a higher resolution. To calculate resolution values, first we exported the ABI 3130 electropherogram into a CSV file format using Chromagnum V0.98. The elution time and peak widths for wt and mut peaks have been obtained using Gaussian fit (non-linear curve fitting) in Origin 8 (OriginLab, Northampton, MA, USA). Resolution was calculated using the following equation:

$$R = \frac{2 |t_{wt} - t_{mut}|}{(w_{wt} + w_{mut})}$$

where t_{wt} is wt peak elution time, t_{mut} is mutation peak elution time, w_{wt} is wt peak width at half max, w_{mut} is mutation peak width at half max (Kourkine *et al*, 2002a).

All the results are presented as means with error bars representing standard deviation. An unpaired t-test was performed using the statistical software GraphPad QuickCalcs (<http://www.graphpad.com/quickcalcs/ttest1.cfm>, La Jolla, CA, USA) to evaluate significant differences between the sample resolution values, migration time difference values and peak widths. The p values are listed in the legend of the appropriate figure.

4. Results and Discussion

A typical CE-SSCP electropherogram for mutation detection in the *rpoB* gene and *mabA-inhA* promoter region are shown in Figure 1. In these samples, both the forward (fwd) or sense DNA strand and the reverse (rev) or antisense DNA strand have been labeled to achieve the most sensitive mutation detection. The fwd strand is labeled with FAM (black) while the rev strand is labeled with HEX (gray). A wildtype (wt) or unmutated sample is heat denatured and snap cooled to form unique conformational folds depending on the DNA sequence. These samples are analyzed by CE-SSCP to determine a baseline for comparison. Next, an unknown or mutated sample is mixed with wt, heat denatured and snap cooled and then run on CE-SSCP. The presence of additional peaks in the electropherogram, indicate the presence of mutation (as highlighted by the thick gray or black arrows in Figure 1). The sensitivity and resolution of the CE-SSCP depends on parameters such as separation polymer, capillary coating, temperature and electric field strength. These parameters often need to be investigated extensively for the optimal mutation detection. Therefore, we have analyzed these parameters as discussed in detail below.

4.1 Interaction of Separation and Coating Polymer

The chemical (hydrophilicity) and physical properties (concentration and molecular weight) of the separation polymer define the interaction of the DNA fragments with the polymer, thus influencing DNA separation resolution. A variety of different polymer chemistries have been explored for SSCP including linear polyacrylamide (LPA) (Kourkine *et al*, 2002a; Hestekin *et al*, 2006; Ren *et al*, 1997), hydroxy methylcellulose (Nishimura and Tsuchioka, 2000), and Genescan (Applied Biosystems) (Woolley and Mathies, 1995; Kourkine *et al*, 2002b). Ren *et al*. showed that viscous short chain LPA, short chain PDMA and short chain LPA-PDMA are excellent separation polymers for DNA and SSCP due to their hydrophilic nature which tends to form more open structures for separation (Ren *et al*, 1997; Ren *et al*, 1999; Ren and Fang, 2001). Previous studies have also shown that increasing the

concentration of the polymer can lead to better separation of the single strand conformers (Kourkine *et al*, 2002a; Hestekin *et al*, 2006; Ghozzi *et al*, 1999; Wenz *et al*, 1998; Hestekin and Barron, 2006). While higher polymer concentrations typically produce better separation and mutation detection capabilities, they also lead to increased difficulty in loading the polymer into capillary and increased analysis times.

Polymers also play an important role in coating the silica capillaries and suppressing electroosmotic flow (EOF) and analyte adsorption. The separation efficiency and reproducibility of DNA separations in CE are decreased by the presence of EOF. Polyethylene oxide, polydimethylacrylamide (PDMA) and polyvinylpyrrolidone are the more hydrophobic polymers while methylcellulose, polyvinyl acrylamide and poly-N-hydroxyethylacrylamide (PHEA) are the more hydrophilic polymers used for adsorbed capillary wall coating (Albarghouthi *et al*, 2002; Fung and Yeung, 1995; Madabhushi, 1998; Gao and Yeung, 1998; Albarghouthi *et al*, 2003). PDMA and polyvinylpyrrolidone coatings are hard to desorb in aqueous solutions due to their hydrophobic properties (Gao and Yeung, 1998; Madabhushi *et al*, 1997). A previous study by Doherty *et al.*, showed that as the hydrophobicity of the coating polymer increased, the thickness of the absorbed coating layer decreased until the coating became ineffective for EOF suppression (Doherty *et al*, 2002). However, a slightly hydrophobic polymer, like PDMA, was still found to be able to significantly suppress EOF. Recent studies have also indicated that the polymer PHEA provides superior performance to PDMA for CE-SSCP (Kourkine *et al*, 2002a) and a long-lifetime coating for microchip electrophoresis – SSCP (Hestekin *et al*, 2006).

In previous studies comparing coating effectiveness, performance has either been based on EOF suppression calculations or on SSCP detection at only one separation polymer concentration. Therefore, for this study the resolution of PDMA and PHEA coated capillaries at different concentrations of separation matrices 3, 3.5, 4 and 4.5% (w/v) PDMA for the rpoB gene and mabA-inhA promoter region mutations detection using CE-SSCP. As mentioned in the previous paragraph, PHEA has previously been reported to be a highly effective hydrophilic coating polymer for mutation identification by SSCP/heteroduplex analysis. PDMA was used as the separation matrix, but it also has the ability to self-coat the capillaries and thus eliminate the additional steps of preparing and coating the capillary with another polymer. Polymer concentrations were selected based on the minimum polymer concentration (3% w/v) to separate single strand conformations to the highest polymer concentration (4.5% w/v) that did not produce significant problems with bubbles and difficulty in loading the polymer into the capillary, which led to current failures in the experiments. Mutation detection for one rpoB gene and one mabA-inhA promoter region sample were tested at each separation polymer and coating polymer condition. Resolution of mutation detection was determined from this data.

When observing the effects of separation polymer concentration alone, both capillary coatings show an increase in resolution with an increase in polymer concentration for all mutations, in agreement with previous studies (Kourkine *et al*, 2002a; Hestekin *et al*, 2006; Ghozzi *et al*, 1999; Inazuka *et al*, 1997). Interestingly, when comparing the effects of the different capillary coatings with increasing polymer concentrations, Figure 2 shows that as the concentration of PDMA increases, the PDMA coated capillary works better than the PHEA coated capillary. The superior performance of the PDMA coating at the higher separation polymer concentrations may be due to a replenishing of the PDMA coating by also using PDMA as the separation polymer.

To further explore the effectiveness of the two coating polymers, the sum of the peak widths ($w_{wt}+w_{mut}$) for the PHEA and PDMA coated capillaries was compared. Poor coating can result in broad peaks due to the slower elution in the presence of EOF as well as interactions

between the capillary wall and sample. There was no statistically significant trend between PHEA and PDMA coated capillaries. However, we noted that there were lower peak widths with less variance for the PDMA coated capillary compared to the PHEA coated capillary at higher concentrations of PDMA (data not shown). When we retrieved the run dates for the experiments, it was apparent that as the PHEA coated capillary aged the variability of the data increased. This could be due to fouling or degradation of the PHEA coating over time. A previous study by Hestekin *et al.* observed that PHEA coated capillary appeared to have better resolution and longer coating lifetime with the inclusion of a small amount of PHEA into the LPA separation polymer (Hestekin *et al.*, 2006).

4.2 Temperature

The effect of temperature on SSCP analysis has been previously determined to have a significant influence on mutation identification, which is highly dependent on the DNA sequence (Ren and Ueland, 1999; Ren *et al.*, 1997; Atha *et al.*, 2001; Arakawa *et al.*, 1996; Sekiya, 1993). At higher temperatures (from 30 to 45 °C), the conformers may become unstable, leading to a loss of CE separation on the basis of their small conformation differences (Ren and Ueland, 1999). At lower temperatures (15 to 30 °C), ssDNA fragments can maintain more stable structures and therefore migrate according to the differences in their conformations (Arakawa *et al.*, 1996; Ren, 2000). In addition to the effect of temperature on the ssDNA conformations' stability, it also can affect the entanglement and viscosity of the polymer matrix inside the capillary and thus influence the migration time of the conformations (Ren and Ueland, 1999). Therefore, 20-30°C has been previously reported as the best temperature for mutation detection by SSCP (Ren and Ueland, 1999; Arakawa *et al.*, 1996; Hestekin, 2006). Based on this information, CE-SSCP analysis was compared at 20, 25 and 30°C using a *rpoB* gene and a *mabA-inhA* promoter region mutation.

Figure 3 shows the effect of temperature on mutation detection for the *rpoB* gene. As the temperature increased from 20 to 30°C, the separation between the *rpoB* wt and mut rev strands significantly decreased, until 30°C where the mutation was no longer detectable. Figure 4 shows the effect of temperature on resolution of mutation detection for the fwd and rev DNA strands of the *rpoB* gene and *mabA-inhA* promoter region mutations. Interestingly, for each gene one mutation detection is improved with increasing temperature, while the other is significantly reduced. For both the *rpoB* fwd and *inhA* rev mutation detection, resolution increased with increasing temperature, while for the *rpoB* rev and *inhA* fwd the resolution decreased with increasing temperature. This again highlights the variable effect of temperature on the mutations and underlines the importance of the sequence-specific conformation of the single strands in understanding the effects of temperature.

In order to better understand the interplay of sequence-specific conformations and temperature, we used the program MFOLD to predict secondary structure of the wt and mut samples. Glavac *et al.* have previously reported that free energy differences between the wt and mut samples correlated with differences in migration times for fragments smaller than 100 bp, but not significantly for larger fragment sizes (Glavac *et al.*, 2002). In analyzing our data, we found no significant correlations between the lowest free energy difference and the resolution of mutation detections which was not surprising given the >200 bp length of our samples. In addition, we used the MFOLD predicated structures to calculate each conformer's melting temperature but were unable to correlate it with any of the mutation detection parameters (i.e. resolution, migration time). In addition to these effects, increasing the temperature also decreased the migration time of the wt and mut peaks, as shown in Figure 3. The faster migration times are probably due to a decrease in the viscosity of the separation polymer with an increase in temperature (Ramaswamy and Musser, 1998). Ultimately, the lowest temperature (20°C) was selected as the best electrophoretic condition

because it was able to achieve mutation detection in the most samples with the highest overall resolution.

4.3 Electric Field Strength

Electric field strength is a factor that influences the mutation detection abilities of CE-SSCP. At higher electric fields, there is a higher current leading to Joule heating, which in turn increases in capillary temperature. This can change the conformations of the single stranded DNAs and increases dispersion and peak widths, therefore resulting in lower resolution (Heller, 2000; Ren, 2000). Low electric fields can cause the slower migration of the conformations (e.g. longer analysis times), increased sample diffusion, and therefore increased peak width and reduced resolution. Due to the complex effects of the electric field strength on sample analysis, it is very important to select an electric field that can balance both of these effects.

Although electric field strength is an important variable for mutation detection, only a limited number of previous studies have investigated its effect. Ren *et al.* studied the effect of electric field strength on the C677T mutation detection using CE-SSCP and observed 500 V/cm as the optimum electric field strength when using short chain polyacrylamide as the separation polymer (Ren *et al.*, 1997). Hestekin *et al.* observed the effect of electric field strength on p53 exon 5-9 mutation detection using microchip electrophoresis-SSCP/HA and identified 350-450 V/cm as optimum electric field strength when using LPA as the separation polymer (Hestekin *et al.*, 2006). For this study, the effect of electric field strength at applied voltage of 5, 10, and 15 kV (139, 278 and 417 V/cm) on the resolution of mutation detection in the *rpoB* gene and *mabA-inhA* promoter region was examined, as shown in Figure 5. For mutation detection in the *rpoB* fwd and *inhA* fwd single DNA strands, the resolution increases with separation voltage. In contrast, the *rpoB* rev strand mutation detection showed a decrease in resolution with an increase in the electric field strength. For both cases, the most significant change occurred between 139 and 278 V/cm while 278 and 417 V/cm had similar values.

In order to better understand the underlying causes for these changes in resolution, we separately examined the differences in migration times and sum of the peak widths for the wt and mut samples. For all the mutation detections, the difference in migration times decreased with increasing electric field strength with the most significant change between 139 and 278 V/cm (decreased by >50%). This would be expected to decrease resolution; however, an increased electric field strength also decreased peak widths, especially from 139 to 278 V/cm (decreased by up to 75%), which leads to the observed increase in resolution. Heller has previously described that higher electric fields can cause either an increase in peak width due to the higher Joule heating or a decrease in peak width due to the faster migration of the DNA (Heller, 2000). This would seem to indicate that in our experiments, it is the faster migration of the conformers rather than Joule Heating which is the dominant effect. Since the 278 and 418 V/cm electric field strengths produce similar resolution values, either could be selected as the optimum. We selected 278 V/cm because of intermittent problems with bubbles at the highest applied voltage (data not shown).

4.4 Detection of complex TB sample

Once the optimized conditions were determined (4.5% PDMA in PDMA coated capillary at 20°C and 278 V/cm), the CE-SSCP system was ready to detect mutations in a complex TB sample. To create a complex sample, we amplified the non-TB bacteria (*Mycobacterium gordonae* and *Mycobacterium smegmatis*) DNAs using the *rpoB* and *mabA-inhA* amplification protocols. These protocols did not appear to amplify the non-TB DNA as initially determined by slab gel electrophoresis. However, when these samples were

included in the complex sample mixture analyzed by CE-SSCP, we observed the peaks for the *mabA-inhA* promoter region from *M. gordonae* as shown in Figure 5. This is most likely due to the ability of CE to detect lower concentrations than slab gel electrophoresis. Figure 5 also shows that CE-SSCP with the optimum conditions was able to simultaneously detect a single mutation from the *rpoB* gene, two different mutations from the *mabA-inhA* promoter region, and to identify the presence of a non-TB mycobacteria (*M. gordonae*). This indicates the potential for a multiplex assay in which multiple drug resistance genes could be tested in the same CE-SSCP analysis.

5. Conclusion

There is increasing need for a rapid and accurate method to identify mutations leading to drug resistant diseases. For tuberculosis, mutations in the *rpoB* and *inhA* genes have been linked to drug resistance for two of the most commonly used antibiotics. CE-SSCP has been previously unexplored for its potential to be a rapid, inexpensive method to meet these criteria. Previous work with CE-SSCP for other mutation detections has indicated the importance of examining analysis conditions before a high sensitivity of the method can be achieved. Therefore, we have investigated the effect of polymer separation matrix concentration, capillary wall coating, electric field strength, and temperature on resolution. In agreement with previous studies we found that the highest polymer concentration (4.5% w/v) provided the highest resolution of mutation detection. Interestingly, the optimum capillary coating varied depending on the concentration of separation polymers and was determined to be highest when the coating and separation polymer were the same. While temperature has a highly complex effect on mutation detection by SSCP, the overall best value was determined to be the lowest (20°C). We also found that 278 V/cm was the best electric field strength as it produced the best balance between resolution, analysis time, peak width and migration time differences. Based on the results of the optimization study, we were able to use these conditions to demonstrate the ability of CE-SSCP to simultaneously identify a *rpoB* gene mutation and two *mabA-inhA* promoter region mutations in addition to differentiating the TB-causing bacteria from a non-TB causing bacteria (*M. gordonae*) in less than 60 minutes. The ability of CE-SSCP to multiplex the analysis of different drug resistance regions as well as to differentiate TB-causing from non-TB causing mycobacteria could lead to lower costs and lower analysis times, especially compared to traditional culture-based methods, for the diagnosis and analysis of treatment for TB.

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*Abbreviations

CE	capillary electrophoresis
EOF	electroosmotic flow
INH	isoniazid
LPA	linear polyacrylamide
MDR-TB	Multi Drug Resistance Tuberculosis
mut	mutation

PHEA	poly- <i>N</i> -hydroxyethylacrylamide
PDMA	poly-dimethylacrylamide
RIF	rifampicin
SGE	slab gel electrophoresis
SSCP	single-strand conformation polymorphism
TB	tuberculosis
wt	wildtype

Highlights

first report of CE-SSCP for the detection of mutations leading to TB drug resistance
explored optimum CE-SSCP conditions including polymer, temperature and voltage
noted separation polymer concentration affected choice of best polymer coating
simultaneous identification of mutations in 2 regions and non-TB causing mycobacteria
analysis complete in hours as opposed to the days-week for culturing samples

Figure 1.

Typical electropherograms demonstrating mutation detection by CE-SSCP for the *rpoB* gene (a, b) and *mabA-inhA* promoter region (c, d). Mutations and their numbering correspond to those shown in Table 1. Large arrows highlight the mutation detection peaks. Samples were injected at 333 V/cm for 15 s at 20°C with 278 V/cm separation voltage using 4.5% (w/v) PDMA separation polymer in a PDMA coated capillary.

Figure 2.

Effect of separation polymer concentration and coating type on resolution of mutation detection by CE-SSCP. Mutations were detected by CE-SSCP in PDMA (black) and PHEA (gray) coating capillaries using PDMA separation polymer coating concentrations of 3%, 3.5%, 4% or 4.5% w/v. Mutations were detected in DNA from the *rpoB* gene for the forward (▲) and reverse (●) strands and from the *mabA-inhA* promoter region forward strand (■). Samples were injected at 333 V/cm for 15 s with 278 V/cm separation voltage at 25°C. Error bars represent standard deviation with $n = 6$ for *rpoB* fwd, $n = 2$ for *rpoB* rev and $n = 4$ for *inhA*. A * indicates a statistically significant difference between polymer coating resolution values at the same polymer separation matrix concentration with a $p < 0.0015$.

Figure 3.

Representative electropherograms demonstrating the effect of temperature on mutation detection by CE-SSCP for the *rpoB* gene at 20°C (a, b), 25°C (c, d), and 30°C (e, f) for wildtype alone and mutation detection, respectively. Arrows highlight the mutation detection peaks. Samples were injected at 333 V/cm for 15 s with 278 V/cm separation voltage using 4.5% (w/v) PDMA separation polymer in a PDMA coated capillary.

Figure 4.

Effect of temperature on the resolution of mutation detection using CE-SSCP for the rpoB forward (▲) and reverse (●) strands and from the inhA gene forward (■) and reverse (X) strands. Error bars represent standard deviation with $n = 6$ for rpoB fwd, $n = 2$ for rpoB rev and $n = 6$ for mabA-inhA. All samples had statistically significant differences in resolution values with a $p < 0.005$ except for the rpoB reverse strand samples and inhA forward strand between 25 and 30°C. Samples were injected at 333 V/cm for 15 s with 278 V/cm separation voltage using 4.5% (w/v) PDMA separation polymer in a PDMA coated capillary.

Figure 5.

Effect of electric field strength on resolution of mutation detection using CE-SSCP. Resolution of mutation detection was determined in the forward DNA strand of the mabA-inhA promoter region (black), the forward DNA strand of the rpoB gene (white) and the reverse DNA strand of the rpoB gene (gray) at applied electric field strengths of 139 V/cm (5 kV), 278 V/cm (10 kV) and 417 V/cm (15 kV). Error bars represent standard deviation with $n = 5$ for all samples. The 139 V/cm resolution value was statistically different ($p < 0.05$) than either the 278 V/cm or 417 V/cm resolution values. Samples were injected at 333 V/cm for 15 s at 20°C using 4.5% (w/v) PDMA separation polymer in a PDMA coated capillary.

Figure 6.

Electropherograms demonstrating the ability of CE-SSCP to simultaneously detect mutations in the *rpoB* gene and *mabA-inhA* promoter region while also detecting a non-TB causing mycobacterium (*M. gordonae*). Samples were injected at 333 V/cm for 15 s with 278 V/cm separation voltage at 20°C using 4.5% (w/v) PDMA separation polymer in a PDMA coated capillary. Wildtype controls for the *rpoB* gene and *mabA-inhA* promoter region are the same as Figure 1.

Table 1
RpoB and mabA-inhA sequences with mutations

Sample	Fragment Length (bp)	Sequence
rpoB	157	TCGCCGCGATCAAGGAGTTCTTCGGCACCAGCCAGCTGAGCCA ATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCC ^T ACAA GCGCCGACTGTCGGCGCTGGGGCCCGGCGGTCTGTACGTGA GCGTGCCGGGCTGGAGGTCCGCGACGTGCA
mabA-inhA	248	CCTCGCTGCCCAGAAAGGGATCCGTCATGGTCTGAAGTGTGCTGA GTCACACCGACAAACGTCACGAGCGTAACCCCAGTGCGAAAG TTCCCGCCGAAATCGCAGCCACGTTACGCTCGTGGACATAC CGATTTCGGCCCGGCGCGGCGAGAC ^{T1} GATAGGT ^{C2} TGTCGG GGTGACTGCCACAGCCACTGAAGGGGCCAAACCCCATTCGTATC CCGTTCACTCCTGGTTACCGGAGGAAACCGGGGGAT

The location and type of mutation is represented by a superscript to the right of the altered base. Primers are identified by gray shading. For the rpoB gene, the underline bolded text highlights the RRDR region, while for the mabA-inhA sample it represents the mabA-inhA promoter region (Telenti *et al*, 1993; Telenti *et al*, 1997; Guo *et al*, 2006).