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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 *rpo*B 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 *rpo*B 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.

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1. Introduction

Multi-drug resistant tuberculosis (MDR-TB) is still a major emerging health problem in developed and developing countries (WHO, 2007). In the 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 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 *rpo*B gene, which encodes RNA polymerase, in *Mycobacterium* (*M.*) *tuberculosis*. The RIF resistance determining region (RRDR), from codons 507 to 533 in the *rpo*B gene, accounts for 95% of the mutations (McCammon et al., 2005). 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

Abbreviations: CE, capillary electrophoresis; EOF, electroosmotic flow; INH, isoniazid; LPA, linear polyacrylamide; MDR-TB, multi drug resistance tuberculosis; mut, mutation; PHEA, poly-*N*-hydroxyethylacrylamide; PDMA, poly-dimethylacrylamide; RIF, rifampicin; SGE, slab gel electrophoresis; SSCP, single-strand conformation polymorphism; TB, tuberculosis; wt, wildtype.

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resistance in less than 24 h. 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, 2000a; 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, 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 *rpo*B, mabA-*inh*A regulatory region, katG, *inh*A, 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 *rpo*B 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), *Mycobacterium gordonae* (ATCC 35760D-5) and *Mycobacterium 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 *rpo*B and mabA-*inh*A fragments using PCR as described below. Mutant (mut) DNA for the *rpo*B gene and mabA-*inh*A 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 are outlined in Table 1. 20 μg of DNA was 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 $MgCl_2$, 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-*inh*A, the forward (fwd) and reverse (rev) primers were labeled with FAM and HEX respectively. These primers amplify a 157 bp fragment in the *rpo*B gene and a 248 bp fragment in the mabA-*inh*A promoter region which contain the regions where mutations commonly occur (Telenti et al., 1993, 1997). The amplification was performed using 20 μl of PCR mixture, containing 1.4 μl of 5* Go Taq Flexi Buffer, 2 mM $MgCl_2$, 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 *rpo*B, 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-*inh*A 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, and 3 μl of each TB and non TB bacterium samples for testing complex sample. After loading

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