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Diagnostics

Reverse line probe assay for cheap detection of Single Nucleotide Polymorphisms in *Mycobacterium tuberculosis*

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ABSTRACT

More and more Single Nucleotide Polymosrphisms of interest among pathogenic organisms are described with the advent of Whole Genome Sequencing but WGS approach is still too expensive, time consuming, and relying on bioinformatical means that are not available in many developing countries. This study presents a low-cost reverse hybridization line probe technique for detecting SNPs in *Mycobacterium tuberculosis*. The proposed test is able to detect mutations in the RRDR of *rpoB* gene in *M. tuberculosis* with specificity and sensitivity of 98% and 100%, respectively and for an average cost of less than €3 per sample. The technique proved efficient not only on pure DNA samples extracted from culture isolates but also on crude extracts from clinical samples. The flexibility of the platform allows to get it transformed to any kind of test detection, hence, building a bridge between rich countries performing SNP discovery and countries with high burden that can target these SNPs on the collected samples.

1. Introduction

Molecular epidemiology of bacterial pathogens enters a new era: Whole Genome Sequencing becomes a common technique in rich countries [1] that enables to gather maximized information to infer transmission chains and assessing drug resistance when SNPs accounting for resistance are almost or all known. For instance, *Mycobacterium tuberculosis* reads can be analyzed using Phyresse interface [2, 3]. Methods specifically designed to detect resistance are also beginning to be developed (Deeplex by Genoscreen, P. Supply, personal communication, and ongoing clinical trial NCT03303963). However, many infectious diseases concern low-income and middle-income countries that have massive amounts of patients and cannot benefit from WGS. Alternative methods are thus still needed.

The rising issue for tuberculosis is the continuous increase of multiple drug resistance (MDR) *i.e.* the resistance to most potent first-line drugs; rifampicin (RIF) and isoniazid. In 2014, there were 480,000 (range: 360,000–600,000) estimated new cases of MDR-TB worldwide with approximately 190,000 deaths [4]. The spread of MDR could be controlled by rapid drug sensitivity testing as it plays an important role in timely and accurate initiation of effective therapy and surveillance of drug resistance [5, 6]. RIF resistance is found to be coupled with isoniazid resistance in more than 90% cases [7, 8] and hence RIF resistance can be used as a surrogate marker for the detection of MDR-TB. Mutations that confer resistance to this drug are frequently found in "RIF Resistance Determining Region (RRDR)", an 81 bp "hot-spot" region consisting of 27 amino acids encompassing codon 426 to 452 of *M. tuberculosis* [9] which corresponds to 507 to 533 in *Escherichia coli* [10].

We propose "Reverse Hybridization Line Probe Assay", using tailing of the oligonucleotides with deoxyribose thymidine (dT) and Nylon membrane as cheap option to immobilize probes. The present study aimed to identify key parameters to build up in-house reverse hybridization assays to screen mutations in bacterial pathogens such as those leading to RIF resistance in *M. tuberculosis*.

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Table 1
–Effect of hybridization and washing conditions on the signal intensity.

Combination	Hybridization Temp. (°C)	Washing Temp. (°C)	Pre-hybridization solution	Probes with efficient detection
1	42	50	Yes	None
2	45	53	Yes	All
3	50	58	Yes	Only W430, W434, W445, W450, W452
4	55	63	Yes	Only W430, W434, W450, W452
5	42	50	No	High background
6	45	53	No	All
7	50	58	No	High background
8	50	58	No	High background

2. Materials and methods

2.1. Oligonucleotides used to screen mutations

A total of 40 oligonucleotides derived from already published literature [11] were used in this study. These correspond to 5 wild type probes spanning the mutation hotspot region of *rpoB* gene of *M. tuberculosis* and 35 mutant probes complementary to different mutations (Table S1). All these oligonucleotides were poly(dT) tailed.

2.2. Immobilization of oligonucleotides on filters

All the oligonucleotides were applied on Hybond N + Nylon membrane (Amersham RPN 303N) using Miniblotter 45 (Immunetics, Inc. USA). They were immobilized by exposing membrane twice to UV radiation using UV cross linker at auto-crosslink mode (1200 μJ). The unbound nucleotides were removed by washing with 5XSSPE/0.5%SDS for 20 min at room temperature followed by washing with double distilled water and stored at room temperature till further use.

2.3. M. tuberculosis samples

A total of 125 bacterial samples enriched in MDR were included in this study. Eighty-one (81) bacterial cultures on Lowenstein Jensen (LJ) medium came from different regions of Pakistan (Peshawar = 20, Rawalpindi = 13, Quetta = 1, Faisalabad = 2, Lahore = 25, Karachi = 8, Islamabad = 12), collected during 2009–2010. The rest (44) were clinical specimens (blood; n = 35 and sputum; n = 6). All the cultures and clinical specimens were confirmed as *M. tuberculosis* complex by PCR amplification using primers specific for IS*6110*.

2.4. Extraction of genomic DNA

DNA from *M. tuberculosis* cultures on LJ slants was extracted by CTAB method (van Soolingen et al., 1991) while the extraction of DNA from clinical samples was performed using SDS/proteinase K method (Goldenberger et al., 1995). Sputum samples were decontaminated by NaOH/N-acetyl-L-cystein solution before extraction of DNA. About 40 ng of DNA from culture isolates, while 5 μL of DNA from clinical specimens, was used for subsequent PCRs.

2.5. Amplification of RRDR of rpoB gene

Amplification of RRDR of *rpoB* gene of *M. tuberculosis* was achieved by nested PCR using primers reported in literature [12]. The outer and inner pair of primers generated a fragment of 395 bp and 257 bp, respectively. The PCR was performed in a final volume of 25 μL containing 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs (Fermentas Cat. #R0181) and 1U of Taq DNA polymerase (Fermentas Cat. # EP0402). Outer pair of primers (10 pmol) was used in the first round of PCR cycle with amplification program consisted of: 5 min at 95 °C for 5 min; 30 cycles at 95 °C for 60 s, 58 °C for 30 s and 72 °C for 30 s. The second round of PCR was performed using inner set of primers (20 pmol and biotin labelled 5′ end of reverse primer) and 1 μl of PCR product from the first round of PCR cycle. The cycling parameters were; 95 °C for 5 min followed by 30 cycles of 95 °C for 60 s, 65 °C for 30 s and 72 °C for 30 s.

2.6. Sequencing of RRDR region of rpoB gene

To assess the accuracy of in house developed reverse hybridization assay, DNA sequencing was used as “Gold Standard”. The 257 bp PCR amplified fragments of hotspot region of *rpoB* gene for 81 isolates were commercially sequenced (Macrogen, Korea). Sequence information was stored, assembled, and analyzed using the Lasergene sequence analysis package (DNAStar Inc., Madison, WI, USA) and SeqScape (Version 2.6; Applied Biosystems) software.

2.7. Reverse hybridization

For hybridization, varying volumes of PCR product were mixed with a hybridization solution (5XSSPE /0.5% SDS) of 150 μL final volume and heat denatured for 2 min. This solution was applied to Hybond N + nylon membrane at right angle to the oligonucleotides probes, using Miniblotter 45. Hybridization conditions were optimized for quantity of PCR product, hybridization temperature, hybridization time and washing temperature (Table 1). Detection of hybridization signals was carried out using Biotin Chromogenic Detection kit (Fermentas Cat. #K0662) according to the suppliers' instructions. Color development on membrane indicated the positive hybridization signal.

3. Results and discussion

Sequencing analysis of 81 culture isolates revealed that 46 had 48 mutations of 10 different kinds in the RRDR of *rpoB* gene, while 35 isolates did not carry any mutation. Codon 450 was found to be most frequently mutated (65%) followed by codons 445 (14%), 435 (12%), 437 (2%), 430 (2%) and 439 (2%). Mutations in codon 450 were TCG to TTG[Ser→Leu] or TGG[Ser→Trp] while codon 445 exhibited four different types of SNPs: CAC to TAC/GAC/CTG/AAC[His→Tyr/Asp/Leu/Phe]. Two isolates showed double mutation: 450(TCG→TTG) [Ser→Leu] along with codon 445(CAC→AAC) [His→Asn].

Using these reference samples, we set up and measured the quality of a test aiming at detecting *rpoB* mutations potentially linked to RIF resistance. Five wild type probes and 30 probes corresponding to the most frequent mutations were coupled to a membrane and used to detect mutations in a subset of samples. Some rare mutations for which resistance was not proven, such as 445(CAC→AAC)[His→Asn] and 445(CAC→CTG)[His→Leu] mutation were not included. H37Rv was used to identify and to assess the sensitivity and specificity of wild-type probes while sequenced isolates allowed assessing 17 of 30 mutant probes. To find out the common parameters to get high sensitivity and specificity of hybridization signals, several conditions were tested where hybridization using 20 μL of PCR product, at 45 °C for 20 min with washing at 53 °C gave better results (Table 1). Worthy of note is that varying conditions provided varying levels of background or