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IDENTIFICATION OF DRUG-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* BY SINGLE STRAND CONFORMATION POLYMORPHISM AND CLEAVASE FRAGMENT LENGTH POLYMORPHISM

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ABSTRACT

Twenty isolates of *Mycobacterium tuberculosis* resistant to rifampicin(RIF), isoniazid(INH) and streptomycin(STR) were analysed by Polymerase Chain Reaction (PCR) amplification of rpoB, katG and rrs genes to evaluate comparative diagnostic significance of genetic assays. Mutations were identified by single strand conformation polymorphism (SSCP) and cleavase fragment length polymorphism (CFLP) and were confirmed by DNA sequencing. SSCP of 4 RIF resistant and 14 INH resistant isolates showed an extra peak at the level of 75-bp and 85-bp respectively, while 2 STR resistant isolates showed 2 peaks with 9 bases difference. CFLP showed a different pattern among RIF, INH and STR sensitive and resistant isolates Thus SSCP and CFLP can be used as alternative diagnostic methods for identification of mutations in RIF, INH and STR resistant strains of M.tuberculosis.

KEY WORDS : Single strand conformation polymorphism, Cleavase fragment length polymorphism, *Mycobacterium tuberculosis*, Drug resistance.

INTRODUCTION

In the last 10 years there has been an increase in the number of cases of tuberculosis in many countries of the world including U.S.A with a significant increase among certain population groups. Factors contributing to this increase are HIV infection, homelessness, poverty, deterioration in the public health services and immigration from countries with high incidence of tuberculosis (1). Concurrent with the recent increase in the incidence of tuberculosis there has been a steady increase in drug resistance in *M. tuberculosis* with serious consequences in hospitals(2) and prisons especially in individuals infected with HIV(3) Important advances in the understanding of the molecular basis of drug resistance in M. tuberculosis have been achieved (4), particularly with regard to the association of resistance to isoniazid , deletion (5) and mutation (6) in the katG gene. Mutations also have been described in other genes such as rpoB gene encoding for the ß subunit of RNA polymerase associated with rifampicin resistance (7) ; rrs encoding for 16 S RNA gene and gene S 12 encoding for ribosomal protein associated with streptomycin resistance (8). Understanding of the molecular basis of drug resistance has contributed to the development of rapid diagnostic tests. A single strand (SS) DNA molecule reassociates itself forming hairpin structure with characteristic secondary structure depending on the nucleotide sequence. The presence of a mutation leads to the formation of a different secondary structure that can be detected by the rate of mobility in a gel, which is the basis of SSCP analysis (9). Mutations in rifampicin-resistant *M.tuberculosis* isolates have been detected using a line probe assay (10). Recently cleavase analysis for detecting mutations in *M. tuberculosis* KatG genes has been described (11) .In the present study we applied SSCP and CFLP analysis to the rapid identification of genes encoding resistance to INH, RIF and STR.

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MATERIALS AND METHODS

Twenty isolates of drug resistant *M.tuberculosis* were obtained from the British Columbia Centre for Disease Control (BCCDC). All samples were tested for drug-susceptibility using the radiometric method BACTEC 460 (Becton Dickinson, Mississauga, Ontario) with isoniazid concentrations of 0.1 mg/ml, rifampicin 2mg/ml and streptomycin 2mg/ml. Three large full loops of mycobacteria culture grown in solid Middlebrook media were harvested and resuspended in 500 µl TE buffer (10 mM Tris Hcl, 1 mM EDTA, pH 8.0). The samples were heat inactivated for 30 min at 95 °C . An equal volume of chloroform was added, and the samples were mixed by rotation for 20 min. The tubes were placed in an ultrasonic cleanser (Bransonic, Branson Cleaning Equipment, Shelton CT) at 65 °C for 20 min. The samples were centrifuged in a microcentrifuge for 1 min. The aqueous phase was recovered and the DNA concentration was determined using a mini fluorometer TKO 100 (Hoefer scientific Instruments, San francisco CA).

Oligonucleotide synthesis

Oligonucleotide primers katG 1 5'-GCCCGAGCAACACCC-3' and katG 2 5'-ATGTCCCGCGTCAGG-3' from the katG gene from *M.tuberculosis* with GenBank accession number S42739; primers rif 139 5'-ATCCGGCCGGTGGTCGC-3' and rif 296 5'-CGGGTGCACGTCGCGGA-3' for rpoB gene (ß subunit of RNA polymerase) with GenBank accession number L05910 and primers m16S309 5'-GGCAGCAGTGGGGAATA-3' and m16S658 5'-ACCGGTGTTCCTCCTGA-3' from the rrs gene that codes for 16S RNA with GenBank accession number X52917 were selected and synthesized at UBC Biotechnology Laboratory. Sequencing primers were synthesized and labeled at the 5' end with fluorescein amidite (FluorePrime, Pharmacia LKB Biotechnology Uppsala, Sweden). Primers were purified by HPLC in a Pep S reverse phase column (Pharmacia).

DNA amplification

Polymerase chain reaction (PCR) was done in 25 µl volume reaction mixture containing (final concentrations) 1.25 mM $MgCl_2$, 25 mM KCl, 10 mM Tris-HCl pH 8.3, 0.01% gelatine, 20 mM of each of the four d NTPs, 20 mM of each of the 2 primers, 3 ng of template DNA, and 1 unit of Taq DNA polymerase (BRL), covered with 15 µl mineral oil. Amplification was done for 35 cycles with denaturing at 94 °C for 1.5 min, annealing at 68 °C for katG, 70°C for rpoB and 57 °C for rrs and extension at 72°C for 1 min.

Gel electrophoresis

Ten percent of the PCR reaction was analysed by electrophoresis in a 2 % agarose gel using a 100 bp DNA marker ladder (Pharmacia). Gels were stained with ethidium bromide and photogragphed under a 300 nm UV transilluminator (Fotodyne, New Berlin, Wisconsin). Positive amplification was identified by detection of 237 bp fragment for katG, 174 bp for rpoB and 366 bp for 16S RNA rrs gene.

DNA sequencing

PCR products were purified in a QIA quick-spin PCR purification column (QIAGEN, Chatsworth, CA). Both strands of the PCR product were sequenced using fluorescein labeled primers and cycle sequencing reactions (Autocycle Sequencing Kit, Pharmacia) in the presence of Thermus thermophilus DNA polymerase in the Perkin-Elmer Cetus DNA Thermal cycler. The cycles were : 94 ° C for 1 min, annealing at 68 ° C for katG, 70 ° C for rpoB and 57 ° C for rrs for 1 min, extension at 72 ° C for 1.5 min for 17 cycles and then 13 cycles of denaturing at 94 ° C for 1 min and extension at 72 ° C for 1.5 min. The sequencing reactions were analysed in an Automatic Laser Fluorescent (ALF) DNA sequencer from Pharmacia (Uppsala, Sweden).

Single strand conformation polymorphism

PCR amplification of the isolates was performed as above except that 6.26 µM of fluorescein-11-dUTP (FdUTP, Pharmacia) was added to the PCR reaction. The PCR products were purified in a QIA quick-spin PCR purification column. The purified PCR products were denatured at 95 ° C for 5 minutes prior to analysis on a 6 % acrylamide, 7 M urea gel, using a 50-500 bp fluorescein labeled DNA ladder marker in the ALF DNA sequencer (Pharmacia). A fluorogram was obtained at 40 ° C.

Cleavase Fragment Length Polymorphism (CFLP)

CFLP reactions were prepared using the CFLP Evaluation Kit Polymorphism Detection System (Third Wave Technologies, Madison WI). PCR reactions were made in the presence of 6.25μM FdUTP. The PCR products were purified in a QIA quick spin column and then were treated with 50 Units of Exonuclease I (USB Cleveland OH) at 37 ° C for 15 min. The reaction was heat inactivated at 70 ° C for 15 min and precipitated with 25 μl of 4M ammonium acetate and 50 μl isopropanol. The sample was incubated at room temperature for 10 min and then centrifuged in the microcentrifuge for 10 min. The pellet was washed 2 times with 70 % ethanol. DNA was quantitated and diluted to 4 ng/ μl. Twenty nanograms of DNA in 5 μl and 7 μl mineral oil were added for incubation at 95 ° C for 10 sec and then cooled to 55 ° C. A master mix containing, 2 μl of 10X CFLP buffer 100 mM MOPS, pH 7.5, 0.5 % Tween-20. 0.5 % Nonidet P-40), 2 μl of 2mM $MnCl_2$ and 1 μl of Cleavase (25 units) was added to the DNA and incubated at 60 ° C for 4 min. The reaction was terminated by adding 5 μl of sequencing stop solution (95 % formamide, 20 mM EDTA and 10 mg/ml Dextran Blue 2000). The reaction was incubated at 95 ° C for 5 min and then placed on ice before loading 10 μl to a 6 % acrylamide, 7M urea denaturing gel for electrophoresis and detection in the ALF DNA sequencer (Pharmacia). A fluorogram was obtained at 40 °C.

Computer analysis

Multiple sequence alignments were made with the program Clustal V ; a phylogenetic tree was constructed using the DeSoete Tree fit; secondary structure of the SS DNA was studied with the program M FOLD-RNA secondary prediction; all these programs were implemented in genetic Data Environment. The fluorograms were analysed with the fragment Manager software (Pharmacia).

RESULTS

Four resistant and 3 rifampicin sensitive isolates were analysed by PCR amplification and a 174-bp fragment was obtained from the rpoB gene. The presence of the katG gene in 14 INH-resistant and 3 INH-sensitive isolates from *M.tuberculosis* was confirmed by PCR amplification of a 237- bp fragment of the 5' end (position 3-239) of the katG gene. Two streptomycin resistant isolates and one sensitive isolate produced the expected 366-bp fragment of the rrs gene. Using fluorescence-based-PCR-SSCP, it was possible to distinguish RIF, INH and STR resistant isolates from sensitive isolates (Figure 1.). All resistant RIF isolates showed an extra peak at the level of 75-bp rather than a single 174- bp characteristic of RIF- sensitive isolates. The fluorescein labelled PCR product from the INH-sensitive isolates was detected as a single peak at the level of the 237- bp; the 14 samples from INH-resistant isolates showed an extra peak located at the level of the 85 bp. SSCP analysis was able to

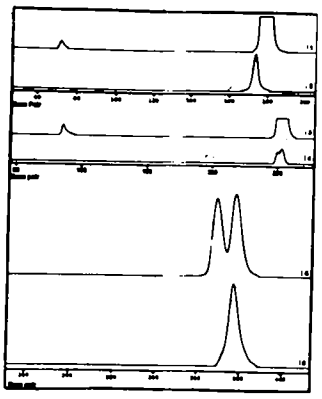

Figure 1. SSCP analysis. *M.tuberculosis* isolates 1-2 RIF; 3-4 INH; 5-6 STRE. 1,3 and 5 resistant strains. 2, 4 and 6 sensitive strains. The rifampicin-resistant isolate shows an extra band located in the 75-bp.(1) In the INH-resistant strain the extra peak is located at 85-bp (3) and in STR there are 2 peaks with 9 bases difference (5).

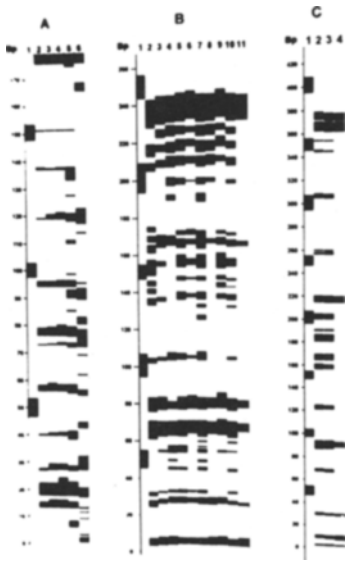

Figure 2. CFLP analysis of *M.tuberculosis* isolates. Line 1; DNA marker. RIF-sensitive isolate is located in frame A, line 6; INH-sensitive in frame B, line 11 and STR-sensitive in frame C, line 4. Resistant isolates show different CFLP pattern to the sensitive isolates. The presence of a 95bp band in lines 2-5 and a 110-bp band in line 5 that correlate with mutations corresponding to amino acid number 526 and 531 of the whole sequence. INH-resistant isolates in lines 2-10 show a more complex pattern than the sensitive isolates. STR-resistant isolates in line 2-3 show a band of 166-bp that correlates with a mutation.

differentiate between two sreptomycin resistant isolates and a sensitive one. The sensitive isolate showed a single peak at 366 bp and the resistant had two peaks with 9 bases difference.

DNA sequence of the 174- bp amplified portion of the rpoB gene showed a mutation in base 95 (A to G) corresponding to amino acid number 526, (histidine to arginine) in all 4 resistant isolates, one of these isolates (r 2424) also had a mutation in base 110 (C to T), corresponding to amino acid 531 (serine to leucine). The DNA sequence of the INH-resistant cases showed mutations distributed at random in all 14 cases with 1 to 5 base mismatches. Alterations included point mutations, deletions and insertions up to two bases. In four isolates there was a mutation in amino acid 65 (alanine to serine). The phylogenetic analysis showed a cluster of six isolates with small differences from the INH-sensitive isolates; there were two, samples with more

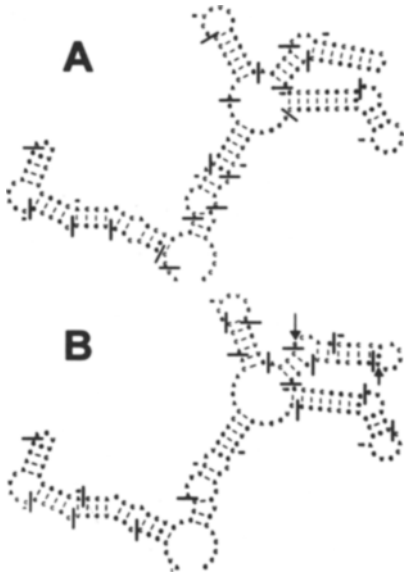

Figure 3. Secondary structure of rpoB gene. A; rifampicin-sensitive. B; Rifampicin-resistant. Inside the frame are shown differences in the secondary structure. Mutations in RIF-resistant are indicated by arrows. Bars represent cutting places by Cleavase as shown in figure 2.

mutations and were located distant to the reference isolate (data not shown).

CFLP analysis showed a different pattern among rifampicin, INH and streptomycin-sensitive and resistant isolates (Figure 2). The rpoB sequences of rifampicin-sensitive and resistant isolates have very similar secondary structures with some differences at the level of the position of the mutations present in the rifampicin-resistant isolates (Figure 3). Cleavase cuts the single stranded DNA molecule at the beginning or at the end of the hairpin structure. Inside a hairpin, Cleavase cuts when there is no base pairing, or if there is a mismatch (G-T). We found that Cleavase recognised a single mutation localised at position 95 of our sequence (amino acid 526 of the whole sequence) in isolates r2418, r2422 and r2430. Cleavase also recognised the location of two mutations in isolate r2424 associated with rifampicin resistance. These mutations were located in position 95 and 110 of our sequence corresponding to amino acid 526 and 531 of the whole sequence (Figures 2-3).The secondary structure of the katG gene sequence from INH-resistant isolates showed great variability when compared with pattern from the INH-sensitive isolates (data not shown); this variability correlated with the difference in patterns observed in the CFLP analysis

(Figure 2). Streptomycin-resistant isolates showed a band in position 166bp that correlated with a mutation A to C in position 513 of the original description (8)

DISCUSSION

Important advances in the understanding of molecular basis of drug resistance in *M.tuberculosis* have been achieved (4,12)leading to the identification of 12 genes associated with drug resistance in this organism (13). Thus molecular basis of drug resistance has contributed to the development of rapid diagnostic tests. Nucleic acid assays for rapid susceptibility testing are based on the detection of differences between the genetic material of a resistant and a susceptible strain. It has been observed that SSCP is simple, accurate and suitable for analysis of a large number of samples. CFLP has similar characteristics and is also able to provide information on the location of single base mutation. We have compared the difference by amplifying portions of rpoB, katG and rrs genes (both sensitive and resistant) by DNA sequence, SSCP and CFLP. Each of these procedures gave specific information to make distinction. SSCP is a procedure based on the detection of changes in the rate of mobility of the SS DNA due to the presence of a single base mutation that produced conformational changes in the molecule. In our study PCR-SSCP of all the resistant RIF and INH isolates showed an extra peak at the level of 75-bp and 85-bp respectively. While, STR resistant isolate showed double peak with a difference of 9 bases.Isolates with abnormal SSCP could be the subject of further analysis including DNA sequencing and CFLP. CFLP analysis is based on the use of the enzyme Cleavase I that recognises and cleaves the SS DNA molecule at the level of a conformational change or the presence of mutations. The method is highly sensitive in that it is able to identify a single base mutation and generate series of DNA fragments with a characteristic pattern for the mutated (resistant) and non-mutated (sensitive) organism.. Analysis of CFLP certainly looks littlle more complicated than SSCP ; however , it may yield more information about the resistance gene. While the DNA sequencing is highly accurate method for detection of mutations associated with drug resistance in tuberculosis but is more expensive and requires special equipment and personnel. Thus SSCP and CFLP are simple and sensitive methods, suitable for analysis of large number of samples and the results are available in less than 72 hours.

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