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Molecular Biological Techniques for Detection of Multidrug Resistant Tuberculosis (MDR) and Extremely Drug Resistant Tuberculosis (XDR) in Clinical Isolates of *Mycobacterium tuberculosis*

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1. Introduction

Tuberculosis (TB) is a contagious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). When people infected with tuberculosis cough, sneeze, talk or spit, the bacilli are propelled into the air. Each person with active TB disease will infect on average between 10 and 15 people every year. But people infected with TB bacilli will not necessarily become sick with the disease. The immune system "walls off" the TB bacilli which, protected by a thick waxy coat, can lie dormant for years. When the immune system is weakened, the chances of the infection progressing to disease are higher.

Overall, one-third of the world's population is currently infected with the TB bacillus. 5 - 10% of people who are infected with TB bacilli (but who are not infected with HIV) become sick or infectious at some time during their life. People with HIV and TB infection are much more likely to develop TB [WHO, 2010].

Globally, there were an estimated 14 million prevalent cases of TB in 2009, equivalent to 200 cases per 100 000 population. Most of the estimated number of cases in 2009 occurred in Asia (55%) and Africa (30%); 3 smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and the Region of the Americas (3%) [WHO, 2010]. In 2009, an estimated 1.3 million deaths (range: 1.2 – 1.5 million) occurred among HIV-negative cases of TB, including 0.38 million deaths (range: 0.3–0.5 million) among women. This is equivalent to 20 deaths per 100 000 population [WHO, 2010].

Until 50 years ago, there were no medicines to cure TB. Now, strains that are resistant to a single drug have been documented in every country surveyed and strains of TB resistant to all major anti-TB drugs have emerged. Drug-resistant TB is caused by inconsistent or partial treatment, when patients do not take all their medicines regularly for the required period because they start to feel better, or the doctors and health workers prescribe the wrong treatment regimens, or the drug supply is unreliable. A particularly dangerous form of drug-resistant TB is multidrug-resistant TB (MDR-TB), which is defined as the disease

caused by TB bacilli resistant to isoniazid and rifampicin, the two most powerful anti-TB drugs. Rates of MDR-TB are high in some countries, especially in the former Soviet Union, and threaten TB control efforts [WHO, 2010].

While drug-resistant TB is generally treatable, it requires extensive chemotherapy (up to two years of treatment) with second-line anti-TB drugs which are more costly than first-line drugs, and which produce adverse drug reactions that are more severe, though manageable. Quality assured second-line anti-TB drugs are available at reduced prices for projects approved by the Green Light Committee [WHO, 2010].

The emergence of extensively drug-resistant (XDR) TB, particularly in settings where many TB patients are also infected with HIV, poses a serious threat to TB control, and confirms the urgent need to strengthen basic TB control and to apply the new WHO guidelines for the programmatic management of drug-resistant TB.

The important ramifications in the laboratory diagnosis of tuberculosis and drug resistant tuberculosis are: the delay in the isolation of the bacilli in culture, low sensitivity/detection limit of the direct smears and lack of technically trained personnel. Identification of tuberculosis using molecular techniques namely, polymerase chain reaction (PCR), PCR based restriction fragment length polymorphism (PCR-RFLP) and PCR based DNA sequencing are very rapid, more sensitive and reliable when compared to the conventional culture. There are several in house nested PCR (nPCR) standardized for the detection of *M. tuberculosis* from clinical specimens targeting MPB64 [Therese KL et al, 2005], IS6110 [Wang et al, 2004] and 16S rRNA [Ninet et al, 1996] genes. MPB64 is an immunogenic protein produced by *M. tuberculosis* and a few strains of *M. bovis* and BCG strain. IS6110 is an insertion element present in single or multiple copies in *M. tuberculosis* complex isolates. There are studies which reported on the lack of IS6110 insertion element in strains isolated from Asian population. Also there are strain variations among the *M. tuberculosis* isolates. Thus multiplex PCR or nPCR targeting more than one gene target will be a better tool for the detection of *M. tuberculosis* from direct clinical specimens.

Drug resistance in *M. tuberculosis* is caused by mutations in relatively restricted regions of the genome. Mutations associated with drug resistance occur in *rpoB* for rifampicin (RIF) *katG* and the promoter region of the *mabA* (*fabG*1)-*inhA* operon for Isoniazid (INH), *embB* for ethambutol (EMB), *pncA* for pyrazinamide (PZA), *rpsL* and *rrs* for streptomycin (STR) and *gyrA, gyrB* for fluoroquinolones (FQs) such as ofloxacin (OFX) and levofloxacin (LVX) [Musser et al, 1995; Zhang et al, 2000].

2. MDR-TB

2.1 Worldwide reports on MDR-TB

In 2008, there were an estimated 440,000 (range, 390 000-510 000) MDR-TB cases emerging worldwide. About 250,000 of these cases (range: 230 000–270 000) should have been reported to WHO, if countries had tested all the TB patients that they notified for drug resistance. However, only just over 30,000 MDR-TB cases (12%) were actually notified globally in 2009 [WHO, November 2010].

Almost 50% of MDR-TB cases worldwide are estimated to occur in China and India. In 2008, MDR-TB caused an estimated 150,000 deaths. Since 1994, 114 countries have reported

surveillance data on MDR-TB: 42 perform continuous surveillance of anti-TB drug resistance based on routine testing of all TB patients; 72 rely on periodic surveys of representative samples of TB patients. The Russian Federation, which was able to provide high-quality continuous surveillance data from 12 of its oblasts and republics, reported proportions of 23.8–28.3% MDRTB among new TB cases in three of its oblasts in the northwest part of the country. Other Russian oblasts were found to have proportions of MDR-TB as low as 5.4% among new TB cases. Tajikistan, in its first ever survey, found proportions of 16.5% MDR-TB among new TB cases and 61.6% MDR-TB among previously treated TB patients in Dushanbe city and Rudaki district, the highest proportion ever reported among previously treated TB patients. To date, 12 countries have reported nationwide or subnational proportions of MDR-TB of 6% or more among new TB cases [WHO, November 2010].

Five of these countries also report MDR-TB proportions of 50% or more among previously treated cases. All of these settings are located in the eastern part of Europe or in Central Asia.

2.2 Recently developed molecular techniques for the detection of drug resistance in tuberculosis

2.2.1 Solid-phase Hybridization techniques

There are currently two commercially available solid-phase hybridization techniques: the Line Probe Assay (INNO-LiPA Rif TB Assay; Innogenetics, Ghent, Belgium) for the detection of rifampicin resistance and the GenoType MTBDR assay (Hain Lifesciences, Nehren, Germany) for the simultaneous detection of isoniazid and rifampicin resistance. The LiPA assay was introduced several years ago and is based on the hybridization of amplified DNA from cultured strains or clinical samples to 10 probes covering the core region of the *rpoB* gene of *M. tuberculosis*, immobilized on a nitrocellulose strip. The GenoType MTBDR on the other hand, detects resistance to isoniazid and rifampicin in culture samples based on the detection of the most common mutations in the *katG* and *rpoB* genes respectively. Both assays have now been evaluated in different settings, giving encouraging results. In a recent study Hillemann *et al* evaluated the GenoType MTBDR assay and found that 99% of MDR strains with mutations in the *rpoB* gene and 88.4% of strains with mutations in the codon 315 of the *katG* gene were correctly identified. Correlation with DNA sequencing was 100% and compared with conventional tests good sensitivity and specificity were also obtained. Both solid hybridization methods have shown to be relatively simple to perform although basic expertise in molecular biology and PCR techniques is required. As with other genotypic methods the sensitivity of the test depends on the amount of DNA present in the sample and also the presence of inhibitors could cause false-negative results.

INNOLiPA Rif. TB kit: INNOLiPA Rif. TB kit simultaneously detects the *M. tuberculosis* complex and the presence of mutations in the *rpoB* gene associated with resistance to rifampicin which is considered a marker for MDR-TB strains. The strip contains 5 probes for detection of sensitive genotypes (S1-S5) [Morgan et al, 2005; Makinen et al, 2006] and 4 probes for detection of resistance genotypes (R2,R4a, R4b and R5). Rifampicin resistance is indicated by the absence of one or more sensitive probes, possible accompanied by the appearance of one or more mutant probes.

The **GenoType MTBDR** test is able to detect mutations in the *rpoB* gene for RIF resistance, and the most frequent mutation at codon 315 of the *katG* gene for INH resistance, either in isolates or clinical specimens. The specificity and sensitivity of the assay for RIF resistance were nearly 100%; for INH-resistance, despite a high specificity (approximately 100%), the sensitivity of the test ranged from 70% to 90%, depending on the prevalence of the particular mutation at the *katG* locus [Hilleman et al, 2007].

GenoType MTBDRplus (Hain Lifescience, Germany), an advanced version of the assay, includes probes for the identification of other mutations in the hotspot region of the *rpoB* gene for RIF resistance, and probes to detect mutations in the promoter region of the *inhA* gene involved in INH resistance. These improvements facilitate the detection of another 10% to 20% of INH-resistant cases, with an enhancement in rapid MDR-TB diagnosis.

The line probe assays are accurate and useful for rapid detection of drug resistance directly in clinical specimens. However, the number of genes that can be analyzed remains limited and the test fails to distinguish insertion mutations. Furthermore, they retain a lower sensitivity among acid fast bacilli-negative samples. In general, line probe assays are expensive and require sophisticated laboratory infrastructure. Their role and utility in low-income, high-burden countries needs to be evaluated in field studies.

2.2.2 Real-time Polymerase Chain Reaction techniques

Real-time PCR techniques have also been introduced for rapid detection of drug resistance. Different probes have been used like the TaqMan probe, fluorescence resonance energy transfer (FRET) probes, molecular beacons and bioprobes. The main advantages of real-time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be the requirement for expensive equipment and reagents, and the need for skilled technical personnel. Real-time PCR was initially applied to *M. tuberculosis* strains but more recently it has been successfully applied directly in clinical samples. Results could be obtained in an average of 1.5-2.0 h after DNA extraction. Real-time PCR could eventually be implemented in reference laboratories with the required capacity to properly set up the technique and in settings where it can contribute to the management of TB patients.

Detection of antitubercular drug resistance is vital to effective patient management. Real-time PCR offers the potential to detect gene mutations responsible for drug resistance within hours from patient specimens compared with the average of 2 weeks required for traditional susceptibility test methods. The *rpoB* and *katG* genes are the most common *M. tuberculosis* targets utilized in real-time PCR methods and well-known mutations in these genes correlate with resistance to rifampin and isoniazid, respectively [Edwards et al, 2001; El-Hajj et al, 2001; Garcia de Viedma et al, 2002; O'Mahony et al, 2002; Piatek et al, 2002; Torres et al, 2000; Torres et al, 2003; van Doorn et al, 2003]. The significance of other gene targets such as *kasA*, *ahpC-oxyR*, and *inhA* for the prediction of isoniazid resistance is somewhat controversial [Piatek et al, 2000]. Torres et al used two sets of FRET hybridization probes to detect *rpoB* mutations in 24 rifampin-resistant strains *of M. tuberculosis* and another set of FRET hybridization probes to detect *katG* mutations in 15 isoniazid-resistant *M. tuberculosis* strains [Torres et al, 2000]. Additionally, Garcia de Viedma et al. used two sets of *rpoB* probes and one set of *katG* probes to detect *rpoB* and *katG* mutations, but in a single tube, for

29 resistant *M. tuberculosis* isolates [Garcia de Viedma et al, 2002]. Since not all gene mutations conferring drug resistance are well characterized and are thus not amenable to PCR assay development, traditional culture-based susceptibility testing methods are still required. However, the ability to predict rifampin and isoniazid resistance up to 2 weeks sooner than current methods for some isolates should have significant benefit for patient care.

2.2.3 Microarray

An array is an orderly arrangement of samples where matching of known and unknown DNA samples is done based on base pairing rules [Heyman et al, 1999]. An array experiment makes use of common assay systems such as microplates or standard blotting membranes. The sample spot sizes are typically less than 200 microns in diameter usually contain thousands of spots. Thousands of spotted samples known as probes (with known identity) are immobilized on a solid support (a microscope glass slides or silicon chips or nylon membrane [Heyman et al, 1999]. The spots can be DNA, cDNA, or oligonucleotides. These are used to determine complementary binding of the unknown sequences thus allowing parallel analysis for gene expression and gene discovery. An experiment with a single DNA chip can provide information on thousands of genes simultaneously. An orderly arrangement of the probes on the support is important as the location of each spot on the array is used for the identification of a gene [Heyman et al, 1999].

Microarray technology is used in the detection of drug resistant *M. tuberculosis* rather than the detection of *M. tuberculosis* from clinical specimens. The TB Biochip oligonucleotide microarray is the most widely used Microarray for the detection of isoniazid and rifampicin resistance simultaneously [Garcia de Viedma D et al, 2003].

TB-Biochip oligonucleotide microarray for the detection of rifampicin resistant *M. tuberculosis*

The TB-Biochip oligonucleotide microarray system is designed to detect and identify 29 codon substitutions and 1 codon deletion distributed over 10 codon positions (507, 511, 512, 513, 515, 516, 522, 526, 532, 533) within the rifampicin resistant determining region (RRDR). Each element of the microarray contains an immobilized oligonucleotide whose sequence matches that of either a wild-type or mutated segment of the RRDR. The use of acrylamide gel pads increases the robustness of the hybridization reaction. Hybridization of the microarray with fluorescently labeled target DNA produces a spatial pattern of fluorescence intensities corresponding to the efficiencies of hybridization of the labeled target DNA to the various oligonucleotide probes.

In the TB-Biochip system, the fluorescence intensities are recorded using a charge-coupled device camera, and the relative intensities of fluorescence for the elements representing wildtype sequences and mutant sequences for each codon are compared using imaging software and automated computer-assisted interpretation of hybridization results [Garcia de Viedma D et al, 2003; Cavusoglu et al, 2004]. The isolate is designated **RIF susceptible** if the fluorescence of each of the wild-type elements is greater than the fluorescence of any of the corresponding mutant elements. The isolate is designated **RIF resistant** if the fluorescence of any one of the mutant elements is greater than the fluorescence of its corresponding wild-type element.

Advantange and disadvantage of TB- biochip system

The complete TB-Biochip system may be suitable for use in clinical laboratories with molecular biology expertise because it requires relatively little hands-on time for experimental manipulations or data analysis, tests can be run individually or in batches, and specialized training is not required. The observed discrepancies between the results of conventional DST and the TB-Biochip system (all were falsely called susceptible with the TB-Biochip system) likely result from the large number of mutations found in RIF-resistant isolates and the limited range of mutations included on the biochip.

Although technically a solid-phase-type hybridization assay, microarrays, also known as biochips, have been proposed as new molecular methods for detecting drug resistance in *M. tuberculosis*. They are based on the hybridization of DNA obtained from clinical samples to oligonucleotides immobilized in a solid support, such as miniaturized glass slides. They have been mainly used to detect resistance to rifampicin. In a recent evaluation using oligonucleotide microarrays for analysis of drug resistance, Gryadunov *et al*. detected over 95% rifampicin resistant and almost 80% isoniazid resistant *M. tuberculosis* isolates within 12 h in a sample of drug-resistant isolates and clinical samples. For the time being and due to the high cost involved, the use of microarrays for detecting drug resistance in TB is still beyond the reach of most clinical mycobacteriology laboratories.

2.3 DNA sequencing

Sequencing DNA of PCR amplified products has been the most widely used method; it is accurate and reliable and it has become the gold standard for mutation detection. It has been performed by manual and automated procedures although the latter is now the most commonly used. It has been widely used for characterizing mutations in the *rpoB* gene in rifampicin-resistant strains and to detect mutations responsible for resistance to other anti-tuberculosis drugs. It would be rather difficult, however, to implement it routinely for detection of drug resistance mutations for several drugs since it would involve several reactions for each isolate, making the cost high.

2.3.1 DNA extraction

DNA from clinical *M. tuberculosis* isolates can be extracted by using Qiagen kit (Germany) and also by keeping the MGIT suspension at 80˚C for 10 minutes. After 10 minutes, centrifuged at 3000 rpm and the supernatant can be used as template DNA for PCR. The procedure for extraction of DNA using Qiagen kit is mentioned below.

Reagents and other accessories required

a. Proteinase K
b. Lysis buffer (AL buffer)
c. Ethanol
d. Washing buffer-1 (AW1 buffer)
e. Washing buffer-2 (AW 2 Buffer)
f. Elution buffer (AE Buffer)
g. Minispin (eppendorff)
h. Sterile 1.5ml vials

i. Micropipettes (20-200µl)
j. Filter barriers tips (20-200µl)

Procedure

- Pipette 20µl QIAGEN Proteinase k into the bottom of a 1.5ml microfuge tube.
- Add 200µl of the sample to the microfuge tube. Use up to 200µl whole blood, plasma, serum, Buffy coat upto 5 X 10^6 lymphocytes in 200µl PBS
- Add 200µl of AL buffer to the sample. Mix by pulse vortexing for 15 seconds
- Incubate at 56° C for 10 minutes.
- Briefly centrifuge the micro centrifuge tube to remove drops from inside of the lid.
- Add 200µl of ethanol (96-100%) to the sample, mix by pulse vortexing for 15 seconds. After mixing, briefly centrifuge the 1.5ml microfuge tube to remove drops from inside of the lid.
- Carefully apply the mixture from step 6 to the QIAamp mini spin column (in a 2ml collecting tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for 1 min. Place the QIAamp mini spin column in a clean 2 ml collecting tube and discard the tube containing the filtrate.
- Carefully open the QIAamp mini spin column and add 500µl buffer AW1 without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1min. Place the QIAamp mini spin column in a clean 2ml collecting tube and discard the tube containing filtrate.
- Carefully open the QIAamp mini spin column and add 500µl buffer AW2 without wetting the rim. Close the cap and centrifuge at 14,000 rpm for 3min, followed by an empty spin at 14,000 rpm for 1min.
- Place the QIAamp mini spin column in a clean 1.5 ml microfuge tube and discard the tube containing filtrate. Carefully open the QIAamp mini spin column and add 200µl AE Buffer. Incubate at room temperature for 1min, and then centrifuge at 8,000 rpm for 1min. Discard the column and store the DNA at -20°C.

2.3.2 PCR protocol

Reagents and other accessories required:

- Stock dNTPs dilution: 100 mM concentration of dNTPs- dATP, dCTP, dTTP and dGTP
- Working standard dNTP (200µM): 2 µl of each of the stock dNTP made up to 400 µl using MilliQ water.
- Forward and reverse primers
- *Taq* DNA polymerase (3 units)
- PCR Thermal cycler
- Cyclomixer
- Sterile 0.5 ml or 0.2 ml vials
- Micropipettes (20-200µl, 0.5- 10µl)
- Filter barrier tips (20-200µl)
- Gel casting tray/trough
- Gel combs
- Electrophoresis tank
- Powerpack
- Gel documentation system

The PCR cocktail contained the following:

dNTP	8 µl
10X buffer (15mM Mg2+,Tris, Kcl(500 mM)-pH 8.3)	5 µl
Forward Primer (1 pM)	1 µl
Reverse Primer (1 pM)	1 µl
MilliQ water	30 µl
Taq polymerase	0.3 µl

2.3.3 PCR protocol for amplification of *embB* gene using primers targeting 640-1577 region

PCR targeting *embB* gene was standardized with a specialized Taq DNA polymerase enzyme called "Z Taq" enzyme (Takara Bio, Ohtsu, Shiga, Japan). The *Z-Taq* polymerase offers unmatched PCR productivity, with a processing speed five times faster than those of other commercially available *Taq* polymerases. The total PCR cycle takes only 29 minutes. All the reagents for PCR (dNTP, 10X, Z Taq) will be provided along with the buffer. Each 50 µl reaction contained 2.5mM dNTP, 10X, 1pM of forward and reverse primers and 2.5 Units of Z taq enzyme. The details of primer targeting drug resistance genes, their thermal profile used and the expected amplicon size are given in the Table2.

Detection Of Amplified Products: The amplified product is subjected to electrophoresis on 2% agarose gel incorporated with 0.5 µg/ml ethidium bromide for visualization by UV transilluminator (Vilber Lourmat – France).

2.3.4 DNA sequencing of amplified products

The term **DNA sequencing** refers to sequencing methods for determining the order of the nucleotide bases—adenine, guanine, cytosine, and thymine—in a molecule of DNA. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of all: the sequence of nucleotides. The classical chain-termination method requires a single-stranded DNA template, a DNA primer, a DNA polymerase, radioactively or fluorescently labeled nucleotides, and modified nucleotides that terminate DNA strand elongation. *Dye-terminator sequencing* utilizes labelling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than four reactions as in the labelled-primer method. In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labelled with fluorescent dyes, each of which with different wavelengths of fluorescence and emission. Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing. Its limitations include dye effects due to differences in the incorporation of the dye-labelled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequence trace chromatogram after capillary electrophoresis. This problem has been addressed with the use of modified DNA polymerase enzyme systems and dyes that minimize incorporation variability, as well as methods for eliminating "dye

Target genes / Primer sequence (5′-3′ Direction)	**Thermal profile**	**No. of Cycles**	**Expected Amplicon Size (bp)**
rpoB for rifampicin CCACCCAGGACGTGGAGGCGATCACAC AGTGCGACGGGTGCACGTCGCGGACCT	95 °C-5 min 95°C-30sec 72°C-1min 72°C -5 min	35	286
katG1 for isoniazid GCCCGAGCAACACCC ATGTCCCGCGTCAGG	94˚C - 1min 58˚C –1min 72˚C –2 min	35	237
katG2 for isoniazid CGAGGAATTGGCCGACGAGTT CGGCGCCGCGGAGTTGAATGA	94˚C - 1min 55˚C –1min 72˚C –2 min	35	414
katG3 (targeting 138 codon) for isoniazid CCGGCACCTACCGCATCCAC GCCCCAATAGACCTCATCGG	95˚C - 1min 60˚C –30sec 72˚C –1 min	30	269
katG4 (targeting 315 codon) for isoniazid GAAACAGCGGCGCTGGATCGT GTTGTCCCATTTCGTCGGGG	Same as above		209
inhA for isoniazid CCTCGCTGCCCAGAAAGG A ATCCCCCGGTTTCCTCCGGT	94˚C - 5min 94˚C - 1min 64˚C –1min 72˚C –2 min	40	248
oxyR-ahpC for isoniazid GCTTGATGTCCGAGAGCAT GGTCGCGTAGGCAGTGCCCC	94˚C - 2min 94˚C - 1min 60˚C –1min 72˚C –2 min	35	701
rpsL for streptomycin GGCCGACAAACAGAACGT GTTCACCAACTGGGTGAC	94˚C - 1min 94˚C - 1min 56˚C –1min 72˚C –1 min 72˚C – 7 min	35	505
rrs for streptomycin TTGGCCATGCTCTTGATGCCC TGCACACAGGCCACAAGGGA	94˚C - 1min 94˚C - 1min 56˚C –1min 72˚C –1 min 72˚C – 7 min	35	1140
pncA for pyrazinamide GGCGTCATGGACCCTATATC CAACAGTTCATCCCGGTTC	94˚C – 30 sec 60˚C – 30 sec 72˚C – 30 sec	35	670
embB for ethambutol CCGACCACGCTGAAACTGCTGGCGAT GGTGGGCAGGATGAGGTAGT	95˚C – 5 sec 55˚C – 10 sec 72˚C – 10 sec	35	937

Table 2. Primer sets used in the study to sequence the different loci of target genes with their thermal profile and expected amplicon sizes[Sekiguchi et al, 2007; Siddiqi et al, 2002; Sreevatsan et al 1997]

blobs". The dye-terminator sequencing method, along with automated high-throughput DNA sequence analyzers, is now being used for the vast majority of sequencing projects.

DNA sequencing involves the following steps,

- Amplification of specific sequence from DNA
- Electrophoresis of amplified products in 2% agarose gel
- Elution of amplified products
- Cycle sequencing
- Purification of extension products
- Sequence analysis

Gel elution (Qiagen DNA Elution kit)

1. The amplified product (30 µl) is run on 2% agarose gel
2. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
3. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg is approximately 100 µl).
4. Incubate at 50ºC for 10 mins in thermal cycler (or until the gel slice has completely dissolved with intermittent vortexing).
5. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to buffer QG without dissolved agarose).
6. NOTE: If the color of the mixture is orange or violet, add 10µl of 3 M Sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
7. Add one gel volume of Isopropanol to the sample and mix by repeated pipetting.
8. Place a MinElute column in a 2ml collection tube provided in a suitable rack.
9. Apply the sample to MinElute column, and centrifuge at 13,000 rpm for 1 minute.
10. Discard the flow through and place the MinElute column back in the same collection tube.
11. Add 500µl of buffer QG to the spin column and centrifuge at 13,000 rpm for 1 minute.
12. Discard the flow through, and place the MinElute column back in the same collection tube.
13. To wash, add 750µl of buffer PE to the MinElute column and centrifuge at 13,000 rpm for 1 minute.
14. Discard the flow through, and place the MinElute column for an additional 1 minute at 13,000 rpm.
15. Place the MinElute column into a clean 1.5ml micro centrifuge tube.
16. To elute DNA, add 10µl of buffer EB (10mM Tris.Cl, pH 8.5) or water (pH 7.0-8.5) to the centre of the membrane, let the column stand for 1 minute, and centrifuge at 13,000 rpm for 1 minute.
17. Store the eluted products at -20 ºC.

2.3.4 Cycle sequencing

Cycle sequencing combines amplification and enzymatic DNA sequencing using 5′ dye labeled terminators.

Requirements for Cycle Sequencing

- Forward primer or Reverse primer at the concentration of 1 picomole/µl each.

- Big Dye Terminator cycle sequencing Ready reaction kit (ABI prism, USA)

Reaction Protocol

- Ready reaction mix (2.5X RR mix) - 2.0µl
- 5x sequencing buffer - 1.0µl
- Forward/Reverse primer (1pmol/µl) - 2.0 µl
- Milli Q water - 2.0 µl
- Amplified PCR product (eluted) - 2.0 µl

Reaction Condition for Cycle Sequencing

Initial Denaturation	- 96˚C for 1 minute.	
Denaturation	- 96˚C for 10 seconds.	25 cycles
Annealing	- 50˚C for 5 seconds.	
Extension	- 60˚C for 4 minutes.	
Holding Temp	- 4˚C	

Purification of Cycle Sequenced Product

The cycle sequenced products are purified to remove the unincorporated dye terminators before the samples are analyzed.

Reagents Required

- 500 mM EDTA
- 3M sodium acetate
- Chilled ethanol
- 70% ethanol

Procedure

- Take 0.5ml sterile PCR vial and add 10 µl of Milli Q water.
- Then add 2 µl of 125mM EDTA, followed by 10 µl of cycle sequenced product, 2 µl of 3M-sodium acetate (pH 4.6) and 50 µl of chilled ethanol. Vortex well and incubate at room temperature (22-28^{o}C) for 15 minutes
- Centrifuge at 12,000 rpm for 20 minutes.
- Pipette out the supernatant and wash the pellet twice with 250 µl of 70% ethanol at 12,000 rpm for 10 minutes. Care should be taken not to touch the sides of the eppendorf vial during pipetting.
- The vials are then dried at 37˚C (incubator) until ethanol completely evaporates. Presence of ethanol will prevent complete dissolving of DNA in formamaide.

2.3.5 Loading into DNA sequencer

Once the ethanol is completely dried, add 10 µl of formamide. This is denatured at 95˚C (Thermal cycler) for 3 minutes and immediately snap cooled in ice. The sequence of the PCR amplified DNA is deduced with the help of the ABI Prism 3100 AVANT (Applied Biosystems, USA) genetic analyzer that works based on the principle of Sanger dideoxy

sequencing. The amplified products with the dye at the terminated 3'end is subjected to capillary electrophoresis by an automated sample injection. The emitted flurorescence from the dye labels on crossing the laser area are collected in the rate of one per second by cooled, charge-coupled device (CCD) camera at particular wavelength bands (virtual filters) and stored as digital signals on the computer for processing.

2.3.6 Basic Local Alignment Search Tool (BLAST) analysis

The sequences are analysed by sequence analysis softwares such as Bio Edit sequence alignment software or Chromas software. BLAST analysis, using pubmed, http://www.ncbi.nlm.nih.gov/BLAST is done to confirm the sequenced data with the standard strains and to determine the percentage homology.

2.3.7 Multalin analysis

Multalin analysis (http://multalin.toulouse.inra.fr/multalin/) to be done to identify the presence of polymorphism or mutation by comparing with the reference strain from genbank (Accession No. L27989 for *rpoB*, U41314 for *katG*, MTU16243 for *inhA* and *oxyR-ahpC*, X70995 for *rrs* and *rpsL*, AY743320 for *pncA* and MTU68480 for *embB*). An example of multalin analysis targeting *rpoB* and *katG* are shown in the figure 1&2.

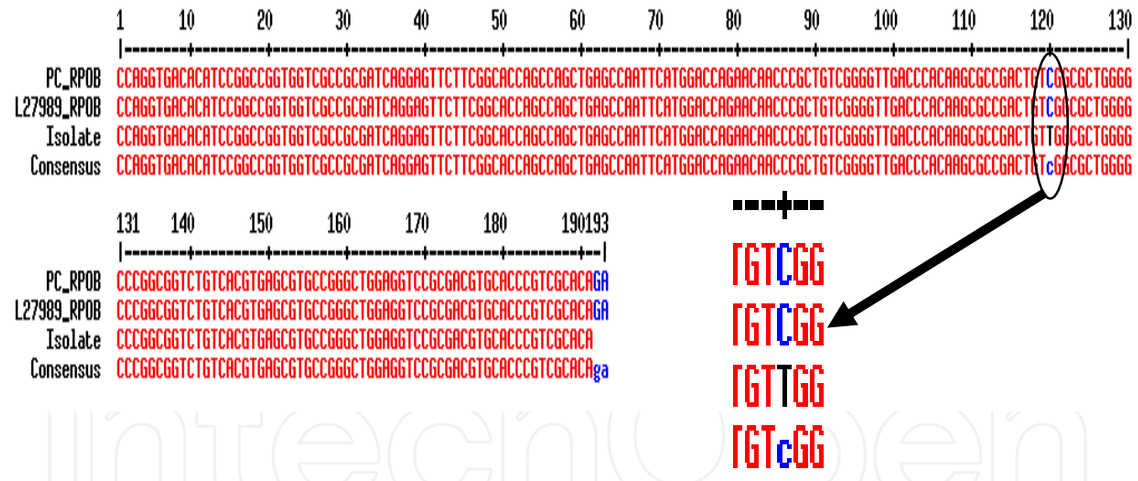

Fig. 1. An example of multalin result targeting *rpoB* gene using forward primer showing the presence of most commonly reported mutation TCG→TTG (Ser531Leu). Inset enlarged view of the mutation.

2.3.8 PCR based DNA sequencing for XDR-TB strains

If an MDR-TB strain has the above-mentioned mutations, it should be screened for resistance to Amikacin, capreomycin, kanamycin and the fluoroquinolones by PCR based DNA sequencing targeting *rrs, tlyA, thyA*, *gyrA* and *gyrB*. The details of primer targeting drug resistance genes, their thermal profile used and the expected amplicon size are given in the Table 3.

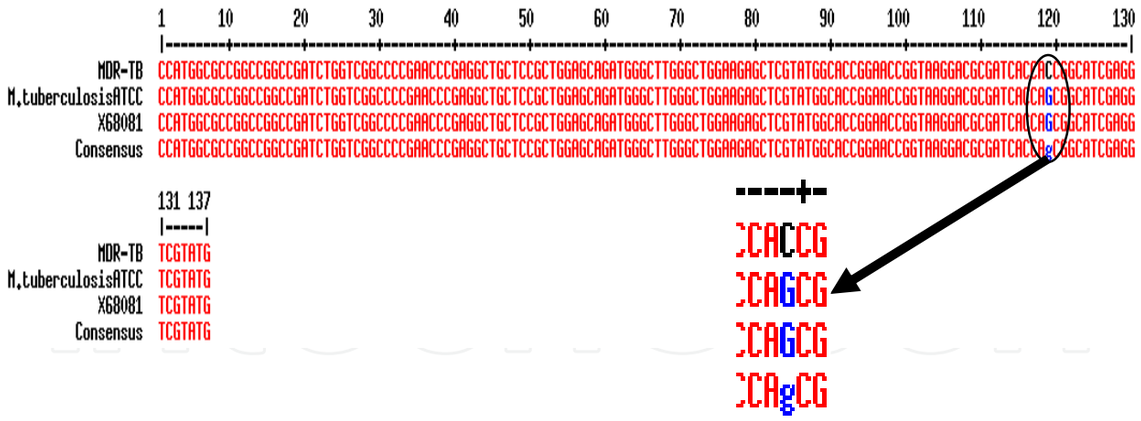

Fig. 2. An example of multalin result targeting *katG* gene using forward primer showing the presence of most commonly reported mutation A**GC**→ACC (Ser315Thr). Inset enlarged view of the mutation.

Target genes / Primer sequence (5′-3′ Direction)	**Thermal Profile**	**No. of Cycles**
thyA (Amikacin, capreomycin, kanamycin) ATCGTGTGCCCCATGGTGATCT CTCGGTGTATTCCCGTCGACT	95˚C – 1 min 60˚C – 1 min 72˚C – 1 min	30
tlyA(Amikacin, capreomycin, kanamycin) CATCGCACGTCGTCTTTC AATACTTTTTCTACGCGCCG	95˚C – 1 min 60˚C – 1 min 72˚C – 1 min	30
gyrA for Moxifloxacin,Ofloxacin, Ciprofloxacin CAGCTACATCGACTATGCGA GGGCTTCGGTGTTACCTCAT	94˚C – 1 min 52˚C – 1 min 72˚C – 1 min	40
gyrB for Moxifloxacin,Ofloxacin, Ciprofloxacin CCACCGACATCGGTGGATT CTGCCACTTGAGTTTGTACA	94˚C – 1 min 57˚C – 1 min 72˚C – 1 min	40

Table 3. Primer sets used in the study to sequence the different loci of target genes with their thermal profile and expected amplicon sizes [Sekiguchi et al, 2007; Siddiqi et al, 2002; Sreevatsan et al 1997]

For PCR protocol and DNA sequencing protocol, please refer to procedures 2.3.1 to 2.3.7.

3. Conclusion

The expectation that molecular techniques would surpass conventional methods for diagnosis of TB or phenotypic susceptibility testing has not yet been realized. The genetic basis of resistance must be understood before achieving such a goal. However, the clinician now has a variety of new tools to improve the diagnosis of TB and drug resistance. Most of them still require detailed and systematic evaluations using standard techniques as references before their widespread application in clinical settings. Most of these techniques require trained personnel and specialized equipment, hindering their application in field conditions, but they can be used in reference laboratories as part of the TB control programs.

The physician must be cautious when using results obtained by these techniques, especially when diagnosing drug resistance. Although it is not recommended, these molecular methods might be used as a complement to the standard methods in situation of difficult diagnosis, but never should be used solely to base such decisions.

4. Acknowledgement

The authors gratefully acknowledge the infrastructure facility and support provided by Vision Research Foundation, Chennai and for the financial support through a research grant by Chennai Willingdon Corporate Foundation, Chennai, India.

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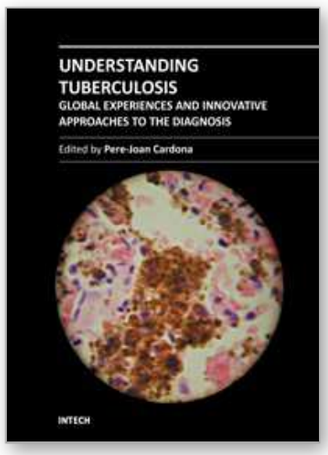

Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis

Edited by Dr. Pere-Joan Cardona

ISBN 978-953-307-938-7
Hard cover, 552 pages
Publisher InTech
Published online 15, February, 2012
Published in print edition February, 2012

Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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