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Review Article

Molecular Diagnostic Methods for Multidrug Resistance in Tuberculosis: A Review

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ABSTRACT

Tuberculosis (TB) has existed for millennia and remains a major global health problem. Novel technologies for rapid detection of anti-TB drug resistance have therefore become significant in TB research and development MDR- TB and XDR – TB is a persistent threat to the public health. Various molecular tests have been developed to detect mutations associated with resistance. The advantages of molecular methods of DST include rapid turnaround time, but the disadvantages include a low sensitivity for some compounds, however a major concern is cost. This leads to the need of rapid and robust diagnostic techniques to detect the drug resistance in TB. Though culture-based methods currently remain the reference standard for drug susceptibility testing, molecular diagnostic methods helps in prompt detection of the drug resistance, quicker diagnosis, surmounting the programmatic management and surveillance of drug-resistant TB, potential for high through put, and also have fewer requirements for ensuring laboratory biosafety. Line probe assay and Gene Xpert MTB/RIF assay are the most widely used methods and furthermore these techniques are endorsed by world health organization (WHO). Newer advances in these two technologies as SL-LPA and Ultra assay respectively also have promising future in the diagnostic field.

1. INTRODUCTION

Tuberculosis (TB) has existed for millennia and remains a major global health problem. It leads to ill-health in approximately 10 million people each year.[1]This is despite the fact that, the timely diagnosis and correct treatment of most people who develop TB disease can be cured. Anti-TB drug resistance is a major public health problem that threatens the progress made in TB care and control worldwide. A patient who develops an active disease with drug resistant TB strains can transmit this form of TB to other individuals. Resistance to TB drugs is a formidable obstacle to effective TB care and prevention globally.

TB drug resistance types include: a) Mono-resistance: resistance to one first-line anti-TB drug only b) Poly-resistance: resistance to more than one first-line anti-TB drug, other than both isoniazid and rifampicin c) Multidrug resistance (MDR): resistance to at least both isoniazid and rifampicin d) Extensive drug resistance (XDR): resistance to any fluoroquinolone, and at least one of three second-line injectable drugs (capreomycin, kanamycin and amikacin), in addition to multidrug resistance d) Rifampicin resistance (RR): resistance to rifampicin detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs. It includes any resistance to rifampicin, in the form of mono-resistance, poly-resistance, MDR or XDR. About 9% of MDR-TB patients develops XDR-TB, which is even more difficult to treat.

MDR-TB is multifactorial and fuelled by improper use of antibiotics in chemotherapy of drug

susceptible TB patients, poor management of supply and quality of drugs, and airborne transmission of bacteria in public places. Case management becomes difficult and the challenge is compounded by catastrophic economic and social costs that patients incur while seeking help and on treatment. In most resource-poor countries with a high TB-burden, patients with symptoms suggestive of TB seek care from a wide array of health-care providers. [1] Evidence suggests that failure to involve all care providers used by TB suspects and patients hampers case detection, delays diagnosis, leads to inappropriate and incomplete treatment, contributes to increasing drug resistance and places an unnecessary financial burden on patients.

EPIDEMIOLOGY

According to World Health Organization (WHO), TB is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS. In 2016, there were an estimated 1.3 million TB deaths among HIV-negative people (down from 1.7 million in 2000) and an additional 374 000 deaths among HIV-positive people.[1]An estimated 10.4 million people fell ill with TB in 2016: 90% were adults, 65% were male, 10% were people living with HIV (74% in Africa) and 56% were in five countries: India, Indonesia, China, the Philippines and Pakistan. Drug-resistant TB is a continuing threat. In 2016, there were 600,000 new cases with RRTB, the most effective first-line drug, of which 490 000 had MDR-TB. Almost half (47%) of these cases were in India, China and the Russian Federation.[1]In 2016, 6.3 million new cases of TB were reported (up from 6.1 million in 2015), equivalent to 61% of the estimated incidence of 10.4 million.A total of 129,689 people were started on treatment for drug-resistant TB, a small increase from 125,629 in 2015 but only 22% of the estimated incidence; treatment success remains low, at 54% globally.[1]

As per the Global TB report 2017 the estimated incidence of TB in India was approximately 28,00,000 accounting for about a quarter of TB cases in the world. In 2017 India re-estimated its national figures of the burden of Tuberculosis; incorporating information from a wider range of sources and thus is more accurate than previous estimates. The major additional information source is the private sector notification seen throughout the country and in certain project locations with interventions targeted at private sector notification. The current statistics of TB and MDR/RR TB incidence, HIV TB Co-morbidity and TB related mortality is shown in Table 1. First National Drug Resistance Survey results showed the rates of MDR among new TB patients to be 2.84% and that in previously treated to be 11.60 %.[2]

Looking at the trend of drug resistance in TB, use of rapid diagnostic techniques for detection of the drug resistance is the need of time. Conventional methods for mycobacteriology culture and drug susceptibility testing (DST) are slow and cumbersome, demanding successive procedures for isolation of mycobacteria from clinical specimens, identification of *M. tuberculosis* complex, and in vitro testing of strain susceptibility in the presence of anti-TB drugs. During this time, patients may be inappropriately treated, drugresistant strains may continue to spread, and augmentation of resistance may occur.[3] Novel technologies for rapid detection of anti-TB drug resistance have therefore become significant in TB research and development. This review highlights the different molecular diagnostic methods for multidrug resistance in tuberculosis.

MOLECULAR BASIS OF DRUG RESISTANCE

Drug resistance in TB is a natural phenomenon, due to random mutations in *M. tuberculosis*, classically occurring at rates ranging from 3 x 10-7 to 1 x 10-9 per organism per generation for first-line anti-tuberculosis drugs (rifampicin, isoniazid, ethambutol, streptomycin).[3,4] Thus, the *M. tuberculosis* population within a given human host probably contains at least a small proportion of naturally occurring drug resistant mutants which are selected under conditions of inadequate or ineffective chemotherapy. This process not only expands the drug resistant population within the infected individual, but also allows for resistance to additional drugs to develop, forming a cycle of amplification of drug resistance.

Rifampicin acts by binding to the beta-subunit of the RNA polymerase (coded for by the rpoB gene), inhibiting RNA transcription.[3,4,5]Subsequent DNA sequencing studies have shownthat more than 95% to 96% of rifampicin resistant strains have

mutations in an 81-bp (base pair) region(codons 507 to 533) of the rpoB gene.[3, 5-8] Automated DNA sequencing has been employed and more than 50 mutations within this region have been characterized; however, the majority are point mutations incodons 516, 526, or 531.[3-5, 9] Mutations in other regions of the rpoB gene have also beenreported, however much less commonly. Isoniazid inhibits InhA andenoyl-ACP-reductase which is involved in mycolic acid biosynthesis.[3, 4]Mutations causing isoniazid resistance are located in different regions of several genes. Isoniazid is said to be the 'pro-drug' which is converted to its active form by the catalase peroxidase enzyme KatG.[3] Therefore resistance can be due to several factors, including the binding of the pro-drug to its InhA target, by increased expression of the target InhA or by the activation of the pro-drug by KatG (encoded by the katG gene).[3] Mutations in katC, oxyR, ahpC, furA genes have also been reported in IHN resistance.[4] Streptomycin resistance emerges through mutations in rrs and rpsL that produce an alteration in the streptomycin binding site.[10,11] Pyrazinamide resistance emerges through mutations in pncA, IS6110 insertion.[4] Ethambutol resistance is associated with mutation in embBgene.[4]

Fluoroquinolones resistance is associated with mutation in gyrA, gyrB genes.[4]Although the molecular mechanisms of resistance to the furtherchief anti-tuberculosis drugs (including isoniazid, pyrazinamide, streptomycin, ethambutol and fluoroquinolones) have been elucidated, the molecular basis for resistance is still not fully understood.[3]

MOLECULAR DIAGNOSTIC METHODS

Since resistance arises from genetic mutations, the approach to detect the mutations themselves is applied. Various molecular tests have been developed to detect mutations associated with resistance. The advantages of molecular methods of DST include rapid turnaround time, but the disadvantages include a low sensitivity for some compounds, however a major concern is cost. It is generally observed that specialist staff is required to perform molecular assays. However, some assays such as Gene Xpert are extremely easy to use. They can even be taken out of the laboratory setting and used as a “near” point of care test. Various molecular methods available are discussed in this section.

Line Probe Assays

These tests use PCR and reverse hybridization methods for the rapid detection of mutations associated with drug resistance. These are designed to identify *M. tuberculosis* complex and simultaneously for detection of mutations associated with drug resistance. These assays are an open-tube format, which can lead to cross contamination and an increased risk of false positive results and hence is a disadvantage.[12] LPA technology is suitable for use at national/central reference laboratories, or at laboratories where there is proven capacity to conduct molecular testing. [3] Adequate and appropriate laboratory infrastructure and equipment is a must for these assays. The necessary biosafety precautions and the prevention of contamination should also be maintained.

LPA technology involves the following steps: First, DNA is extracted from *M. tuberculosis* isolates or directly from clinical specimens. Next, polymerase chain reaction (PCR) amplification of the target resistance-determining region of the gene is performed using biotinylated primers. Following amplification, labelled PCR products are hybridized with specific oligonucleotide probes and immobilized on a strip. Captured labelled hybrids are then detected by colorimetric development, enabling detection of the presence of *M. tuberculosis* complex, as well as the presence of wild-type and mutation probes for resistance. If a mutation is existent in one of the target regions, the amplicon will not hybridize with the relevant probe. Mutations are thus detected by lack of binding to wild-type probes, as well as by binding to specific probes for the most commonly occurring mutations. The post hybridization reaction leads to the development of coloured bands on the strip at the site of probe binding and is observed macroscopically.

Various LPAs which are available includes INNO-LiPARif.TB (Innogenetics, Zwijndrecht, Belgium) and Genotype MTBDR and MTBDRplus (Hain Life science, GmbH, Germany). The Genotype MTBDRplus assay detects both rifampicin and INH resistance, whereas the INNO-LiPARif.TB detects rifampicin resistance only. The choice of

technology for line probe assays should take into account the prevalence of MDR-TB and rifampicin mono-resistance as well as country guidelines for management of rifampicin mono-resistant and MDR-TB patients.

The use of commercial line probe assays instead of in-house assays is recommended to ensure reliability and reproducibility of results, as in-house assays have not been used outside limited research settings or sufficiently validated. Any new or generic line probe assays must be subjected to adequate validation, so that the adequate data allows systematic review and meta-analysis (including assessment of data quality).

Results from field demonstration projects documenting feasibility and consistent performance equal to conventional methods and commercial line probe assays should also be included.

New or generic line probe assays for MDR-TB should have these characteristics: a) A specific probe to identify *M. tuberculosis* complex; b) Multiple probes to detect the most common mutations in rpoB (codons 531, 526 and 516); c) Multiple overlapping wild-type (susceptible) probes covering the RRDR region of rpoB; Preferably, multiple probes to detect both high-level (katG mutations) and low-level isoniazid resistance (inhA mutations); d) Strip technology, with appropriate assay procedure controls, allowing visual detection of results; e) Line probe test production under ISO 13485:2003 standards; f) Performance characteristics equal to those of conventional DST methods; g) Performance characteristics equal to those of current commercial line probe assays.[3]

In May 2016, new recommendations were issued by WHO on the use of a novel diagnostic test - called MTBDRsl – a line probe assay to detect resistance to second-line anti-TB drugs (SL-LPA).[13] It is a DNA-based test that identifies genetic mutations in MDR-TB strains which makes them resistant to fluoroquinolones and injectable second-line TB drugs. This is the first and only WHO recommended rapid test for detection of additional resistance in MDR-TB patients as well as XDR-TB. Thisrapid diagnostic test is recommended by WHOfor identifying those MDR- or rifampicin-resistant TB patients who can be placed on the shorter MDR-TB regimen. The results of this test will also be critical in assigning patients on targeted conventional MDR-TB regimens with improved outcomes.[13]

The SL-LPA produces results in just 24-48 hours, a huge improvement over the 3 months or longer currently required. It permits quick triage of confirmed rifampicin resistant or MDR-TB patients into either the shorter MDR-TB regimen or the conventional longer regimen. Detection of any second-line resistance by the SL-LPA suggests that MDR-TB patients should not be enrolled on the shorter regimen as this could jeopardise their treatment outcome and stimulate the development of XDR-TB. Patients detected with XDR-TB by the SL-LPA should be enrolled on the carefully designed individual regimens to optimise their chances of success.[13]

Countries with existing LPA capacity can instantaneously adopt the SL-LPA as the laboratory methods are the same as for first-line LPA. Laboratory facilities for LPA need at least three separate rooms - one each for DNA extraction, pre-amplification procedures, and amplification and post-amplification procedures. Restricted access to molecular facilities, unidirectional work flow, and stringent cleaning protocols are must and should be established to avoid contamination.

Proficient laboratory staff should be trained to conduct LPA procedures. An external quality assessment programme for involved laboratories should be developed as a priority. System for rapid reporting of LPA results to clinicians must be established to provide patients with the benefit of an early diagnosis.

As reported to WHO, by 2014 approximately 400 LPA laboratories had been established in low and middle-income countries.[13]WHO recommends the use of SL-LPA for patients with confirmed RR-TB or MDR-TB as an initial test to detectto fluoroquinolones and the second-line injectable drugs resistance, instead of phenotypic culture-based DST.[13]

The use of SL-LPA is recommended for direct testing of sputum specimens as well as indirect testing on culture isolates from RR-TB or MDR-TB patients, including adults and children (irrespective of the smear status). These recommendations do not eliminate the need for phenotypic DST to confirm resistance to other drugs and to monitor the

emergence of additional drug resistance during treatment.[13]

GeneXpert MTB/RIF assay

This is a cartridge based nucleic acid amplification test (CB - NAAT). The development of the Xpert® MTB/RIF assay was completed in 2009 and is considered an important revolution in the battle against TB. In 2016, the programme has extended TB care services and made breakthrough changes in the strategy of diagnosis and treatment of TB. An additional 500 CBNAAT machines were installed over the year, expanding the rapid molecular diagnostic facilities to 628 laboratories. With an upsurge in CBNAAT laboratory network, there is exponential rise in drug resistant TB case finding. In 2016, more than 33,820 drug resistant TB patients diagnosed as compared to 29,057 in 2015.[14]It is for the first time that a molecular test is sufficiently simple and robust to be introduced and used outside conventional laboratory settings. Xpert MTB/RIF assay detects *M. tuberculosis* as well as mutation that cause rifampicin resistance using three specific primers and five unique molecular probes to ensure a high degree of specificity. The assay provides results directly from sputum in less than 2 hours.[15] The GeneXpert MTB/RIF assay remain the solitary self-contained cartridge based fully automated DNA testing system that can accurately detect both TB and resistance to rifampicin in less than 2 hours, and it is the only established technology among a new generation of automated molecular diagnostic platforms.[15]

The Xpert MTB/RIF has high sensitivity (88%)in detecting TB.The negative predictive value (NPV) is greater than 98% both in settings with a low prevalence of TB and in those with a high prevalence of TB which implies that a negative result accurately excludes TB in most situations. However, the ability of any diagnostic test using sputum specimens to detect TB depends on the quality of the specimen collected; therefore, an individual with a negative result from Xpert MTB/RIF could still have TB. An individual still suspected of having TB after a negative Xpert MTB/RIF test may consequently, require further clinical management and another diagnostic test which includes a repeated Xpert MTB/RIF test using a different sputum specimen.[15] TheXpert MTB/RIF has high specificity(99%)for detecting TB and false-positive results are probably to be linked to the detection of dead *M. tuberculosis* bacilli by Xpert MTB/RIF that would not be detected by culture, which is the present reference standard. The positive predictive value (PPV) of Xpert MTB/ RIF testing is adversely affected in settings with a low prevalence of disease or in populations with a low prevalence as the specificity of Xpert MTB/RIF is not 100%. [15]

The Xpert MTB/RIF assay has high sensitivity (95%) in detecting rifampicin resistance and the NPV (the NPV for rifampicin resistance is the proportion of cases diagnosed as rifampicin-susceptible that are truly susceptible) is greater than 98% both the settings, low prevalence and high prevalence of rifampicin resistance. Therefore a negative result accurately excludes the possibility of rifampicin resistance and, generally, no further testing is required to confirm negative results. In rare instances, when a patient is strongly suspected of having MDR-TB even after a negative result from Xpert MTB/RIF, a follow-up test may be done using phenotypic culture-based DST to detect rifampicin resistance due to mutations in regions outside of the rpoB region which is detected by Xpert MTB/RIF. [15]

The Xpert MTB/RIF has a very high specificity (98%) in detecting rifampicin resistance, and increasing evidence has shown that the infrequent occurrence of so-called false-positive results may be linked to the detection by Xpert MTB/RIF of strains that are truly resistant to rifampicin, but which are not detected by the phenotypic culture based DST(the present reference standard). Such strains seem to have clinically relevant mutations in the region conferring resistance to rifampicin, causing disease for which first-line treatment is likely to fail. The PPV for detecting rifampicin resistance (the PPV for rifampicin resistance is the proportion of cases diagnosed as rifampicin-resistant that are truly resistant) using Xpert MTB/RIF exceeds 90% in settings where the underlying prevalence of rifampicin resistance is more than 15%, and the PPV is probably even higher considering the limitations of the present reference standard. In settings where rifampicin resistance is rare, the PPV is adversely affected nevertheless it can be greatly improved by undertaking a careful risk assessment of individual patients and targeting testing carefully to increase the pre-test probability of rifampicin resistance.[15]

On 20 January 2017, WHO convened a Technical Expert Consultation to assess the performance of the new Ultra assay in comparison to the Xpert MTB/RIF assay in a multi-centric diagnostic accuracy study coordinated by FIND in ten study sites in eight countries. The Technical Expert Group found that the Ultra assay is non-inferior to the Xpert MTB/RIF assay for the detection of *Mycobacterium tuberculosis* (MTB) and for the detection of rifampicin resistance. [16]In certain populations, the Ultra performs better for MTB detection especially for individuals whose specimens are frequently paucibacillary. The Ultra cartridge showed better performance for the detection of MTB in smear-negative culture-positive specimens, paediatric specimens, extra-pulmonary specimens (notably cerebrospinal fluid) and in testing smear-negative culture-positive specimens from HIV-positive individuals.[16]
The current WHO recommendations for the use of Xpert MTB/RIF now also apply to the use of Ultra assay as the initial diagnostic test for all adults and children with signs and symptoms of TB and in the testing of selected extra-pulmonary specimens (CSF, lymph nodes and tissue specimens).[16]

DNA microarray

DNA microarray technology can be very effectively used to detect the drug resistance in TB based on the associated mutations. In a blinded analysis of 153 clinical isolates by Yvonne Linger et al.[17], microarray sensitivity for first-line drugs compared to phenotypic DST (true resistance) was 100% for rifampin (RIF) (14/14), 90.0% for isoniazid (INH) (36/40), 70% for ethambutol (EMB) (7/10), and 89.1% (57/64) combined. Microarray specificity (true susceptibility) for first-line agents was 95.0% for RIF (132/139), 98.2% for INH (111/113), and 98.6% for EMB (141/143). Overall microarray specificity for RIF, INH, and EMB combined was 97.2% (384/395). The overall positive and negative predictive values for RIF, INH, and EMB collectively were 84.9% and 98.3%, respectively. For the second-line drug streptomycin (STR), overall concordance between the agar proportion method and microarray analysis was 89.5% (137/153). Sensitivity was 34.8% (8/23) because of limited microarray coverage for STR-conferring mutations, and specificity was 99.2% (129/130).

In a study by Peijun Tang et al.[18],sputum samples of 42 patients with TB in the Affiliated Hospital of Infectious Diseases of Soochow University (Soochow, China) were collected. GeeDom MTB drug detection kits were used to create a DNA microarray chip and examine the RFP-resistance associated gene mutation points rpoB RRDR 511, 513, 516, 526, 531 and 533, and the INH-resistance associated gene mutation points katG315 and inhA 15 of the sputum samples. The conventional Lowenstein-Jensen culture medium was used gold standard to assess drug sensitivity using the absolute concentration method. Compared with the results from the absolute concentration method, the susceptibility and specificity of RFP sensitivity detected by the DNA microarray chip were 92.8 and 93.8%, respectively. The susceptibility and specificity of INH sensitivity detected were 66.7 and 81%, respectively. The rpoB RRDR 526, 531 mutations were the primary cause of MTB RFP resistance and the katG315 mutations was the primary cause of INH resistance. According to this study, detection of rpoB and katG gene mutation points by a DNA microarray chip may be used as a rapid, accurate and bulk clinical detection method for RFP and INH resistance in MTB.

Both these studies show promising results for using DNA microarray in diagnosing drug resistant TB.

PCR - based Sequencing

This method includes amplification of targets further for sequencing based screening of drug resistance in *Mycobacterium tuberculosis* can further be extended to rapid identification non tuberculous Mycobacteria (NTM) as *M. avium* complex (MAC).

In Ailyn C. Pérez-Osorio et al. study [19], The MID-DRS (Mycobacterial IDentification and Drug Resistance Screen) assay utilized a single multiplexed PCR with two components, representing both rapid mycobacterial identification and, in cases in which MTBC is detected, targets for DNA sequencing-based screening of mutations associated with resistance to the first-line drugs. The assay was developed and evaluated using both bacterial isolates and respiratory specimens, with complete analysis possible using AFB-positive clinical specimens in as little as 2 days.

Their study concluded that MID-DRS helps in the rapid confirmation of TB, MAC, and other NTM species in smear-positive specimens and also reduces the time and number of steps necessary to assess resistance to first-line TB drugs.

Pyrosequencing (PSQ)

Pyrosequencing is a method of DNA sequencing (determining the order of nucleotides in DNA) based on the "sequencing by synthesis" principle, in which the sequencing is performed by detecting the nucleotide incorporated by a DNA polymerase. It relies on light detection based on a chain reaction when pyrophosphate is released. PSQ has the ability to detect first- and second-line anti-TB drugs associated mutations and has the additional advantage of being rapidly adaptable for the identification of new mutations.

In a study by S.-Y. Grace Lin et al.[20], a total of 130 clinical isolates and 129 clinical specimens were studied. The correlations between the PSQ results and the phenotypic DST results were 94.3% for isoniazid, 98.7% for rifampin, 97.6% for quinolones (ofloxacin, levofloxacin, or moxifloxacin), 99.2% for amikacin, 99.2% for capreomycin, and 96.4% for kanamycin. For testing clinical specimens, the PSQ assay yielded 98.4% sensitivity for detecting MTBC and 95.8% sensitivity for generating complete sequencing results from all sub assays. This study concluded that PSQ assay was able to promptly and precisely detect drug resistance mutations with the sequence information provided, which allows further study of the association of drug resistance or susceptibility with each mutation and the accumulation of such knowledge for future interpretation of results. Thus, reporting of false resistance for mutations known not to confer resistance can be prevented, which is a significant benefit of the assay over existing molecular diagnostic methods endorsed by the World Health Organization.

In a Kanchan Ajbaniet al. study[21], total of 187 archived isolates were run through a PSQ assay in order to identify *M. tuberculosis* (via the IS6110 marker), and to detect mutations associated with M/XDR-TB within small stretches of nucleotides in selected loci. The molecular targets included katG, the inhA promoter and the ahpC-oxyR intergenic region for isoniazid (INH) resistance; the rpoB core region for rifampin (RIF) resistance; gyrA for fluoroquinolone (FQ) resistance; and rrs for amikacin (AMK), capreomycin (CAP), and kanamycin (KAN) resistance. PSQ data were compared to phenotypic mycobacterial growth indicator tube (MGIT) 960 drug susceptibility testing results for performance analysis. The PSQ assay illustrated good sensitivity for the detection of resistance to INH (94%), RIF (96%), FQ (93%), AMK (84%), CAP (88%), and KAN (68%). The specificity of the assay was 96% for INH, 100% for RIF, FQ, AMK, and KAN, and 97% for CAP. This study determines that PSQ is a highly competent diagnostic tool that reveals specific nucleotide changes associated with resistance to the first- and second-line anti-TB drug medications. It has the potential to be linked to mutation-specific clinical interpretation algorithms for prompt treatment decisions. PSQ is a high-throughput, robust and rapid diagnostic sequencing technique.

Both these studies used library containing wild-type sequences and mutant sequences with IdentiFire software (Qiagen, Valencia, CA) to align the sequences.

PCR ELISA

PCR-ELISA combines an immunological method (ELISA) to detect and quantify specific PCR products directly after immobilization of DNA on a microtitre plate. A sensitive and specific PCR–ELISA implicates the labelling of target amplicons in the course of PCR, their hybridization with target-specific probes followed by capture of labelled hybrids onto microtitre plates or tubes and subsequent detection through immunoassay. Labelling of amplicons is often performed with digoxigenin (DIG) molecules, and the DIG-labelled amplicons are easily detected utilizing highly specific anti-DIG antibodies. The immunoassay is principally carried out like a conventional ELISA. PCR–ELISA assays are generally carried out in microtiter plates with colorimetric signal detection. Depending on the specific requirements, it is also promising to perform chemiluminescent detection using microplate luminometers or imaging systems. In Lucia Garcia et al. study[22],forward and reverse primers used were RP4T (5′-GAGGCGATCACACCGCAGACGT-3′)and RP8T (5′-GATGTTGGGCCCCTCAGGGGTT-3′) respectively. The pair of these primers amplified a 255-bp fragment of the rpoB gene. The reverse primer was labelled with digoxigenin at the 5′ end to facilitate detection of the PCR product.Five

overlapping 5′-biotinylated oligonucleotides were designed as capture probes for the detection of the rpoB PCR products. The five probes were specific for the wild-type *M. tuberculosis* rpoB gene and span the region in which mutations conferring rifampicin resistance have been designated. The PCR-ELISA cannot identify the specific mutation instigating rifampicin resistance but does specify the region in which the mutation is located. However, the knowledge of specific mutation conferring resistance is not necessary for efficient patient management. The advantage of the PCR-ELISA system is that it is rapid and is accurate in identifying rifampicin resistant strains.

PCR – SSCP

In this technique, the target sequence is first labelled and amplified simultaneously using labelled substrates by PCR. The polyacrylamide gel electrophoresis is further used to resolve the denatured PCR which leads to detection of mutations as altered mobility of separated single strands in the autoradiogram. Hence, the procedures involved overall are rapid and modest. The mutation can further be characterized by elution of the mutated allele from the gel and its amplification for sequence determination. [23]In SS Negi et al. study [24], Rifampicin resistance was detected successfully by PCR-SSCP in 20/22(90.90%) of rifampicin-resistant strains showing a total of nine different mutations in seven codon positions: codon 513 (CAA→CCA), 516 (GAC→GTC), 507 (GGC→GAC), 526 (CAC→GAC, TAC), 531 (TCG→TTG, TGG), 522 (TCG→TGG) and 533 (GTG→CCG). Two rifampicin-resistant strains showed an identical PCR-SSCP pattern with the wild type H37Rv; 77.27% rifampicin-resistant strains showed a single point mutation and 9.09% had no mutation. Three rifampicin-resistant strains showed characteristic double mutations at codon positions 526 and 531. Sensitivity and specificity calculated was 90.90% and 100%. Rifampicin-resistant genotypes were chiefly found in codon positions 516, 526 and 531. The extracted DNA was amplified in a 25μL reaction mixture containing 11.5μL ddH2 O, 1.5μL MgCl2 , 2.5μL 10X PCR buffer, 200μM dNTPs each, 2.5U Taq Polymerase (GeneAmp (R) Gold PCR reagent kit; PE Biosystem, Foster City, CA, USA) and 5μL DNA. The primers usedwere in a concentration of 10pM each for amplification of the rifampicin-resistant region with primers TR 9 (5'-TCGCCGCGATCAAGGAGT-3') and TR 8(5'-TGCACGTCGCGGACCTCCA-3') (Gen Bank accession number L05910). These primers generated a 157bp amplicon on 2% agarose gel. The SSCP of the PCR products was analysed by electrophoresis with 12% acrylamide gels. This study concluded that, PCR-SSCP appears to be an efficacious method of envisaging rifampicin resistance and substantively reduces the time required for susceptibility testing from 4 to 6 weeks to a few weeks.

CONCLUSION

MDR- TB and XDR – TB is a persistent threat to the public health. This leads to the need of rapid and robust diagnostic techniques to detect the drug resistance in TB. Though culture-based methods currently remain the reference standard for drug susceptibility testing, molecular diagnostic methods helps in prompt detection of the drug resistance, quicker diagnosis, surmounting the programmatic management and surveillance of drug-resistant TB, potential for high through put, and also have fewer requirements for ensuring laboratory biosafety. Line probe assay and Gene Xpert MTB/RIF assay are the most widely used methods and furthermore these techniques are endorsed by WHO. Newer advances in these two technologies as SL-LPA and Ultra assay respectively also have promising future in the diagnostic field.

REFERENCES

1. Global tuberculosis report 2017. Geneva: World Health Organization; 2017. Licence: CC BY-NCSA 3.0 IGO.
2. Central TB Division, Directorate General of Health Services. India TB Report 2018. New Delhi: India Offset Press; 2018 p.9.
3. Molecular Line Probe Assays for Rapid Screening uf Patients at Risk of Multi-Drug Resistant Tuberculosis (MDR-TB). World Health Organisation; 2018 p. 3-33.
4. Gillespie S. Evolution of Drug Resistance in *Mycobacterium tuberculosis*: Clinical and Molecular Perspective. Antimicrobial Agents and Chemotherapy. 2002; 46(2):267-274.
5. Musser J. Antimicrobial Agent Resistance in Mycobacteria: Molecular Genetic Insights. Clinical Microbiology Reviews. 1995; 8(4):496-514.
6. Hirano K., Abe C. And Takahashi M. Mutations in the rpoB Gene of Rifampin-Resistant *Mycobacterium tuberculosis* Strains Isolated Mostly in Asian Countries and Their

Rapid Detection by Line Probe Assay. JClinMicrobiol. 1999;37(8), pp.2663-6.
7. Cavusoglu C, Hilmioglu S, Guneri S, Bilgic A. Characterization of rpoB Mutations in Rifampin-Resistant Clinical Isolates of *Mycobacterium tuberculosis* from Turkey by DNA Sequencing and Line Probe Assay. JClinMicrobiol.2002; 40(12):4435-4438.
8. Bartfai Z, Somoskovi A, Kodmon C, Szabo N, Puskas E, Kosztolanyi L et al. Molecular Characterization of Rifampin-Resistant Isolates of *Mycobacterium tuberculosis* from Hungary by DNA Sequencing and the Line Probe Assay. JClinMicrobiol. 2001; 39(10):3736-3739.
9. DROBNIEWSKI F, WILSON S. The rapid diagnosis of isoniazid and rifampicin resistance in *Mycobacterium tuberculosis* -- a molecular story. JClinMicrobiol. 1998;47(3):189-196.
10. Honore N, Cole S. Streptomycin resistance in mycobacteria. Antimicrobial Agents and Chemotherapy. 1994;38(2):238-242.
11. Sreevatsan S, Pan X, Stockbaue K, Williams D, Kreiswirth B, Musser J. Characterization of rpsL and rrs Mutations in Streptomycin-Resistant *Mycobacterium tuberculosis* Isolates from Diverse Geographic Localities. Antimicrobial Agents and Chemotherapy. 1996; 40(4):1024-6.
12. Chegou N, Hoek K, Kriel M, Warren R, Victor T, Walzl G. Tuberculosis Assays: Past, Present and Future. Expert Rev Anti Infect Ther. 2011;9(4):457-469.
13. Molecular Line-Probe Assay forthe Detection of Resistance to Second-Line Anti-Tb Drugs (Sl-Lpa). World Health Organization; 2016. Available from: http://www.who.int/tb/publications/factsheet_tb_sllpa.pdf
14. Central TB Division, Directorate General of Health Services. India TB Report 2017. New Delhi: Shree Ganesh Associates; 2017;p. -xvii.
15. Xpert MTB/RIF implementation manual: technical and operational 'how-to'; practical considerations. France: GPS Publishing; 2014.(WHO/HTM/TB/2014.1) Available from: http://apps.who.int/iris/bitstream/10665/112469/1/9789241506700_eng.pdf. Accessed on 15/04/2018.
16. WHO meeting report of a technical expert consultation: non-inferiority analysis of Xpert MTF/RIF Ultra compared to Xpert MTB/RIF. Geneva: World Health Organization; 2017 (WHO/HTM/TB/2017.04). Licence: CC BY-NCSA 3.0 IGO.
17. Linger Y, Kukhtin A, Golova J, Perov A, Lambarqui A, Bryant L et al. Simplified Microarray System for Simultaneously Detecting Rifampin, Isoniazid, Ethambutol, and Streptomycin Resistance Markers in *Mycobacterium tuberculosis*. JClinMicrobiol. 2014;52(6):2100-2107.
18. Tang P, Wang X, Shen X, Shi M, Zhu X, Yu X, et al. Use of DNA microarray chips for the rapid detection of *Mycobacterium tuberculosis* resistance to rifampicin and isoniazid. Experimental and Therapeutic Medicine. 2017;13(5):2332–8.
19. Perez-Osorio AC, Boyle DS, Ingham ZK, Ostash A, Gautom RK, Colombel C, et al. Rapid Identification of Mycobacteria and Drug-Resistant *Mycobacterium tuberculosis* by Use of a Single Multiplex PCR and DNA Sequencing. JClinMicrobiol.2011Jul; 50(2):326–36.
20. Lin S- YG, Rodwell TC, Victor TC, Rider EC, Pham L, Catanzaro A, et al. Pyrosequencing for Rapid Detection of Extensively Drug-Resistant *Mycobacterium tuberculosis* in Clinical Isolates and Clinical Specimens. JClinMicrobiol. 2013; 52(2):475–82.
21. Ajbani K, Lin S-YG, Rodrigues C, Nguyen D, Arroyo F, Kaping J, et al. Evaluation of Pyrosequencing for Detecting Extensively Drug-Resistant *Mycobacterium tuberculosis* among Clinical Isolates from Four High-Burden Countries. Antimicrobial Agents and Chemotherapy. 2014Mar; 59(1):414–20.
22. Garcia L, Alonso-Sanz M, Rebollo MJ, Tercero JC, Chaves F. Mutations in the rpoB Gene of Rifampin-Resistant *Mycobacterium tuberculosis* Isolates in Spain and Their Rapid Detection by PCR-Enzyme-Linked Immunosorbent Assay. J Clin Microbiol. 2001 Jan; 39(5):1813–8.
23. Hayashi K. PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. Genome Research. 1991Jan;1(1):34–8.
24. Negi S, Rai A, Gupta S, Khare S, Lal S. Characterization of rpo B Gene for Detection of Rifampicin Drug Resistance by SSCP and Sequence Analysis. Int J Infect Dis. 2008; 12.

Table 1. Estimates of TB Burden in India and Global, 2016

Indicator	No.	No/ Lakhs	Global statistics
Incidence of TB (including HIV)	27,90,000	211	1,04,00,000
Mortality due to TB (Excluding HIV)	4,23,000	32	13,00,000
Incidence of MDRTB/RR	1,47,000	11	6,01,000
Incidence of HIV-TB	87,000	6.6	10,30,000
Mortality due to HIV-TB co-morbidity	12,000	0.92	3,74,000

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