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REVIEW ARTICLE

Molecular Mycobacteriology and Expansion in Disease Diagnosis

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Abstract Molecular diagnostic tools for tuberculosis (TB) have evolved quickly with new innovations which can provide unprecedented opportunities for the rapid, sensitive and specific diagnosis of *M. tuberculosis* in clinical specimens and the status of its drug sensitivity. Microscopy and culture methods can not be replaced but the molecular assays can be applied in parallel with any new molecular tests for the diagnosis of TB. For extra pulmonary specimens, the use of the amplification methods is advocated, since rapid and accurate laboratory diagnosis is critical. Customization of the diagnostic usefulness of a molecular assay, according to the ease, reliability and need for health care sector is of immense value in a modern clinical mycobacteriology laboratory.

Keywords Amplification · Master mix · Multiple drug resistance · Nested PCR

Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is an old and serious infectious disease in humans, and it is still a major public health problem worldwide [1, 2]. It is estimated that nearly 1 billion people will be newly infected with TB between 2000 and 2020 and, furthermore, two hundred million people will develop disease and 35 million will die from TB within this period. In India 1.8 million tuberculosis cases occur annually, accounting for one-fifth of the world's new TB cases and two-thirds of the cases in the South-East Asia Region. This makes India the highest TB burden country in the world [3, 4]. TB remains one of the major causes of death in India. Early diagnosis, together with adequate therapy and prevention measures against further transmission are essential for TB control. Traditional smear microscopy and culture-based routine diagnostics for *M. tuberculosis* are commonly used in clinical mycobacteriology laboratories. However, traditional methods are inadequate for the effective control of TB due to the fact that they are time-consuming, cumbersome and high concentrations of bacteria must be present in the sample to be detected. A rapid, sensitive, and accurate diagnostic tool for detection of *M. tuberculosis* in clinical specimens is essential for the successful diagnosis of TB patients. Over the last few years, new molecular methods have been introduced, including PCR-restriction fragment length polymorphism, real-time PCR, DNA sequencing, DNA strip assays as mycobacterial diagnostic tools, leading to considerable improvement of both speed and accuracy for identification.

The prevalence of TB is further complicated by the appearance of strains with multidrug resistance (MDR) in almost 3 % of all newly diagnosed patients [5]. The conventional phenotypic methods for assessing drug resistance are slow and in order to avoid delays in both therapy and prevention of MDR transmission, various genotypic methods based on line probe assays, DNA sequencing or real-time PCR, have been proposed for the detection of mutations associated with resistance to anti-tuberculosis drugs. The aim of the present report is to review the molecular methods used in Mycobacterial diagnostics and

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to assess their diagnostic usefulness in a modern clinical mycobacteriology laboratory.

Direct Detection of *Mycobacterium* in Clinical Specimens

In-house PCR for the Detection of Mycobacteria from Clinical Specimens

In the last decade, nucleic acid amplification-based techniques (NAAT's) have become accessible to the clinical mycobacteriology laboratory. PCR protocols amplifying a large variety of chromosomal DNA have concentrated on detection of both genus-specific and *M. tuberculosis* complex-specific DNA regions. The insertion element IS 6110 and the 16S rDNA are the most common targets used. Other regions used for amplification include the rpoB gene encoding the β-subunit of the RNA polymerase, the gene coding for the 32 kD protein, the recA gene, the hsp65 gene, the dnaJ gene, the sodA gene and the 16S–23S rRNA internal transcriber spacer [6–8]. In research laboratories, nucleic acid amplification tests (NAATs) are very sensitive and detect as few as 10 bacilli. These tests are highly sensitive in clinical samples and studies have shown that sensitivity and specificity are ranging as high as 90–100 %. NAATs may be tested on any specimen thought to contain bacilli [blood, urine, cerebrospinal fluid (CSF)] but there is even less sensitivity reported in non-respiratory samples. Sensitivity is improved when multiple samples are tested, because not all samples necessarily contain detectable nucleic acid [9, 10]. Most of *in house* PCR procedures achieve a sensitivity never matched by commercial systems but are often burdened by the high incidence of false positive results due to amplicon cross-contamination of specimens. To minimize the risk of specimen-to-specimen contamination, a physical separation of processes, equipment, and reagents is recommended. Four different work areas are suggested, including a reagent preparation area to prepare PCR master mix, a sample processing area where procedures, including nucleic acid extraction, occurs, a target loading area where the specimen is added to the PCR master mix in the reaction vessel, and an amplification area where thermocycling and probe detection occurs [11]. The reagent preparation area should be kept free of all patient specimens and DNA extracts. Protocols for the sample preparation area should minimize the number of tubes that are simultaneously open. Each of the work areas should contain dedicated working materials, reagents, and pipetting devices. Reagents should be prepared and aliquoted into single use or small volumes. This ensures ease of use and less chance for contamination. All working surfaces should be cleaned before and after use, preferably with a reagent that destroys nucleic acid such as a 5 % bleach solution. Gloves should be changed frequently, at least before beginning each of the separate tasks required in a dedicated work area and should always be changed if moving from one work area to another work area. The use of aerosol-resistant pipette tips and pipette tips long enough to prevent specimen contact with the pipette aids in the prevention of specimen contamination [12]. Enzyme contamination control systems such as uracil-*N*-glycosylase (UNG) can be incorporated into the real-time PCR master mix as an added safeguard to sterilize amplified product that may be carried over to subsequent batches of tests [13]. Over the last few years, real-time PCR systems have been increasingly used in routine mycobacteriology laboratories. The technique allows real-time monitoring of a DNA amplification reaction by measuring an accumulating fluorescence signal. Real time PCR provided improved sensitivity and specificity, reducing turnaround time and avoiding the use of ethidium bromide-stained gels. Different real-time instruments are now available in the market.

Real-time PCR detection technology has been widely evaluated. The majority of real-time PCR methods reported to date for mycobacteria focus on detection of the *M. tuberculosis* complex. Several publications address the detection of Mycobacteria at the genus level. The risk of contamination is considerably less with real-time PCR compared to conventional PCR, but it still can occur. Specimen to specimen contamination has become a greater challenge than amplified product contamination. The most obvious situation where specimen-to-specimen contamination can occur is with the transfer of specimen to the PCR vessel or to the DNA extraction tube [14, 15].

Commercially Available Assays

Amplicor MTB Test

The Amplicor MTB Test (Roche Molecular Systems, Basel, Switzerland) relies on standard PCR. A 584 bp fragment of the 16S ribosomal RNA gene, comprising a species-specific region flanked by genus-specific sequences, is amplified using biotinylated primers. In the master mix, an unusual combination of nucleotides is present—as an adjunct to adenine, guanidine and cytosine, uracil is used in place of thymine. As a consequence, the amplification product differs from the target DNA in that it contains uracil instead of thymine. This device is part of a contamination-control system based on the use of uracil-*N*-glycosylase, an enzyme that fragments DNA wherever uracil is present. The enzyme, added to the samples before amplification, destroys any amplicon resulting from

previous amplifications without damaging the uracil-free target DNA. Because of the genus-specific nature of the annealing regions, 16S ribosomal DNA belonging to any Mycobacterial species is amplified by this PCR. The use, in the revealing phase, of magnetic beads coated with *M. tuberculosis* complex-specific probes allows the removal, by washing, of any other DNA. The detection of the specific amplification product is performed by adding an avidin-enzyme conjugate and a chromogenic substrate [16]. The amplification and detection steps are carried out automatically by the Cobas Amplicor instrument. Once the sample extraction has been performed by heating (95 °C), the tube is placed in the thermal cycler integrated in the Cobas instrument. Without further handling, the amplification product will be automatically transferred into the detection station where the chromogenic reaction is developed and read. The turnaround time is 6–7 h. The method is approved by the US FDA for testing smear-positive respiratory samples. It includes an internal control, composed of synthetic DNA characterized by identical annealing sequences as the mycobacterial target; when this is not amplified, it signals the presence of inhibitors. The detection of *M. tuberculosis* complex DNA can also be carried out without the Cobas instrument, using a manual kit that, however, does not include an internal control. Other Amplicor kits are available for detection of *Mycobacterium avium* and *Mycobacterium intracellulare* DNA in clinical samples. From the literature review, specificity is close to 100 % while sensitivity ranges from 90 to 100 % in smear-positive samples and from 50 to 95.9 % in smear negative ones [17].

Amplified MTD

Amplified *M. tuberculosis* Direct Test (AMTD), developed by GenProbe (San Diego, CA, USA), is an isothermal (42 °C) transcriptase-mediated amplification system. A *M. tuberculosis* complex-specific region of the 16S ribosomal RNA gene produces double-stranded ribosomal DNA, due to the combined action of reverse transcriptase and ribonuclease. In turn, RNA polymerase catalyzes the synthesis of multiple stretches of ribosomal RNA from the ribosomal DNA synthesized before. A new cycle starts when the newly produced ribosomal RNA undergoes further transcription by reverse transcriptase. The sensitivity of the method is increased by the presence, in each bacterium, of a high number of 16S ribosomal RNA target molecules (about 2000) compared to only one copy of 16S ribosomal DNA. Another advantage of the amplification from RNA relies on the low stability of such a molecule; this minimizes both the risk of contamination and the incidence of false-positive results due to the persistency of stable nucleic acids (DNA) in the host organism, even after the complete eradication of the infection. The detection of amplification products relies on hybridization with a specific, single-strand DNA probe labeled with a chemiluminescent molecule (Hybridization Protection Assay). The whole process is performed manually, starting with the extraction by means of sonication, continuing with the addition of different reagents until the final reading with the luminometer. Thermal-cyclers are not needed and the whole amplification step is carried out on a heating block at 42 °C. The turnaround time is 2.5 h. No internal control is provided in the kit to monitor the presence of inhibitors. The method is approved by the Food and Drug Administration of the United States of America (US FDA) for testing smear-positive and smear-negative respiratory samples. The overall sensitivity for respiratory specimens was found in the range between 90.9 and 95.2 % and the specificity between 97.6 and 100 % [18, 19].

BD ProbeTec ET

The BD ProbeTec ET (Becton Dickinson, Sparks, MD) uses DNA polymerase and isothermal strand displacement amplification to produce multiple copies of IS*6110*, an insertion element unique to *M. tuberculosis* complex. The rationale of strand displacement amplification is extremely complex; what is presented here is an extreme simplification. In the initial phase (target amplification), amplification is started by two pairs of primers complementary to contiguous sequences delimiting the target. The elongation of the upstream primer, also named bumper, determines the displacement of the simultaneously elongating downstream primer and finally releases the produced amplicon. A restriction site, present in the downstream primer, will also be present in the released amplicon. In the exponential amplification phase, a new primer anneals to the amplicon and, following digestion by the restriction enzyme, the upstream fragment acts as bumper and displaces the downstream fragment. Real-time detection is based on the energy transfer technology. A hair-pin-shaped probe, complementary to IS*6110*, is marked by two fluorescent molecules, one of which, the donor, is quenched by the other, the acceptor; furthermore, it presents a restriction site in the sequence between the two markers. Once its free end has hybridized with the amplification product, the probe undergoes elongation before being displaced by a primer annealed upstream to the same amplicon. The elongation makes the probe able to bind a new primer which, while elongating, stretches out the "hair-pin" and moves the acceptor away from the donor. The nicking of the restriction site by a proper enzyme further separates donor and acceptor and allows the first to free a fluorescence signal. Some manipulation is required before introduction of the sample into the automatic instrument; each

sample is first inactivated at 105 °C, and then sonicated to extract the DNA, transferred into priming well at 72.5 °C, and subsequently into an amplification well at 54 °C. In the BD ProbeTec ET instrument, the microplate containing the samples and the amplification reagents is incubated at 52.5 °C and the fluorescence emitted is continuously monitored. A thermal cycler is not required. The turn-around time is 3.5–4 h. An internal control is present, characterized by the same annealing sequences as the mycobacterial target. In case of amplification failure, this control alerts for the presence of inhibitors.

The system is not yet approved by the US FDA. Kits are also available for the amplification of nucleic acids of *M. avium*, *M. intracellulare* and *Mycobacterium kansasii*. The literature reports a rate of sensitivity ranging from 98.5 to 100 % for smear positive samples and very variable (0.33–100 %) for smear-negative ones [20, 21].

Genotype Mycobacteria Direct Assay

It is for detection of *M. tuberculosis* complex and four atypical mycobacteria (Hain Lifescience, Nehren, Germany). This novel assay is based on the nucleic acid sequence-based amplification (NASBA) applied to DNA strip technology. According to the manufacturer, the assay has three steps. The first step consists of isolation of 23S rRNA, the second step includes amplification of RNA by NASBA method, and the third step involves the reverse hybridization of the amplified products on membrane strips using an automated system. The assay has the ability for simultaneous detection of *M. avium*, *M. intracellulare*, *M. kansasii*, *M. malmoense* and MTBC. Isolation of highly specific RNA is achieved by the use of the "magnetic bead capturing" method. This assay is useful, reliable and rapid, with sensitivity and specificity of 92 and 100 %, respectively [22].

LCx MTBC Assay (Abbott Laboratories, Diagnostic Division, Chicago, USA)

The assay uses the ligase chain reaction for amplification of a target sequence within the chromosomal gene that codes for protein antigen b, which is specific for members of the MTBC. The overall sensitivity and specificity of the assay was 74 and 98 %, respectively. For smear-positive samples the sensitivity reached 100 %, but for smear-negative it was only 57 %. In a multicenter evaluation of Amplicor and LCx, the sensitivity of both methods was significantly better when only respiratory specimens were considered (78 and 88 %, respectively). When non-respiratory samples were used, the sensitivity was reduced to 59 % for Amlicor and 65 % for LCx [23, 24].

Identification of Mycobacterial Species from Culture by Molecular Methods

For many decades, the identification of Mycobacterial isolates was performed on the basis of biochemical reactions and phenotypic characteristics, which are time-consuming and often give ambiguous results. The molecular methods for Mycobacterial identification are now providing rapid and accurate results. Several methodologies have been used.

PCR-based Sequencing

This methodology is considered the "gold" standard for identification of mycobacteria. Initially, PCR amplification takes place followed by sequencing of the amplicons in an automatic sequencer. The identification of an unknown strain is completed by comparison of the nucleotide sequence with a library of known sequences. The databases for this purpose are available in the internet. Such databases are the GenBank, the Ribosomal Differentiation of Medical Microsystems database (RIDOM) and that of the European Molecular Biology Laboratory (EMBL). Several target genes have been used for the procedure but the most common is the 16S rRNA gene [25]. This gene has been widely sequenced because it contains both highly conserved and variable regions. It consists of more than 1500 bp but usually the first 500 bp are adequate for identification of a common *Mycobacterium* species. As previously mentioned, other important target genes are those encoding for the 65-kDa heat shock protein, the 32 kDa protein, the 16S–23S rRNA internal transcribed spacer (ITS) and the recA gene. The MicroSeq System (Applied Biosys-tems, CA) is a commercial 16S ribosomal DNA sequencing system. Evaluations of the MicroSeq System for routine use were performed by and with good results. The system offers the ability to mycobacteriology laboratories to identify many of the recently described mycobacteria [8, 26].

DNA Probe Technology

The DNA probe technology for identification of bacteria is one of the most successful molecular methods. The AccuProbe (Gen-Probe, San Diego, CA, USA) is the assay based on this technology that is used by the majority of clinical mycobacterial laboratories worldwide. It has the ability to identify a series of clinically important mycobacteria. These are *M. tuberculosis* complex, *M. avium* complex, *M. avium*, *M. kansasii*, and *M. gordonae*. The DNA probes are single-stranded DNA oligonucleotides labeled with acridinium ester that are complementary to the target, which is the rRNA. After sonication, the probes are

added to the broken Mycobacterial cells, to form a stable DNA–RNA complex. Following separation of the labeled complex from unhybridized DNA, the hybridization is detected by light emission in a luminometer. The AccuProbe can be used for both solid and liquid cultures. The method is easy to perform and only a sonicator and luminometer are required as equipment. The method has been widely evaluated with good results. The AccuProbe kits are rapid, highly sensitive and specific. The procedure can be completed in less than 2 h [27].

Line Probe Technology (Hybridization in Strips)

The line probe technology includes PCR (with biotinylated primers), reverse hybridization with different specific DNA probes, immobilized in parallel lines on a strip and colorimetric detection in an automated instrument. The banding pattern is indicative of the species of the isolate. The turnaround time is approximately 5 h. Two systems of line probe assay are commercially available: (a) the Inno LiPA Mycobacteria v2 and (b) the GenoType Mycobacterium:

Inno LiPA Mycobacteria v2, (Innogenetics, Ghent, Belgium)

This assay is based on the amplification of the mycobacterial spacer region 16S–23S rRNA for the simultaneous identification, in just one strip test, of the 17 most frequently isolated mycobacterial species: *M. tuberculosis complex*, *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. gordonae*, *M. fortuitum* complex, *M. malmoense*, *M. genavense*, *M. simiae*, *M. smegmatis*, *M. haemophilum*, *M. marinum/M. ulcerans* and *M. celatum*. Moreover, it has the ability to discriminate subgroups within *M. kansasii* and *M. chelonae* on the same strip. Mixed populations are easily identified. The overall sensitivity and specificity was 100 and 94.4 %, respectively. The probes specific for *M. fortuitum* complex, for *M. avium*-intracellular-scrofulaceum group and for *M. intracellulare* type two cross-reacted with several mycobacteria rarely isolated from clinical specimens [28].

GenoType Mycobacterium (Hain Lifescience, Nehren, Germany)

The procedure includes a multiplex PCR, followed by reverse hybridization and line probe technology. There are three kits: (a) the GenoType MTBC for distinguishing members of the *M. tuberculosis* complex, and (b) the GenoType Mycobacterium CM (Common Mycobacteria), and GenoType Mycobacterium AS (Additional Species) for NTM. The GenoType MTBC is based on the gyrB gene

polymorphism. The AS and CM assays use 23S rDNA as their target, thus the amplicon generated in the CM assay can be used for the AS assay without the need to perform a second PCR. The combined use of CM and AS can distinguish almost 30 different NTM including the following species: *M. avium*, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. ulcerans*, *M. peregrinum*, *M. xenopi*, *M. simiae*, *M. mucogenicum*, *M. goodii*, *M. celatum*, *M. smegmatis*, *M. genovense*, *M. lentiflavum*, *M. heckeshor-nense*, *M. szulgai*, *M. phlei*, *M. hemophilum*, M. gastri, *M. asiaticum* and *M. shimoidei*. The GenoType assays are rapid, easy-to-perform and easy-to-interpret. They have allowed clinical mycobacteriology laboratories to detect infrequent mycobacterial species, without the need of sophisticated techniques. The sensitivity and the specificity compared with 16S rRNA gene sequencing, were 97.9 and 92.4 % for CM and 99.3 and 99.4 % for AS, respectively [29, 30].

PRA Method (Polymerase Chain Reaction and Restriction Enzyme Analysis for Identification of Mycobacteria from Culture)

Telenti et al. [31] developed a rapid method, based on the amplification of the gene encoding the 65-kDa heat shock protein, followed by restriction-fragment-length polymorphism, using two restriction enzymes BstEII and HaeIII. Isolates from both solid and liquid cultures can be used. The fragments of the restriction enzyme digestion are analyzed by agarose gel electrophoresis and compared. The test can be completed within a day. It is a cost-effective and reliable assay that can be used by low-budget laboratories as well [31].

Pyrosequencing

Pyrosequencing^{TM} (Biotage, Uppsala, Sweden) technology is a novel method of nucleic acid sequencing-by-synthesis that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. The cascade of enzymatic reactions generates visible light. The generated light is proportional to the number of incorporated nucleotides. The method is optimal for determining short sequences (typically 20–30 bases of a DNA) rapidly and in a semi-automated format [32].

DNA Microarrays (DNA Chips)

The method is based on hybridization of fluorescently labeled PCR amplicons of an unknown strain to a DNA array, containing nucleotide probes for 16S ribosomal RNA gene. The hybridization pattern and intensity is determined

by scanning the chip using laser confocal fluorescence microscopy. The process of generating the target, its hybridization and reading on the chip requires approximately 2 h. It allows the identification of a large number of strains in one reaction. Sequences of regions from the 16S rRNA and rpoB loci had been developed. Unique hybridization patterns allowed for the identification of *Mycobacterium* species and the RMP-resistant alleles. A great disadvantage is, however, the current high cost of the required equipment [33].

Molecular Methods for Detecting Drug Resistance in Mycobacterial Strains

The emergence of strains of *M. tuberculosis* that are resistant to antimicrobial agents is a global problem. MDR-TB strains are generally defined as resistant to at least isoniazid (INH) and rifampin (RIF). Drug resistance develops either due to infection with a resistant strain, or as a result of inadequate treatment such as when a patient is exposed to a single drug, or because of selective drug intake, poor compliance, use of inappropriate non-standardized treatment regimens, irregular drug supply, poor drug quality, or rarely erratic absorption of medications. Knowledge of the susceptibility pattern of the isolate is crucial for successful therapy [34].

Existence of MDR-TB strains poses a serious threat to TB control programs in many countries. As per the estimates from the State representative drug resistance surveillance (DRS) survey in Gujarat and various district level DRS studies, the prevalence of MDR-TB in new smear positive pulmonary TB (PTB) cases is 1.1–5.3 % and 12–17 % amongst smear positive previously treated PTB cases. These data from India on MDR-TB had been collected from referral centers and institutions, and did not reflect the overall status of drug resistance problem in India. Prevalence of drug resistant tuberculosis varied considerably throughout the world and particularly in India. The reasons for this variation in different studies were poor study design, inadequate laboratory support and reporting systems [35]. The detection of resistant *M. tuberculosis* strains is generally performed by phenotypic assays, which require the isolate to be cultured in the presence of the different drugs. Despite the use of new liquid medium cultures [BACTEC TB-460 (Becton Dikinson, Sparks, MD), BACTEC MGIT 960, or Bact/Allert 3D (bioMerieux, Durham, NC)], the isolation of *M. tuberculosis* is still time consuming (i.e. 1–2 weeks) and leads to delays in obtaining susceptibility patterns. Rapid methods to detect resistance are necessary to optimize antituberculous treatment and avoid the transmission of resistant strains. Several molecular methods to detect resistance mutations in *M. tuberculosis* have been described as the molecular basis of

resistance to anti-TB drugs is becoming clearer. Resistance to anti-tuberculosis drugs is primarily due to mutations in a series of genes. The most frequently found mutations in RMP resistant isolates (96 %) are mutations in an 81-bp segment of the rpoB gene that encodes the β-subunit of RNA polymerase. In 75–85 % of INH resistant *M. tuberculosis* strains there are mutations in two genes, katG encoding catalase-peroxidase and inhA that takes part in fatty acid elongation. Mutations in the embB gene, which plays a role in the synthesis of lipoarabinomannan and arabinogalactan, are connected with ethambutol resistance. More than 70 % of pyrazinamide resistance is due to mutations in the pncA gene, which encodes for pyrazinamidase that converts pyrazinamide to its active form. Mutations in the 16S rRNA gene or the rpsL gene that encodes for the ribosomal protein 12S cause approximately 65–75 % of resistance to streptomycin. Molecular assays have the ability to detect these mutations and reveal the underlying resistance mechanism within hours [36–39].

PCR-DNA Sequencing

Among the many techniques used to identify drug resistance-associated mutations, automated DNA sequencing of PCR products has been the most widely applied. This is considered as the reference method for detection of drug resistance mutations. One important advantage of sequence-based approaches is that the resulting data are virtually unambiguous because a resistance-associated mutation is either present or absent. Initially, the region that is most frequently associated with resistance mutations is amplified. Then, the amplicons are sequenced in order to determine the presence or absence of a specific mutation. The expensive equipment and the expertise needed are probably the most serious drawbacks of the method.

Hybridization-based Techniques

Line Probe Technology

There are two commercially available assays: the Inno-LiPA Rifotuberculosis (Inno-LiPA RFTB; Innogenetics, Belgium) and the GenoType MTBDR plus, (HAIN, Lifescience; Nehren, Germany). These assays use probes specific only for the *M. tuberculosis* complex and additionally for the detection of the mutations responsible for drug resistance.

Inno-LiPA RifTB (Innogenetics, Ghent, Belgium)

The kit contains 10 oligonucleotide probes: one specific for *M. tuberculosis* complex, five wild type probes (S1–S5), and four probes (R) for the detection of the most frequent

mutations that cause resistance to RMP. More than 95 % of the RMP-resistant strains have mutations within an 81-bp hot spot region (codons 507–533) of the rpoB gene. The R probes used are: R2: Asp516Val, R4: His526Tyr, R4b: His526Asp, R5: Ser531Leu. All the probes are immobilized on a nitrocellulose strip. A *M. tuberculosis* isolate is considered susceptible to RMP, if all the wild type probes give a positive signal and all the probes for resistance are negative. The absence of hybridization of one or more of the S probes is indicative of a mutation that may be identified by one of the R probes. Inno-LiPA Rif TB to be a reliable, simple and informative tool with absolute correlation (100 %) between its results and those obtained by the classic susceptibility testing, and the *M. tuberculosis* probe to be completely specific. Although the assay is recommended for use only on isolates where the amount of DNA is large, it can be used directly on clinical specimens after modifications of the protocol (nested PCR). Studies evaluating the line probe assay directly to clinical samples are limited. Inno LiPA assay provides a rapid and reliable detection of RMP resistance in 78.3 % of clinical specimens, compared to Bactec 460 and to rpoB gene sequencing [40, 41].

GenoType MTBDR plus (Hain Lifescience, Germany)

This assay offers the simultaneous identification of *M. tuberculosis* complex and detection of the most common resistance mutations in rpoB (RMP resistance), katG and inhA gene (INH resistance). This assay is the newer version of the GenoType MTBDR assay, which did not have the ability to detect INH resistance, caused by mutation in inhA. The previous and the new version of the assay could correctly identify rifampicin-resistance in 98.7 % of the cases, when compared to conventional susceptibility testing. Furthermore, the new GenoType MTBDR plus achieved better sensitivity for INH resistance (92 vs. 88.0 % of the previous version). GenoType MTBDR plus is a reliable tool for the detection of INH and RMP resistance either in strains or directly in smear positive specimens [42, 43].

Hybridization on DNA Chips

The DNA microarrays can also be used for rapid detection of mutations responsible for drug resistance. It can simultaneously detect different drug resistant mutations of *M. tuberculosis*. The DNA chip technology seems to be the most promising method for future investigation on drug resistance. A drug resistance detection DNA chip (CombiChip Mycobacteria, GeneIn, Pusan, South Korea) is available for identifying mutations associated with

resistance to INH and RMP (katG, inhA and rpoB genes). It is an oligonucleotide microchip coupled to PCR for the detection of mutations. The results were compared to DNA sequencing and culture based drug susceptibility tests. The CombiChip detected all RMP resistant isolates by screening 7 codons in the rpoB hot spot region and it correctly identified 84.1 % of INH resistant isolates by screening the katG codon 315 and inhA [44].

PCR-SSCP (Single-Strand-Conformation-Polymorphisms)

SSCP is based on the conformational distortion that a nucleotide substitution can cause in a single strand DNA fragment. This conformational change leads to an electrophoretic mobility different to that of the wild-type single-strand fragment. The procedure involves amplification of a DNA fragment including the region of interest by PCR, denaturation and running of this fragment in a polyacrylamide gel together with a denatured wild-type reference sample. Mobility shifts in the clinical sample indicate presence of mutation. This method has 100 % specificity for RMP and INH resistance and sensitivity for RMP 96 % and for INH 87 %, using four genetic regions (rpoB, katG, inhA, ahpC). A nested PCR- linked SSCP analysis is also used directly on sputum samples, to detect *M. tuberculosis* and determine RMP susceptibility. In this study, the target was a 157 bp portion of rpoB gene, which has been widely used for PCR-SSCP. The results were concordant with those of conventional drug susceptibility testing and DNA sequencing of culture isolates. Furthermore, the nested PCR-SSCP method enabled the direct detection of RMP resistance from primary clinical specimens. However, it should be noted that the assay does not identify the precise mutation and, consequently, the method is significantly less precise than sequencing. Its usefulness is restricted by extensive labour required and high level of technical skills [40].

Pyrosequencing

Rapid detection of rifampicin resistance using Pyrosequencing technology is available. The target was an 180-bp region of the *rpoB* gene, amplified by PCR and subjected to Pyrosequencing analysis, using four different sequencing primers in four overlapping reactions. The results were compared to other molecular methods (line probe assay and cycle sequencing) and the phenotypic BACTEC 460 method. There was full agreement with the molecular methods showing that pyrosequencing analysis offers high accuracy [45].

Real-time PCR Methodology

Real-time PCR has been used for detection of mutations responsible for INH and RMP resistance. This method

exhibited 85 and 98 % sensitivity for the detection of mutations responsible for INH and RMP resistance respectively and complete specificity for both antibiotics [46].

Cartridge-based Nucleic Acid Amplification Testing (CB-NAAT)

The Xpert MTB/Rif test is a cartridge-based fully automated NAAT (nucleic acid amplification test) for TB case detection and rifampicin resistance testing. It simplifies molecular testing by fully integrating and automating the three processes (sample preparation, amplification and detection) required for real-time PCR-based molecular testing. The Xpert MTB/RIF test is currently the only molecular test of its kind and uses a cartridge containing all elements necessary for the reaction, including lyophilized reagents, liquid buffers and wash solutions [47]. Revised National tuberculosis control programme (RNTCP) in association with Foundation for Innovative New Diagnostics (FIND), launched CBNAAT (cartridge based nucleic acid amplification testing) which aims at a multi-site programme-based phased-implementation of collecting evidence for scale-up of cartridge based nucleic acid amplification testing for the diagnosis of tuberculosis and multi-drug resistant tuberculosis in India. Focus has been made the project available at district, sub-district, medical colleges or private sector in the near by future. Advantages of this highly-automated system requires only a minimally trained staff to generate drug sensitivity testing. The results, can be applied to any pulmonary specimen (irrespective of smear status), which are free of cross-contamination risk and are internally quality-assured, available within 2 h. Where RNTCP has deployed CBNAAT for TB/MDR-TB detection, results can be used for patient management as per the latest available guidance from the programme [48]. The RNTCP National Strategic Plan 2012–2017 also proposed to buy 1000 CBNAAT machines and testing all confirmed TB cases for RIF resistance at the district level. It is unknown whether a larger investment and deployment could accelerate reductions in incidence. This project is expected to inform the real impact of new rapid diagnosis on future TB control programs.

Conclusion

Laboratory diagnostic tests for TB have evolved rapidly as new technology has been introduced and provide unprecedented opportunities for the rapid, sensitive and specific diagnosis of *M. tuberculosis* itself in clinical samples and the status of its drug sensitivity. Some of the molecular tests have now been incorporated into routine laboratory usage allowing the physicians to more rapidly initiate proper drug regimens. Turnover time, specificities and sensitivities of various commercially available assays for molecular diagnosis of tuberculosis differ. Amplicor MTB test (Roche Molecular Systems, Basel, Switzerland) is with 100 % specificity whereas sensitivity ranges between 90 and 100 % with 6–7 h of turnover time. On the other hand, sensitivity of Amplified MTD (GenProbe, San Diego, CA, USA), for respiratory specimens is between 90.9 and 95.2 % and specificity stuck between 97.6 and 100 %, with 2.5 h of turnaround time. BD probe Tec ET (Becton Dickinson, Sparks, MD) is with 98.5–100 % sensitivity and specificity is variable (0.33–100 %) for smear negative cases, with an estimation of 3.5–4 h for the completion of test. Genotype Mycobacteria direct assay (Hain Lifescience, Nehren, Germany) shows, 92 and 100 % sensitivity and specificity respectively, where as LCx MTBC (Abbott Laboratories, Diagnostic Division, Chicago, USA) assay is with 74 and 98 % of sensitivity and specificity respectively, but sensitivity is 100 and 57 % for smear positive and smear negative cases respectively. For Inno LiPA Mycobacteria v2 assay (Innogenetics, Ghent, Belgium), overall sensitivity and specificity is 100 and 94.4 % respectively. Sensitivity and specificity by Geno-Type *Mycobacterium* assay (Hain Lifescience, Nehren, Germany) for common *Mycobacterium* is 97.9 and 92.4 %, whereas for additional species, it is 99.3 and 99.4 % respectively, with 7–8 h as turnover period. CB-NAAT results can be obtained within 2 h as compared to solid LJ media and Liquid Culture (MGIT) which takes around 84 and 42 days respectively. Due to certain limitations in these molecular tests, however, conventional tests such as those based on microscopy and culture should be applied in parallel with any new molecular tests for diagnosis of TB. In addition, particular emphasis should be applied to quality control and quality assurance programs in clinical laboratories which employ any new diagnostic approaches. It should be noted that, although the traditional methods for diagnosis of tuberculosis, such as microscopy and culture, cannot be replaced by direct amplification tests, these assays provide a major improvement in terms of speed. They could be used for rapid confirmation in patients with smear-positive samples. In smear-negative patients, the amplification tests are recommended only when suspicion for TB is high and always in relation to clinical data. For extra-pulmonary specimens, the use of the amplification methods is advocated, since rapid and accurate laboratory diagnosis is critical (e.g. tuberculosis meningitis). The specificities of amplification methods are very high, whereas, the sensitivities vary greatly. Multiple specimens from the same patient, proper decontamination procedures, improved extraction methods and use of internal controls decrease the frequency of false-negative results.

References

1. Pablos-Mendez A. Working alliance for TB drug development, Cape Town, South Africa, February 8th, 2000. Int J Tuberc Lung Dis. 2000;4:489–90.

2. World Health Organization (WHO). Anti-tuberculosis drug resistance in the world third global report. The WHO/IUATLD Global project on anti-tuberculosis drug resistance surveillance 1999–2002. Geneva: WHO; 2004 (WHO/HTN/TB/2004.343).

3. Amdekar Y. Tuberculosis-persistent threat to human health. Ind J Pediatr. 2005;72:333–8.

4. Centers for Disease Control and Prevention (CDC). Trends in tuberculosis-United States, 2005. MMWR Morb Mortal Wkly Rep. 2006;55(11):305–8.

5. Dye C, Espinal MA, Watt CJ, Mbiaga C, Williams BG. World-wide incidence of multidrug-resistant tuberculosis. J Infect Dis. 2002;185:1197–202.

6. Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon JL, Gicquel B. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. J Clin Microbiol. 1990;28:2668–73.

7. Kirschner P, Rosenau J, Springer B, Teschner K, Feldmann K, Bottger EC. Diagnosis of mycobacterial infections by nucleic acid amplification: 18-month prospective study. J Clin Microbiol. 1996;34:304–12.

8. Kox LF, van Leeuwen J, Knijper S, Jansen HM, Kolk AH. PCR assay based on DNA coding for 16S rRNA for detection and identification of mycobacteria in clinical samples. J Clin Microbiol. 1995;33:3225–33.

9. Shinnick TM, Good RC. Diagnostic mycobacteriology laboratory practices. Clin Infect Dis. 1995;21:291–9.

10. Noordhoek GT, Kolk AH, Bjune G, Catty D, Dale JW, Fine PE, et al. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. J Clin Microbiol. 1994;32:277–84.

11. Newton CR. Setting up a PCR laboratory. In: Newton CR, editor. PCR essential data. New York: Wiley; 2005. p. 216.

12. Kwok S, Higuchi R. Avoiding false positives with PCR. Nature. 1989;339:237–8.

13. Thornton CG, Hartley JL, Rashtchian A. Utilizing uracil DNA glycosylase to control carryover contamination in PCR: characterization of residual UDG activity following thermal cycling. Biotechniques. 1992;13:180–4.

14. Drosten C, Panning M, Kramme S. Detection of *Mycobacterium tuberculosis* by real-time PCR using pan-mycobacterial primers and a pair of fluorescence resonance energy transfer probes specific for the *M. tuberculosis* complex. Clin Chem. 2003;49:1659–61.

15. Parashar D, Chauhan DS, Sharma VD, Katoch VM. Application of real time PCR technology in mycobacterial research. Indian J Med Res. 2006;124:385–98.

16. Bergmann JS, Woods GL. Clinical evaluation of the Roche AMPLICOR PCR *Mycobacterium tuberculosis* test for detection of *M. tuberculosis* in respiratory specimens. J Clin Microbiol. 1996;34:1083–5.

17. Soini H, Musser JM. Molecular diagnosis of mycobacteria. Clin Chem. 2001;47:809–14.

18. Pfyffer GE, Kissling P, Wirth R, Weber R. Direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens by a target-amplified test system. J Clin Microbiol. 1994;32:918–23.

19. Gamboa F, Fernandez G, Padilla E, Manterola JM, Lonca J, Cardona PJ, et al. Comparative evaluation of initial and new versions of the Gen-Probe amplified *Mycobacterium tuberculosis* direct test for direct detection of *Mycobacterium tuberculosis* in respiratory and non respiratory specimens. J Clin Microbiol. 1998;36:684–9.

20. Barrett A, Magee JG, Freeman R. An evaluation of the BD ProbeTec ET system for the direct detection of Mycobacterium tuberculosis in respiratory samples. J Med Microbiol. 2002;51:895–8.

21. Piersimoni C, Scarparo C, Piccoli P, Rigon A, Ruggiero G, Nista D, et al. Performance assessment of two commercial amplification assays for direct detection of *Mycobacterium tuberculosis* complex from respiratory and extra- pulmonary specimens. J Clin Microbiol. 2002;40:4138–42.

22. de Luna FFA, Ruiz P, Gutierrez J, Casal M. Evaluation of the GenoType mycobacteria direct assay for detection of *Mycobacterium tuberculosis* complex and four atypical mycobacterial species in clinical samples. J Clin Microbiol. 2006;44:3025–7.

23. Moore DF, Curry JI. Detection and identification of *Mycobacterium tuberculosis* directly from sputum sediments by ligase chain reaction. J Clin Microbiol. 1998;36:1028–31.

24. Tortoli E, Tronci M, Tosi CP, Galli C, Lavinia F, Natili S, et al. Multicenter evaluation of two commercial amplification kits (Amplicor, Roche and LCx, Abbott) for direct detection of *Mycobacterium tuberculosis* in pulmonary and extrapulmonary specimens. Diagn Microbiol Infect Dis. 1999;33:173–9.

25. Harmsen D, Rothganger J, Frosch M, Albert J. RIDOM: ribosomal differentiation of medical micro-organisms database. Nucleic Acids Res. 2002;30:416–7.

26. Cloud JL, Neal H, Rosenberry R, Turenne CY, Jama M, Hillyard DR. Identification of *Mycobacterium* spp. by using a commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. J Clin Microbiol. 2002;40:400–6.

27. Badak FZ, Goksel S, Sertoz R, Nafile B, Ermertcan S, Cavusoglu C, et al. Use of nucleic acid probes for identification of *Mycobacterium tuberculosis* directly from MB/BacT bottles. J Clin Microbiol. 1999;37:1602–5.

28. Tortoli E. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. Clin Microbiol Rev. 2003;16:319–54.

29. Russo C, Tortoli E, Menichella D. Evaluation of the new GenoType *Mycobacterium* assay for identification of mycobacterial species. J Clin Microbiol. 2006;44:334–9.

30. Neonakis IK, Gitti Z, Petinaki E, Maraki S, Spandidos DA. Evaluation of the Genotype MTBC assay for differentiating 120 clinical *Mycobacterium tuberculosis* complex isolates. Eur J Clin Microbiol Infect Dis. 2007;26:151–2.

31. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J Clin Microbiol. 1993;31:175–8.

32. Tuohy MJ, Hall GS, Sholtis M, Procop GW. Pyrosequencing as a tool for the identification of common isolates of *Mycobacterium* sp. Diagn Microbiol Infect Dis. 2005;51:245–50.

33. Gingeras TR, Ghandour G, Wang E, Berno A, Small PM, Drobniewski F, et al. Simultaneous genotyping and species identification using hybridization pattern recognition analysis of generic Myco-bacterium DNA arrays. Genome Res. 1998;8:435–48.

34. Sharma SK, Mohan A. Multidrug-resistant tuberculosis. Indian J Med Res. 2004;120:354–76.

35. Thomas A, Ramachandran R, Rehaman F, Jaggarajamma K, Santha T, Selvakumar N. Management of multi-drug resistance tuberculosis in the field: tuberculosis Research Centre experience. Indian J Tuberc. 2007;54:117–24.

36. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis. 1998;79:3–29.

37. Zhang M, Yue J, Yang YP, Zhang HM, Lei JQ, Jin RL, et al. Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. J Clin Microbiol. 2005;43:5477–82.

38. Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR Jr, et al. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of embB mutations. Antimicrob Agents Chemother. 1997;41:1677–81.

39. Fukuda M, Koga H, Ohno H, Yang B, Hirakata Y, Maesaki S, et al. Relationship between genetic alteration of the rpsL gene and streptomycin susceptibility of *Mycobacterium tuberculosis* in Japan. J Antimicrob Chemother. 1999;43:281–4.

40. Kim BJ, Lee KH, Park BN, Kim SJ, Park EM, Park YG, et al. Detection of rifampin-resistant *Mycobacterium tuberculosis* in sputa by nested PCR-linked single-strand conformation polymorphism and DNA sequencing. J Clin Microbiol. 2001;39:2610–7.

41. Morgan M, Kalantri S, Flores L, Pai M. A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. BMC Infect Dis. 2005;5:62.

42. Johansen IS, Lundgren B, Sosnovskaja A, Thomsen VO. Direct detection of multidrug-resistant *Mycobacterium tuberculosis* in clinical specimens in low- and high-incidence countries by line probe assay. J Clin Microbiol. 2003;41:4454–6.

43. Somoskovi A, Dormandy J, Mitsani D, Rivenburg J, Salfinger M. Use of smear- positive samples to assess the PCR-based genotype MTBDR assay for rapid, direct detection of the *Mycobacterium tuberculosis* complex as well as its resistance to isoniazid and rifampin. J Clin Microbiol. 2006;44:4459–63.

44. Kim SY, Park YJ, Song E, Jang H, Kim C, Yoo J, et al. Evaluation of the CombiChip mycobacteria drug-resistance detection DNA chip for identifying mutations associated with resistance to isoniazid and rifampin in *Mycobacterium tuberculosis*. Diagn Microbiol Infect Dis. 2006;54:203–10.

45. Jureen P, Engstrand L, Eriksson S, Alderborn A, Krabbe M, Hoffner SE. Rapid detection of rifampin resistance in *Mycobacterium tuberculosis* by pyrosequencing technology. J Clin Microbiol. 2006;44:1925–9.

46. Piatek AS, Telenti A, Murray MR, El-Hajj H, Jacobs WR Jr, Kramer FR, et al. Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob Agents Chemother. 2000;44:103–10.

47. Marlowe EM, Novak-Weekley SM, Cumpio J, Sharp SE, Momeny MA, Babst A, Pandori M. Evaluation of the Cepheid Xpert MTB/RIF assay for direct detection of *Mycobacterium tuberculosis* complex in respiratory specimens. J of Clin Microbiol. 2011;49:1621–3.

48. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, et al. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. The lancet. 2011;377:1495–505.