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Advent in technologies for molecular diagnosis of tuberculosis

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ABSTRACT

The incidence of Tuberculosis varies considerably around the world and most Mycobacterial infections in developing nations are still being caused by Mycobacterium tuberculosis members. A quick and correct diagnosis is of great importance because of the high morbidity. Unfortunately, conventional bacteriological methods are time consuming, their sensitivity is low, and so treatment occasionally becomes empirical. PCR method has high specificity in identifying M. tuberculosis in various specimens. 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: Master Mix, Nested PCR, In-House PCR assays, Nucleic acid Amplification.

INTRODUCTION

Mycobacterium tuberculosis (MTB) is a fairly large non motile, rod-shaped bacterium distantly related to the Actinomycetes. Many non pathogenic mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The rods are 2-4 µm in length and 0.2-0.5 µm in width [1]. *M. tuberculosis* is an obligate aerobe. MTB complexes are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, a physiological characteristic that may contribute to its virulence. MTB colonies are small and buff colored when grown on either medium. Tuberculosis is a major socioeconomic burden in India, affecting 14 million people, mostly in the reproductive age group (15–45 years). It is involved in about 5–16% of cases of infertility among Indian women. Current review focuses on the advents in the PCR for the molecular diagnosis of tuberculosis.

In-house PCR for the detection of Mycobacterium from clinical specimens

Diagnostic methods for *M. tuberculosis* have recently improved, and nucleic acid-based amplification techniques (NAATs) now allow for rapid and sensitive detection in clinical diagnostics settings. The insertion sequence IS6110 has been successfully used as a target for PCR amplification in different clinical samples by many investigators. The IS6110, a molecular target amplifying 123 base pairs amplicon is found only in Mycobacterium tuberculosis and not in other members of MTB complex. Therefore, IS6110 plays a crucial role only in differentiating Mycobacterium tuberculosis from its own members. Although this sequence is only used in foreign countries where there is chance of infection by every member of MTB complex. However one of the predominant genes in MTB complex is mpb64 gene (encoding for mannose binding protein) that is present in every member of MTB complex and has the potential

for diagnostic use by differentiating the MTB from MOTT in different clinical samples. In India, only Mycobacterium tuberculosis infects in the majority of cases. MPB64 gene as well as IS6110 can be used in amplification for the molecular diagnostic purposes. PCR has nearly about 98% sensitivity in detecting the pathogen in extra-pulmonary specimens. Only small numbers of bacilli are needed to cause tuberculosis in extra-pulmonary specimens but PCR amplification of for MTB complex in extra pulmonary samples like synovial fluid, CSF, pleural fluid and bone marrow renders the method capable of detecting such small numbers of bacilli in specimens which would otherwise be undetectable using other conventional methods for the diagnosis of tuberculosis. 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 IS6110 and the 16S rDNA are the most common targets used. Other regions used for amplification includes 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[2-4]. In research laboratories, nucleic acid amplification tests (NAATs) are very sensitive and can detect as few as 3 bacilli. These tests are highly sensitive in clinical samples and studies have shown that sensitivity and specificity ranging as high as 90-100%. NAATs may be tested on any specimen thought to contain bacilli (blood, urine, cerebro spinal fluid (CSF) but there is even less sensitivity reported in extra pulmonary specimens. Sensitivity is improved when multiple sampling are tested, because not all samples necessarily contain detectable nucleic acid [5, 6]. 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 [7]. 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 [8]. 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 [9]. 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

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 *Mycobacterium 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 [10, 11].

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 [12]. 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 hours. 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[12].

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.

Spacer Oligonucleotide Typing or Spoligotyping

Spacer oligonucleotide typing or spoligotyping was the first widely adopted PCR-based method for genotyping spoligotyping based on the direct repeat (DR) region of *M. tuberculosis*, has advantages over 1*S6110*: (i) Small amount of DNA sample is needed for clinical examination and strain testing from liquid culture. Only digital number is used to express the results. It can be used for genotyping of isolates with less than 6 copies of IS6110. Spoligotyping data which is represented in absolute terms (digitally) can be readily shared among laboratories there by enabling the creation of large international database (SpolDB). The method has been very successful in providing a tool for the rapid acquisition of MTB genotyping information and for the establishment of a global picture of MTB diversity It is highly reproducible and has been developed into a high –throughput assay for large molecular epidemiology surveys [13].

Amplified MTD

Amplified *Mycobacterium 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 2,000) 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 hours. 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%.

CONCLUSION

Some of the molecular tests have now been incorporated into routine laboratory usage allowing the physicians to more rapidly initiate proper drug regimens. 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 extrapulmonary 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.

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