POLYMERASE CHAIN REACTION IN THE DIAGNOSIS OF TUBERCULOSIS

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ABSTRACT

A rapid, sensitive, specific and yet economical method for the diagnosis of *M. tuberculosis* and other mycobacteria in clinical specimen is a desperate and urgent requirement of the day in the laboratory diagnosis and hence management of tuberculosis. This need is further accentuated by emerging diseases like multi drug resistant tuberculosis, tuberculosis in AIDS patients and opportunistic mycobacterial infections, which do not respond to conventional anti TB therapy. Molecular methods, particularly PCR based detection of *M. tuberculosis*, has come a long way since it was first described about fifteen years ago. Several probes have been developed and some of them, particularly the IS6110 and TB400 have been validated on several clinical samples. The latter has been validated on a variety of clinical specimens along with a simple sample processing method. Polymerase chain reaction based diagnosis of *M. tuberculosis* has been introduced as one of the routine/confirmatory tests in clinical microbiology laboratory in some countries like Canada, the United States and the United Kingdom several years ago. The possibility of introducing PCR based direct diagnosis of drug resistance is being explored in some laboratories, particularly for drugs like rifampicin. The evolution and application of PCR for diagnosis of *M. tuberculosis* is being analysed and discussed in this review.

KEY WORDS: Polymerase chain reaction, tuberculosis, DNA probes.

Tuberculosis and its impact on public health

Tuberculosis (TB) is an important public health problem worldwide. About a third of the world's population (about 1.7 billion) is infected with *M. tuberculosis*. The great majority of the world's population and thus the majority of the infected people reside in developing countries. As 75% of infected people are less than 50 years of age (the most productive period in life), TB has the most devastating effect in the developing world. According to WHO, TB causes 25% of avoidable adult deaths in the developing world and worldwide

about 2.5 million people died of TB in 1992 alone of which 4,50,000 deaths are from children less than 15 years old from developing countries.

The magnitude of the problem is enormous in view of the epidemic of HIV infection that has radically changed the epidemiology of TB (1). Eradication of TB in most developed countries will not be substantially influenced by HIV infection because the percentage of individuals in the 20-50 years age group with *M. tuberculosis* infection is low. The close connection between HIV infection and TB was shown in the USA (2) and particularly in 1992 in Africa (3) where 15-70% of patients presenting with TB are HIV infected. Further, TB as a concurring problem in HIV infection is thought to be due to endogenous reactivation of the tubercle bacilli, as a result of the loss of immune control

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mechanisms in HIV infected individuals. This, coupled to outbreaks of multiple drug resistant TB warrants stringent measures to combat this disease which is increasing at alarming rates.

Control of the disease should not only mean to treat the clinically proven cases of TB but more important to identify early those individuals who are in the early stages or at risk of developing the disease. An important key to successful control of TB is, thus, timely diagnosis. Additionally, because of the differences in the drug sensitivity pattern, it has become necessary to identify *M. tuberculosis* from other species, as a number of non-tuberculous mycobacteria such as *M. avium* and *M. intracellulare* have become opportunistic pathogens in AIDS patients. The timely identification of persons infected with *M. tuberculosis* and the rapid laboratory confirmation of tuberculosis are two key ingredients of effective public health measures to combat the resurgence of tuberculosis and the outbreaks of nosocomially transmitted tuberculosis (4).

The timely identification of infected persons is important because only about 10% of infected immuno competent persons will develop active tuberculosis during their lifetime and the overt disease symptoms appear fairly late in the infection. Appropriate preventive chemotherapy for persons whose infection is progressing toward overt disease can dramatically reduce the development of infectious tuberculosis (5). In fact, preventive therapy for these infected persons may be the most cost-effective way to reduce the general public health impact of tuberculosis in populations in which the incidence is low. On the other hand, rapid diagnosis and treatment/monitoring of infected individuals would help to reduce the development of active disease but also help to reduce the spread of the disease in an endemic population.

Conventional methods of laboratory diagnosis of tuberculosis

Current procedures for identifying infected persons are limited by a lack of sensitivity or specificity or both and the laboratory confirmation of an infection may require several weeks. Indeed,

a workshop of tuberculosis experts conducted a few years ago concluded that all "current techniques for the diagnosis of *M. tuberculosis* are beset by serious limitations" and that "the rapid and specific diagnosis of *M. tuberculosis* is one of the most pressing needs in efforts to eradicate the disease (6).

The usefulness of the historic tuberculin test is limited by its lack of specificity for tuberculosis and by its inability to distinguish between active disease, prior sensitisation by contact with *M. tuberculosis*, BCG vaccination and cross sensitization by other *Mycobacterium* species (7, 8). The tuberculin skin test also fails to detect a substantial proportion of persons co-infected with HIV and *M. tuberculosis* and of persons with advanced tuberculosis. Despite these limitations, the tuberculin skin test is still a very useful tool, especially when conversion to a positive skin test is used to identify recently infected persons for preventive therapy or to help confirm a physicians' suspicion of tuberculosis.

Confirmatory diagnosis of TB requires the identification of *M. tuberculosis* in the patients' samples. Conventionally laboratory diagnosis starts with microscopic examination of smears for the presence of acid fast bacilli followed by culture isolation and biochemical testing of the isolated mycobacterium for the species identification. The entire process often requires 4-6 weeks from specimen collection to species identification, primarily because of the slow growth rate of mycobacteria. Determination of drug susceptibility of an culture isolate can add 2-4 weeks to this already long process.

Acid fast bacilli smear examination is time tested and remains extremely valuable in testing sputum for *M. tuberculosis* while other specimen like bronchial lavage, blood and other aspirates may also be used. The diagnostic yield of this technique is limited primarily because the number of organisms required for a positive smear is between 5000 and 10,000 per ml for reliable detection. The immediate diagnosis of tuberculosis by direct sputum examination ranges from 40 to 75%. However, today with the widespread nature of TB

worldwide and occurrence of other mycobacterial infections, the AFB smear test does not offer any definitive advantage in terms of species identification and differential diagnosis (9).

Rapid tests for the diagnosis of tuberculosis

Several methods that allow rapid determination of cultured organisms have been developed recently. Though traditional culture methods take 4-6 weeks and has only 40-60% sensitivity, modern improved culture methods like BACTEC TB 460 which uses a special liquid media for rapid identification of growth of *M. tuberculosis* (Becton Dickinson, USA) have reduced the time lag to two weeks and have also increased the sensitivity up to 80%. Another rapid method depends on HPLC analysis of the mycolic (10) components of the isolated mycobacteria and this method can provide definitive species identification for more than 50 members of the genus in less than four hours after the culture is made available. The test is thus not only rapid but can also potentially replace an entire battery of biochemical tests.

A second family of rapid test involves nucleic acid hybridisation (11) which are now commercially available. These assays display sensitivity and specificity close to 100% for the detection and species identification of culture isolates and can usually be completed in a few hours. One drawback is that they require 10^5 organisms to give clear-cut results and therefore can mostly be applied only after culture isolation. Combining nucleic acid identification system with a rapid culture system such as BACTEC can shorten the time required for detecting and identifying *M. tuberculosis* to as little as a week (12).

Of the numerous immunological methods for diagnosis of TB, one promising test is the ELISPOT test (13). It measures the antibody secreting cells in response to stimuli from a specific antigen. However, owing to its cost and requirement for special expertise, this test though useful, would not be suitable for use in a routine clinical laboratory. The current methods of laboratory diagnosis of tuberculosis have been reviewed by Heifetes and Good recently (14).

Why molecular methods?

Acid fast smear, culture and radiological examination have been and still remain the methods of diagnosis of human TB in many developing countries even today. In the western world, molecular methods have come to occupy an important niche in the diagnosis and management of tuberculosis, in addition to being used in the diagnosis of other bacterial, viral and parasitic pathogens. Molecular methods, mainly probe based assays and PCR are of great value today in providing a timely diagnosis due to their sensitivity and speed in providing results. Even though they are expensive currently they will help in the overall savings to the patient by providing a timely diagnosis. By providing reliable and definitive identification, these methods would help in the patient management while monitoring drug therapy and prophylactic measures. Molecular methods of diagnosis are being provided as a routine in many government hospitals and laboratories in countries like Canada and USA.

In India, conventional methods are being followed widely in many hospitals and laboratory. This is because knowledge about the clinical application of nucleic acid probes based assays is limited and consequently the use of these probes is also limited to a few premier national and referral hospitals and some corporate diagnostic service centres. Thus, culture isolation and characterisation is still the gold standard for the diagnosis of many diseases in India though in the west the nucleic acid based assays are becoming routine for laboratory diagnosis of fastidious pathogens like *M. tuberculosis*, *Chlamydia* and HIV.

With TB meningitis and HIV infection quick diagnosis is important as a delay of several weeks may prove fatal. Thus there is still a critical need for assays to detect *M. tuberculosis* infection prior to the onset of clinical symptoms. Such assays should be applicable to immuno compromised and immuno competent adults and children. Such a test should be able to differentiate persons with active infection from persons who have protective immunity against any prior infection. With the great progress

made recently in the understanding of the molecular and genetic basis of drug resistance in mycobacteria, supported by complete knowledge of genomic sequence of *M. tuberculosis* (15). As the incidence of resistance to currently available drugs is increasing, PCR can also be used to rapidly determine the presence of mutations in drug targets.

Polymerase chain reaction in tuberculosis management

A rapid diagnostic method of *M. tuberculosis* should enable diagnosis directly from the clinical specimen and often the specimen contains very small number of bacilli. Thus, the direct detection of mycobacteria in specimens requires an extremely sensitive and specific assay and this became a reality only a decade ago. With the invention of PCR in 1985, several other amplification technologies have been developed (Table 1) and they have made a major impact on diverse areas of both basic and applied research.

Table 1.

Amplification Method	Reference
Polymerase Chain Reaction (PCR)	(16), (17).
Ligase Chain Reaction(LCR)	(18)
Transcription based amplification reaction	(19), (20)
Cycling probe	(21)
Reaction QB replicase amplification	(22), (23)
Strand Displacement amplification	(24)
Spacer Oligo nucleotide typing	(25)

Among these, PCR has made the greatest impact both as a research and diagnostic tool. In general, diagnostic molecular microbiology, especially with reference to infectious disease, has been taken over by molecular methods of diagnosis including the probe based diagnosis and PCR (for a review see 26). These molecular diagnostic techniques have been applied to a number of

bacterial, viral, fungal and parasitic pathogens and genetic diseases.

Principle of PCR

Polymerase chain reaction uses oligo nucleotide primers to direct the amplification of target nucleic acid sequences via repeated rounds of de-naturation, primer annealing and primer extension by a thermostable enzyme DNA polymerase. The specificity of the amplification process lies in the choice of primers. It enables making millions of copies of a specific DNA sequence which may be a gene, part of a gene or simply a stretch of nucleotides with a known DNA sequence. A specimen that may contain the sequence of interest is heated to denature double stranded DNA. The specific synthetic oligo nucleotide primers bind to the unique DNA sequences of interest and a heat stable DNA polymerase extends the primers to create a complete and complementary strand of DNA. This process is typically repeated 25-40 times thereby creating millions of copies of the target DNA sequence. If the target sequence were not present in the sample, the primers have nothing to bind to and no amplification occurs. Thus the PCR offers potentially unmatched sensitivity and specificity. The diagnostic implications of such a technique is enormous. The amplified sequence can then be detected under ultra violet light by gel electrophoresis after staining with ethidium bromide and/or by hybridisation to radioactive or non-radioactive probes after transfer onto synthetic membranes. Polymerase chain reaction has already undergone enormous improvements since it was first introduced. Particularly so in primer selection criteria, contamination control, amplified product detection methods, equipment design and automation of the assay system. The basic principle of PCR is illustrated in the Fig. 1. and a general scheme for PCR analysis of a sample is given in Fig. 2

PCR for laboratory diagnosis of tuberculosis

A number of PCR based assay systems have been reported for diagnosis of mycobacterial

a: heat denaturation and primer annealing;
b: primer extension and formation of new strand.

Fig. 1 Scheme of Polymerase Chain Reaction

species including *M. tuberculosis*. *M. tuberculosis* specific amplification performed in the laboratory conditions with specific isolates show very good

performance with reference to specificity and sensitivity to a level of 1 - 10 bacilli per ml of the specimen. In general, the amplification process can be completed in 2-4 hrs of obtaining a processed specimen and the detection assay can be completed in an additional 2 to 24 h.

Most of the published PCR based tests have been shown to work well only in the research laboratory. However, there have been several attempts to address the transfer of these PCR tests to the clinical laboratory (27-29). In these studies, the bacteria were pelleted directly from the sputa by simple centrifugation and lysed by boiling in a simple low salt detergent (30) and the lysates were used for PCR amplification using IS6110 probe. The general conclusions of this extensive study was (i) this PCR based assay easily fit into the daily routine of the clinical laboratory with a turn around time of 24-36 h from the time of specimen collection (ii) existing clinical laboratory personnel were able to

Fig. 2 Process flow for PCR analysis

perform the assay and (iii) false positive results due to end product contamination were relatively rare with the use of appropriate precautions. Also in the clinical laboratories, the specificity of the PCR based assay was essentially 100% though its sensitivity was less than that observed in the research laboratory. Nonetheless, the overall positive predictive value of the test was 98% and most important, the PCR based assay correctly confirmed *M. tuberculosis* infection in all patients for whom three or more sputum specimens were tested.

The main problem in using PCR based techniques for sensitive detection of mycobacteria in clinical samples is the quantitative extraction of nucleic acids. Since they contain a unique lipid rich cell wall, mycobacteria are rather difficult to lyse. Several methods have been published for lysing and extracting nucleic acid from mycobacteria (see 26). However, many protocols published so far have disadvantages i.e., they are, either laborious, insensitive, or complicated by the presence of inhibitors. Therefore, a simple method for quantitative extraction or exposure of nucleic acid was still required. One such simple method was reported for the lysis of mycobacteria (30) present in sputum, which involved washing the cells present in the sputum repeatedly with a buffered detergent by simple centrifugation and then boiling the pellet in small volume (not more than 100 μ l) of the same buffered detergent. The entire sample processing could be done in a microfuge tube (1.5 ml capacity). This process yielded lysates suitable for PCR amplification. Later, this method was tested and appropriately adopted in India, successfully for different clinical specimen like blood (31), lymph nodes, paraffin embedded tissues, fresh biopsies and formalin fixed material (32). This sample lysis procedure is simple in terms of technique, ease of application, consistently yielding inhibitor free lysates for PCR amplification and above all, requiring only simple inexpensive equipment and reagents normally available in any laboratory.

Descriptions of numerous PCR based assays for the detection and identification of *M.*

tuberculosis have been published recently. For the purpose of performing PCR assays the functional significance of the amplification target is unimportant as long as the target is shown to be unique to the organism to be studied. Until methods for elimination of contaminating DNA within the Taq polymerase and reagents are developed, application of PCR for identification of unknown microorganisms will be limited. Appropriate controls for the reagents and for the process have to be included for every batch of sample. It has been estimated that commercially available Taq DNA polymerase lots are contaminated with 100-1000 genome equivalents of bacterial DNA per unit of enzyme (33). One way to avoid amplification of the unwanted and contaminating DNA, as visualised on agarose gel electrophoresis, is to restrict the PCR to 25-30 cycles. Thus, it appears as if PCR under these conditions is limited to samples, which show a large number of the organism of interest to ensure an excess of DNA of the target microorganisms (samples which are positive by microscopy indicate the presence of at least 10^4 target organisms per ml). A 100 fold excess of input DNA over unwanted contaminating DNA will ensure that the input DNA will be preferentially amplified. The proportion of amplified unwanted DNA within the total amplified DNA fragment (theoretically 1%) will not interfere with the interpretation of the results on the gel.

Microscopic analysis of acid fast smears of sputum samples or other appropriate clinical specimens, followed by culture confirmation, remains the cornerstone of the diagnosis of tuberculosis. These traditional bacteriological methods are slow, expensive, further, their sensitivity is quite low when clinical samples that contain small numbers of organisms are analysed. Thus, the rapid direct tests that detect *M. tuberculosis* nucleic acid are very attractive for the laboratory diagnosis of tuberculosis. Eventually nucleic acid amplification assays will replace traditional methods and provide a more timely diagnosis. Many target sequences have been used (Table. 2).

Table 2. Primer sequences for the detection of *M. tuberculosis* by PCR

Ref.	Probe Name	Nucleotide sequence 5' to 3'.
(30)	Tb400	TACGCATTCCGTCCACC GTCATGG CACCGGCGGAACACTG CAGG
(34)	IS6110	CCTGCGAGCGTAGGC GTC GG CTCGTCCAGCGCCGC TTCGG
(35)	Mpt40	CAACGCGCCGTCGGTGG CCCCCAGGCACCGC
(36)	Hance (1989)	GAGATCGAGCTGGAGGA TCC AGCTGCAGCCCAAAG GTG TT
(37)	pMAv	GATCAGGACCGCTGCCG CG CACTCGTCGGGAATGCC CTGC

but the most thoroughly evaluated assays target the *M. tuberculosis* specific repeated DNA element IS6110 (38, 34), (36) and RFLP analysis (38).

IS6110 element is a transposon of size 1361 bp. It was reported only in *M. tuberculosis* complex which comprises of *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti* (39, 40). The specificity and repetitive nature of IS6110 make it an ideal target for amplification by PCR in which a 123 bp product is amplified from the insertion sequence. An internal control has been constructed with the same primer set as the target sequence for amplification in PCR. Use of internal control DNA ensures the efficacy of each individual reaction and that the reaction is not inhibited by interfering substances in the reaction mixture. The PCR product of the internal control is 600 bp long and is easily distinguished from the 123 bp product. In a clinical trial this probe was used to

detect *M. tuberculosis* in sputum and other respiratory samples. When compared with bacteriological and clinical data, the results indicated that the assay was very sensitive and specific, (34).

There is variation in the copy number, being higher in (10-20 copies) in many strains of *M. tuberculosis* and lower in (1-5 copies) *M. bovis* strains (39). In addition, geographical variation is seen in the copy numbers and in the number of loci in the genome among the different strains of *M. tuberculosis*. Sampson *et al* (40) showed 66 unique IS6110 loci within the genome of *M. tuberculosis*. Fewer than 10 copies have been found in some isolates from Vietnam (41) and complete absence of the sequence has been reported in an isolate of an immigrant in Canada (42). Though this variability potentially could reduce the sensitivity and hence the reliability of using IS6110 or its primers for the detection of *M. tuberculosis* in specimens, it provides a means for tracking down the strain in an epidemic (43). Despite the wide use of the IS6110 as a useful tool for detection of *M. tuberculosis* in clinical specimens, the universal application of this probe or the primer sequence apparently has some limitations. Kent *et al* (44) reported amplification of homologous IS6110 sequence from several strains of non-tuberculous mycobacteria also. This observation was also substantiated later (45). Githui *et al.*, (46) countered this by showing that appropriate stringent conditions during hybridisation and washing helped to prove the specificity of this probe for *M. tuberculosis* complex. In spite of these limitations and unequivocal reports, the IS6110 based detection of *M. tuberculosis* for diagnosis, prognosis and epidemiology remains more popular than any other assay.

Another probe, TB400 was developed from one of the cloned fragments of a repeat family (47) and reported to be specific and sensitive for the diagnosis of *M. tuberculosis* and other members of its complex (30). From the same laboratory, a genus probe, pMAv17, was also developed (48) and validated on several clinical samples and simultaneously compared with two species specific probes for differential diagnosis of *M. tuberculosis* in those samples. The pMAv17 and TB 400

provided a good correlation and final result in terms of specificity and sensitivity compared to culture. The specificity of IS6110 was good (100%) and comparable (sensitivity 97%) to TB400 in this study.

Application of PCR assay in the management of tuberculosis

The reliability of a diagnostic method or assay for a given disease is expressed in terms of predictive value and the accuracy of positive and negative prediction is very important. The accuracy of positive prediction is of greatest importance to the clinician because a false positive test may lead to both an inappropriate therapy and cessation of further diagnostic efforts. The usefulness of negative predictive value is that it helps to avoid unnecessary toxicity due to drugs. The predictive accuracy of a test varies with the prevalence of the disease in the given population and when used as a screening test in the diagnostic laboratory attached to a clinic (where the prevalence could be 15-40%) a PCR assay could provide valuable information.

Pulmonary tuberculosis

Polymerase chain reaction based detection of *M. tuberculosis* has a role to play in the diagnosis of smear negative cases, early diagnosis of infection, cases which cannot be diagnosed by conventional methods, monitoring response to therapy (quantitative PCR) and hence determination of drug resistance in the patient. A lot of information is available on application of PCR for diagnosis of *M. tuberculosis* in sputum, see Shinnick and Jonas (26) for a review.

Extrapulmonary TB

Diagnosis of extra pulmonary tuberculosis is complicated by the difficulty in obtaining adequate material for examination. This frequently requires invasive or semi invasive techniques for sampling which often yield only low amount of material for laboratory investigations. Tubercular pleurisy and tubercular meningitis are two extra pulmonary diseases that frequently pose diagnostic challenges.

The reliability of AFB smear is very low (22-26%) with CSF samples obtained from tubercular meningitis cases (49-51). An ideal test should be simple, inexpensive and non-invasive. In such clinical circumstances, rapid and sensitive tests like PCR, which yield results even with low microbial load samples, is of great use as shown by De Lessence *et al* (52) in the diagnosis of tubercular pleurisy. When PCR was used on samples of smear negative pleural fluid from pleurisy cases, 60% of the cases were identified positive using IS6110 probe. Therefore, in circumstances like this and for conditions like pericarditis and others (53, 54) where conventional methods of laboratory diagnosis is unreliable, PCR would be a valuable adjunct (55).

Management of TB in children

It is extremely difficult to obtain respiratory samples from children and consequently, often the samples contain very low load of the pathogen. Therefore, an important application of PCR as a diagnostic tool will be to test such samples from children where the load of the pathogen could be below the sensitivity limit of conventional methods. Delacourt *et. al.* (56), Pierre *et. al.* (57) analysed gastric aspirates obtained from 22 apparently healthy children who showed only a positive skin test. The aspirates were negative by traditional microscopy and culture. Out of these samples, 25% (7/22 patients) were positive by PCR. When compared with clinical history all the seven patients had recent contact with a patient of active tuberculosis.

Drug Resistance in TB

With the recent progress in understanding the molecular and genetic basis of drug resistance in mycobacteria, the possibility of using PCR to rapidly detect the presence of drug resistance exists. This application remains a potentially invaluable tool in regions where the incidence of resistance to first line drugs is significantly high. Significant progress has been made recently in identifying mutations related to drug resistance in *M. tuberculosis* (58-60). Given the importance of

drug resistance in tuberculosis, it would be advantageous to determine drug susceptibility directly from the clinical specimen and avoid the time required for both primary culture (4-6 weeks) and subsequent culture for testing drug sensitivity (2-4 weeks) (see Fig.2). Great progress has been made in identifying mechanisms of resistance to isoniazid, ethionamide, rifampicin (61, 62). The mutations that cause rifampicin resistance have been identified and a rapid PCR based single strand conformation polymorphism (SSCP) electrophoresis assay has been developed for detecting rifampicin resistance in isolates of *M. tuberculosis* (63). This assay holds promise for the rapid identification of most rifampicin resistant strains directly from specimens. One critical limitation to this molecular approach of identifying drug resistance is that, a different assay will be required to detect each of the possible mechanisms of drug resistance if the resistance is due to multiple mechanisms as in the case of INH resistance.

In general, the results to date clearly demonstrate that the new systems are feasible and the promise of next generation of rapid diagnostic tests is held by the gene amplification systems particularly PCR. The US FDA approved PCR based kits have entered the market in the last 5-6 years and the market leader is La Roche Inc, USA, with its Amplicor range of products for several pathogens including *M. tuberculosis*, *M. avium* and *M. intracellulare*. Both automated and manual systems are available from the manufacturer. Therefore it is now possible for a clinical diagnostic laboratory to report the confirmation of an *M. tuberculosis* infection to the physician within hours of receiving a specimen. What is more, such rapid identification of *M. tuberculosis* and possibly its drug sensitivity pattern will allow the physician to promptly initiate proper infection control measures and appropriate anti-infective therapy.

Archival material screening and for screening paraffin blocks

Viability of the target organism is not necessary for the detection of its DNA by PCR and this has immense potential in screening fossils,

preserved (in formalin) biopsies, tissues embedded in paraffin blocks etc. Several studies reported the usefulness of PCR in identifying *M. tuberculosis* DNA from paraffin blocks of lymph nodes, intestinal biopsy etc (64, 32).

Screening Blood for cultures

Most blood samples, processed in the clinical microbiology laboratory, are sterile on culture due to various limitations in culture isolation. As the negative predictive value of PCR is almost absolute under ideal conditions (particularly avoiding inhibition of PCR), the primary use of PCR for bacteremia would therefore be to rapidly screen negative samples and avoid culture of such samples. The usefulness of PCR in this area of laboratory diagnosis is ever increasing (65, 66).

The PCR for the genus *Mycobacterium* and for the species *M. tuberculosis* originally developed in Boston (48, 30), were successfully validated along with the IS6110 system in India on samples of blood (31). Blood samples were collected from HIV positive patients with symptoms of pulmonary tuberculosis at the YRG Foundation for AIDS Care and Research, Chennai. These patients were routinely tested for TB and HIV. Results of PCR analysis of blood samples collected from 55 patients who had radiological changes typical of pulmonary tuberculosis, showed that the TB400 probe could pick up all the samples clinically diagnosed as TB positive while the IS6110 assay missed 5 samples. Though this study proves the utility of these probes on the Indian samples, it also reminds us of the implications of the geographic variation in using the IS6110 sequence as a probe as observed by others earlier in other countries (41, 42). An important outcome of this study was that, the sample processing protocol for blood samples proved to be very effective with no inhibition. This is an advantage, as blood samples are easier to obtain than sputa from children and AIDS patients. This PCR assay could be applied for diagnosis of *M. tuberculosis* infection in such patients.

The results of this study have shown that the sample processing procedure is applicable to

blood samples from HIV infected patients and our probe TB400 and pMAv17 may be used for the diagnosis of TB in such patients. In a reference laboratory where culture facility is available, the need to cut down the cost is very important. The cost of PCR based diagnosis has decreased dramatically now. Culture though, less sensitive, is still considered as the gold standard. If culture isolation has to be done for testing the drug sensitivity pattern of the organisms, the genus PCR could be used to quickly screen and select only the positive samples for culture. Further, short course multi drug therapy is being administered to all patients clinically positive to TB even before culture results become available. In this context we would like to recommend the usefulness of PCR to initiate and monitor the effectiveness of the therapy.

The Genus primers and probe are being used for the first time in India since they were developed and validated. The pMAv17 based assay had not missed any one of several samples screened in Boston, USA. It was interesting to note that it missed 3 samples in India which were positive for *M. tuberculosis* by both TB400 and IS6110 assay. Three separate PCR experiments, each done with increasing amounts of target DNA did not yield any amplification (data not shown) indicating the absence of these primer sequences in these strains. It probably indicates variation in the genus primer sequence in *M. tuberculosis* strains present in those samples and this needs to be further studied. This is the first time that variation in the Genus primer sequence in *M. tuberculosis* is being observed. However, this study reiterates the usefulness of using a genus PCR assay for primary screening of all samples that are received in a clinical microbiology laboratory before proceeding with culture.

The excellent correlation seen between the pMAv17 and Tb400 in both the studies suggests that doing a confirmatory PCR on genus positive samples using a species specific PCR enhances the reliability of the PCR assay for final diagnosis. The former assay identifies the microorganism as mycobacteria and the latter assay confirms it as *M. tuberculosis*. This apparently looks like an

expensive proposition. However, this has to be viewed in the context of many labs in the developing countries which do not have facility (expertise, equipment and containment facility) for doing blood cultures from AIDS patients. The PCR assay requires only a laminar flow hood (which anyway is available in a microbiology laboratory) for sample processing apart from the PCR machine and consumables. The cost of such a facility will not be much higher compared to the investment required for establishing and doing blood cultures for mycobacterial isolation and characterisation from AIDS patients and others. In the long term, the cost of repeating cultures and biochemical characterisation for species identification and above all the inordinate time required for a definitive culture based diagnosis make it worthwhile to introduce PCR for diagnosis of *M. tuberculosis* and other mycobacteria. This would also help to advance the drug therapy for such patients.

The following scheme is proposed for a PCR based diagnosis and reporting in a clinical microbiology laboratory for *M. tuberculosis* and other pathogenic mycobacteria. Fig. 3.

The scenario of PCR assays development in India for detection of *M. tuberculosis*

PCR based assays were developed in India (67, 68) also though they were not validated extensively on all types of clinical specimen. Therefore the usefulness of these probes remains unclear. On the other hand, probes developed by others, namely IS6110 and MB64 probes, have been used to determine the etiological role of *M. tuberculosis* in some rare diseases (69).

Commercial kits/cost

At present technical and cost issues should really not be a problem considering the instances where it can be applied. PCR may be a useful technology for the rapid diagnosis of smear negative cases of active tuberculosis (70). PCR should be able to identify 50-60% of smear negative culture positive cases and this should remove a significant amount of clinical diagnostic uncertainty.

Fig. 3. Specimen flowchart in clinical laboratory for diagnosis of *M. tuberculosis*

A number of readily available commercial kits are routinely used in the clinical labs (71) in the western world, as these provide rapid and sensitive results. The sensitivity, specificity of DNA probes and PCR has been combined with the convenience of ELISA and is being called PCR-ELISA. This format of assay in which a nucleic acid probe (instead of an antigen or antibody) is conjugated to an enzyme like peroxidase or alkaline phosphatase is allowed to react and combine with complementary PCR amplified target sequence. The hybrid is then incubated with the appropriate substrate for the enzyme and the final colour is developed as in any ELISA assay. This PCR-ELISA is becoming popular in the laboratory diagnosis of tuberculosis (72,73).

Genprobe's Accuprobe™ species identification system is an isothermal target based amplification system developed by GenProbe Inc (San Diego, CA) and is referred

to as GenProbe Amplified Myco TB Direct Test or MTD in short when applied to clinical specimens. The rRNA is amplified and detected by a species specific DNA probe (74,75); Roche's AMPLICOR *M. tuberculosis* (76,77) test is the only US-FDA approved currently available PCR-ELISA based *M. tuberculosis* kit available in the market though several other companies are working on the development of similar kits based on other probes for *M. tuberculosis*.

The implications of introducing PCR assay as a routine in the clinical microbiology laboratory

The added cost of running PCR would potentially be compensated by the reduced number of samples for culture and the use of antibiotics for unwarranted therapy. As PCR enables identifying the pathogen even if the patient has taken any broad spectrum antibiotic therapy whose samples invariably give

unequivocal results on culture. The rapidity and sensitivity of PCR assays would be immensely useful to save lives of emergency cases like pediatric meningitis.

Despite the obvious power of PCR it would be a mistake to assume that it will trigger a wholesale replacement of conventional techniques in the near future. In fact, it may create many new problems of its own. Undoubtedly, the greatest initial impact of PCR technology will be in the detection of pathogens for which cultivation systems are labor intensive, lengthy, prohibitively expensive or simply unavailable. As a result the diagnostic repertoire of the clinical laboratory will expand significantly as new testing abilities are developed and new disease associations are identified (78).

Tuberculosis is generally thought to develop in an individual as a consequence of one of the three processes:

- 1) Progression of primary infection
- 2) Exogenous re-infection
- 3) Endogenous reactivation.

Until recently, unfortunately, the possibility of differentiating among these types of tuberculosis in individual patients has not existed. Understanding the exact nature of tuberculosis in a patient is important from the point of view of initiating appropriate drug therapy. With the molecular methods of characterising the strains of tubercle bacilli using RFLP finger printing, PCR etc it is now possible to determine whether the

disease in a patient is due to a new infection or due to reactivation of old infection.

Future and applicability of PCR based diagnosis of TB in developing countries:

1. The analysis of PCR results should be reduced to simple dipstick format which is possible.
2. The cost has to be brought down to the level of AFB smears. Development of reagents indigenously will be a step in that direction.
3. A simple method to render the bacterial DNA accessible for amplification. Such a method is already available and has been validated in India also (32).
4. Assays for use in India should preferably be developed in India and tested locally. If assays developed elsewhere are to be applied, a thorough validation of the assay in terms of locally prepared reagents, water, laboratory conditions, sample collection protocols, sample processing protocols, PCR conditions under existing power supply, protocols for analysis and detection, contamination checks at various stages of the assay need to be done.
5. It is important to verify if a selected probe is applicable to Indian strains and determine its predictive value before applying it as a routine test. Such an evaluation should include large number of experimental controls, controls drawn from the population in which the test will be used rather than among healthy laboratory personnel.
6. Quantitative PCR has a potential for regular use in prognosis, monitoring efficacy of therapy and also in all the anti TB drug development and screening programmes (79).

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