Comparative study for the rapid detection and genotyping of MTB and RIF / INH-Resistant MTB Mutants with Gene Flow Hybridization and Conventional PCR

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A R T I C L E   I N F O

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A B S T R A C T

Tuberculosis (TB) is one of the major infectious causes of morbidity and mortality worldwide. Mycobacteria comprise a diverse group of bacteria that are widespread in nature, some of which cause significant disease in humans. TB is difficult to control due to the time taken for the microbiological diagnosis especially culture on solid media which takes 6–8 weeks. Members of the Mycobacterium tuberculosis complex (MTBC) are the most important human pathogens of the genus Mycobacterium. Traditional methods for detection and identification of mycobacteria include microscopy, culture and phenotypic tests. These methods either lack sensitivity, specificity, or are time consuming. Advances in the field of molecular biology have provided rapid diagnostic tools that have reduced the turnaround times for detecting MTBC and drug resistance in cultures and directly in clinical specimens from weeks to days. The objective of this study was designed to compare Gene Flow with regular PCR and detect the drug resistant mycobacteria in body fluids and tissues besides sputum. The conventional method and PCR detected MTB only in sputum and no sensitivity pattern was observed. In comparison gene flow hybridisation not only detected MTB in sputum, pleural fluid, CSF and tissue samples but it also found the sensitivity patterns. This technology is rapid, cost-effective and the development of the result in an assay is more useful than the single-nested PCR technique for application to diagnosis of a large number of clinical samples.

Pulmonary Tuberculosis is a communicable disease mainly caused by Mycobacterium tuberculosis (MTB) that spreads through the airborne droplet nuclei, whereas extra pulmonary tuberculosis has various other modes of transmission. WHO has declared tuberculosis a global emergency, as there has been a 20% increase in its incidence over the past decade. It is one of the most common infectious diseases of the developing countries. Tuberculosis most commonly attacks the lungs and the associated lymph node but may also affect other tissue or organ in the body. Only people with active disease are vectors and transmit TB from person to person. Approximately two billion people, one-third of the world’s population, are infected with MTB; in which one tenth of the infected people develop active TB during their life.

The diagnosis of mycobacterial infection is accomplished by culture-based identification. Primary culture on Oleic acid-albumin-agar or Lowenstein–Jensen media (LJ Media) for slowly growing mycobacteria without using the BACTEC culture system, usually takes four to six weeks or longer. Conventional bacteriology such as direct microscopy and culture are not sufficient for the early diagnosis of tuberculosis because there are few bacilli in the sputum to be demonstrated. These methods either lack sensitivity, specificity, or are time consuming. Advances in the field of molecular biology have provided rapid diagnostic tools that have reduced the turnaround times for detecting MTBC and drug resistance in cultures, and directly in clinical specimens (tissue, body fluid, CSF, nodule aspiration and sputum), from weeks to days. Advances in recombinant DNA technologies have facilitated the establishment of genomic sequences, the functional and regulatory systems of genes, and protein leading to definitive information on genetic origins of life. The information lying within defective genes and their products from inheritance, sporadic mutations and pathogens can be readily detected. Hence, molecular diagnostics have enjoyed rapid growth.
recently and shall soon become the effective tool for preventive medicine. Undoubtedly, microchip technology has revolutionised the way we study genomes and proteomes because of its ability to detect thousands of sequences, proteins or other analytics simultaneously. However, it has been proven difficult to apply to routine clinical diagnosis and the complex nature of high-density chips will remain difficult to be validated. Moreover, the expensive instruments will only be confined to well-funded laboratories and research institutions where these can be afforded. In practice, definitive diagnosis is possible using hundreds, or less, sequences or analytics deduced from available research data. Thus 'low density chip' is the ideal diagnostic tool because it is much more affordable, efficient and effective for product development worldwide. The ability to amplify a single copy into millions in a short period of time by PCR, or equivalent method, is the key for the advances in DNA analyses. However, the possibility of false amplification requires follow-up analysis for validation. Conventional analysis methods, like fragment analyses or hybridisation, cannot provide adequate means for analysis either because of their specificity or throughput i.e. the ability to simultaneously analyse multiple targets or generating definitive results.

Membrane hybridisation was first developed by Southern and subsequent modification to Reversed Dot Blot (RDB) advanced by Maggio. These techniques are capable of differentiating single base mutations, and the array technology eliminates the difficulty of getting highly specific results for multiple analyses in a single run. Hence, coupled with stringent hybridisation conditions, the highest sensitivity and specificity can easily be achieved. The remaining focus should therefore be on the speed of hybridisation, and the efficient use of the analytics and reagents without the use of sophisticated instrumentation. Considering this background we present the membrane-based 'Flow-through Hybridisation Platform' (US patent number 5741647), which is capable of developing low cost membrane-based macro-arrays for use in molecular diagnostic applications.

The new technology used for the diagnosis is 'flow-through hybridisation'. It allows the recombination reactions to complete in seconds by actively directing the ampiclons toward the probes to form duplexes, thus facilitating a change from a passive to active channelling process. The most potent first line antituberculous drugs used for standard treatment of TB are isoniazid (INH) and rifampicin (R). Resistance of M. Tuberculosis to both these drugs is termed multidrug resistance (MDRTB), and represents an important public health problem in many countries. In addition, sometimes there is resistance to all first line drugs (XDRTB). Therefore objective of this study was designed to compare Gene Flow hybridisation with regular PCR and detect the drug resistant mycobacteria in body fluids and tissues besides sputum.

**MATERIALS AND METHODS**

**Collection of specimens**

This comparative analysis was carried out in the laboratory. A total of 50 cases presenting to chest OPD in a secondary care hospital were selected and samples including sputum, cerebral spinal fluid, pleural fluid and tissues samples were collected. All 50 cases had a positive family history and fulfilled the following criteria: 50 cases had a positive family history and fulfilled the following:

- Patient's selection criteria: Samples of those patients were collected whose chest x-ray showed radiographic consolidations suspicious of pulmonary tuberculosis; either patchy or nodular infiltrate in upper lobes or super or segment of lower lobes, bilateral upper lobe infiltrate with patchy soft shadows with or without cavitations. In some cases, there were bilateral upper lobe infiltrate with patchy soft shadows without cavitations. Besides, on the basis of chest x-rays evaluation of respiratory symptoms (cough > 3 week, hemoptysis, chest pain, and dyspnea), an unexplained illness and flu were also considered. In adults, a multidrug infiltrate above or behind the clavicle (the most characteristic location, most visible in an apical lordotic view) suggest the reactivation of TB. Middle and lower lung infiltrates were nonspecific but prompt suspicion of primary TB in patients (usually young) whose symptoms or exposure history suggested the recent infection, particularly if there was pleural effusion.

The basic screening strategy included AFB smear. After initial investigation for the AFB, the DNA were extracted from all specimens for detecting mycobacterium tuberculosis in these specimens by using PCR, and the significant diagnostic value of PCR were observed by comparing the conventional methods with Gene Flow hybridisation technique. The Gene Flow DRMTB Array Test Kit relies on PCR amplification and flow-through hybridisation technology. The genomic DNA of target pathogen is amplified by biotinylated primers using PCR. The ampiclons are subsequently hybridised to pathogen-specific capturing probes via flow-through hybridisation. By actively directing the ampiclons toward the probes to form duplexes, the flow-through hybridisation facilitates a change from a passive to active channelling process allowing the recombination reactions to complete in seconds. Hybridisation is then followed by a stringent wash and signal development.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Volume (μl)</th>
<th>Duration</th>
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<tbody>
<tr>
<td>46 °C FT p&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Pre-hybridisation (2xSSC + 0.1%SDS)</td>
<td>750</td>
<td>5 min</td>
</tr>
<tr>
<td>Hybridisation</td>
<td>500 + 20 μl PCR pdt</td>
<td>5 min</td>
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<tr>
<td>Stringent wash (0.5xSSC + 0.025%SDS)</td>
<td>750 x 1</td>
<td>-</td>
</tr>
<tr>
<td>Stringent (0.5xSSC + 0.025%SDS)</td>
<td>750 x 2</td>
<td>1 min</td>
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<tr>
<td>25 °C</td>
<td></td>
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<tr>
<td>Blocking</td>
<td>500</td>
<td>5 min</td>
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<tr>
<td>Enzyme Conjugate (1:1,000)</td>
<td>500</td>
<td>5 min</td>
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<tr>
<td>36 °C</td>
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<tr>
<td>A solution</td>
<td>750 x 4</td>
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<tr>
<td>Blocking</td>
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<td>Detection</td>
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<td>B solution</td>
<td>750 x 3</td>
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<tr>
<td>Stop</td>
<td>750</td>
<td>1 min</td>
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PCR was performed according to the conventional procedure. The PCR was carried out in a tube containing 20 μl of a reaction mixture made up of the following components: 10 pmol of each forward and reverse primers for mycobacterium and atypical mycobacteria, 500 μM of four deoxynucleotides, 2 U of Taq polymerase (Promega), 10 x PCR buffer containing and 1.5 mM MgCl₂. The thermal cycler (Master Gradient PCR System, Eppendorf AG, Germany) was programmed to first incubate the sample for 5 min at 95°C followed by 30 cycles consisting of 94°C for 45 s, 56°C for 45 s and 72°C for 1 min with final extension for 7 min at 72°C. The PCR amplified products were identified by electrophoreses on a 2% agarose gel, stained with ethidium bromide, and evaluated under transilluminator. The sizes of PCR amplified product were estimated according to the migration pattern of a 100-bp DNA ladder (Gibco BRL Life Technologies).

**RESULT**

A total of 50 cases were selected on the bases of history and clinical examination on suspicion of tuberculosis. Their samples including pleural fluid, tissue fluid, cerebral spinal fluid (CSF) and sputum were collected. ZN staining for AFB was only positive in sputum sample, whereas in body fluids, as well as in tissue samples, AFB was negative. PCR was performed by two different methods and the result was interpreted.

The PCR was performed on all the samples with gene flow hybridisation and detection was observed in pleural fluid, CSF, endometrial tissue and sputum after DNA extraction and results were observed in all the samples which were suspected for mycobacterium tuberculosis, as shown in figure 1.

In first four blocks the mycobacterium tuberculosis is of wild type. In second lane sample one which is sputum and endometrial tissue were the wild type of tuberculosis. In second lane 2nd and 4th sample were the mutant types at position C3, official name RpoB MUT E3 MTB detected rpoB H526D. The second sample is the pleural fluid and 4th was tissues sample. In third lane the 1st sample was TB isolate culture colonies were the mutant types at position official name KatG E1MTB KatG and second sample was sputum were the mutant types at position C3, official name RpoB MUT E3 MTB detected rpoB H526D as shown in figure 1.

In comparison to gene flow, the conventional method from clinical sputum sample by PCR was detected only in sputum and no sensitivity pattern was observed. The clinical samples including pleural fluid, CSF and tissues sample was not detected as shown in figure 2.

**DISCUSSION**

Before the introduction of molecular typing methods, there was little to aid the distinction between individual strains of M. tuberculosis. Traditional methods of diagnosing tuberculosis have been the isolation of bacilli in culture, or recognition of AFB in clinical specimens. The diagnostic value of various methods widely used in microbiological diagnosis of tuberculosis: direct smear examination for acid-fast bacilli, cultural identification in Lowenstein-Jensen (L-J) medium, the radiometric BACTEC 460 system and PCR, to evaluate the time factor and the sensitivity of the clinical method, has been reported. AFB staining lacks sensitivity. So far, the gold standard has been culture with a dividing time of 48 hours, up to 10 weeks. However, the high number of false-positives that we found suggests that results obtained should be confirmed with BACTEC, which considerably reduces the time required for identification and makes it possible to carry out an antibiotic assay rapidly.

Our findings are consistent with that of Moran Moguel et al. who according to WHO, reported that PCR is a sensitive and
specific technique for detecting the M. tuberculosis complex in both positive and negative bacilloscopy samples. A controlled PCR procedure makes it possible to establish or to exclude the diagnosis of tuberculosis in a time that is reduced from more than six weeks to just 24 to 48 h. This is particularly useful when an early diagnosis is needed to establish a patient’s prognosis or in organ transplant cases. This study was conducted in molecular laboratories for the early and cost-effective diagnosis for the management of TB. We demonstrated the genetic technology, polymerase chain reaction. For identification, and sensitivity patterns new and advanced technique, the gene flow hybridisation test was also performed, and its comparison was done with the conventional technique. In gene flow hybridisation, seven wild-type probes detect major types of mycobacteria in the MTB complex with no cross reaction with major types of non-tuberculosis mycobacteria (NTM). Additional 9-point mutation probes detect mutations causing resistance to Rifampicin (RIF) and Isoniazid (INH) respectively. The Amplification Control (AC) and Hybridisation Control (HC) probes was also used to check for the successful processing of sampling; DNA extraction, PCR amplification and DNA hybridisation steps.

The most potent first-line anti-tuberculous drugs used for standard treatment of TB are isoniazid (INH) and rifampicin (R). Resistance of M. tuberculosis both these drugs is termed multidrug resistance (MDRTB), and represents an important public health problem in many countries.

The development of molecular tools has added a new dimension to the classical epidemiology of tuberculosis and greatly enhanced the understanding of complex transmission dynamics within populations, and between hosts. Advances in the field of molecular biology have provided rapid diagnostic tools that have reduced the turnaround times for detecting MTB and drug resistance in cultures and directly in clinical specimens (tissue, body fluid, CSF, nodule aspiration and sputum) from weeks to days. The flow-through hybridisation is changing from traditional passive, to an active channelling process, allowing the recombination reaction to complete in seconds.

CONCLUSION

The conventional method and PCR detected MTB only in sputum and no sensitivity pattern was observed. In comparison gene flow hybridisation not only detected MTB in sputum, pleural fluid, CSF and tissue samples but it also found the sensitivity patterns.

This technology is rapid, cost-effective and the development of the result in an assay more useful than the single-nested PCR technique for application to diagnosis of a large number of clinical samples.

REFERENCES