

## Application of Polymerase Chain Reaction for Rapid Detection of *Mycobacterium tuberculosis* Complex in Biological Fluids

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**Abstract:** Rapid and accurate diagnosis of *Mycobacterium tuberculosis* complex is essential for the treatment and outcome of the disease as well as prevention of its further transmission. In the present study, a nested Polymerase Chain Reaction (nPCR)-based detection assay is described for rapid detection of tuberculosis. The IS6110 sequence of the *M. tuberculosis* complex was used as a target gene for PCR in two amplification steps. The first round of amplification produced a 567 bp primary PCR product. The second round of amplification, using internal (nested) primers, resulted in a 310 bp nested PCR product. The nPCR provides rapid, sensitive and specific detection of the organism in clinical samples and a variety of different body fluids and aspirates. Clinical samples were collected from infected patients admitted to the National Ribat University Medical Teaching Hospital, Khartoum, Sudan. The sensitivity of the assay indicated that the nPCR detected as little as a single copy of the target DNA. However, application of this nPCR to DNA from other related bacteria including *M. flavescens*, *M. duvalii* and *M. paratuberculosis* did not amplify the primary or the nested PCR products. The described nPCR could be used as a valuable tool for early differential diagnosis of tuberculosis in biological fluids from suspected patients. In addition, the assay should be recommended for inclusion during epidemiological surveys, treatment and control programs of the disease.

**Key words:** Rapid diagnosis, *Mycobacterium tuberculosis* complex, biological fluids, acid fast bacilli, PCR, molecular diagnosis, Sudan

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### INTRODUCTION

Tuberculosis is a chronic debilitating infection caused by *Mycobacterium tuberculosis* complex. The disease affects both humans and animals world-wide and hence it is of public health importance. Currently, the disease is diagnosed by smear microscopy to demonstrate Acid-Fast Bacilli (AFB) and by culture using conventional method of microbiological isolation and identification. The conventional bacteriological methods used for the diagnosis of Pulmonary Tuberculosis (PTB) and Extra-Pulmonary Tuberculosis (EPTB) lack both sensitivity and specificity (Chain, 1995). The sensitivity of a smear microscopy depends largely on the bacterial load or the density of AFB in the submitted sample. The bacterial culture provides a definitive diagnostic method for an active infection but it requires 6-8 weeks for identification of positive specimens. Thus, conventional bacteriological method is time consuming, cumbersome and expensive (Small and Perkins, 2000; Daniel, 1990). In most of developing countries including the Sudan, only smear

microscopy is routinely available in hospitals and clinical centers. The absence of a positive smear in individuals still suspected of having TB is a diagnostic problem, especially, when there are no other tests available (Torrea *et al.*, 2005). Because of the problems associated with early and accurate diagnosis of tuberculosis, treatment is frequently delayed. This delay in diagnosis and treatment contributes to the poor prognosis observed in infected patients. EPTB is difficult to diagnose without cytological and histological examinations, which are frequently not available for routine diagnosis in the majority of the hospitals in the Sudan. In general, sputa are not useful in diagnosing EPTB and handling blood samples for diagnosing TB is hazardous. In addition, blood is not always considered the sample of choice for detection of the organism as the infection may be localized. More over, blood is known to contain PCR inhibitors (Folgueira *et al.*, 1993, 1996). However, body fluids and aspirates including, sputa, pleural fluid, ascitic fluid, bronchoalveolar lavage, cerebrospinal fluid, urine and puss aspirate (from abscess or chronically infected

wounds) are easy to collect and the organism can be concentrated by centrifugation before subsequent DNA extraction.

Despite the development of new sensitive techniques for identification of clinical isolates of mycobacteria, their use is limited to research only and not currently applied on practical scales. These assays include capillary Gas Chromatography (GLC) of low-molecular-weight fatty acids, high-performance liquid chromatography (Tisdall *et al.*, 1982; Butler and Guthertz, 2001). However, high cost of these methods and requirement for sophisticated equipment limit their routine use in developing countries. Semi-automated commercial kits (Cobas Amplicor system from Roche Diagnostic Systems and Abbott LCx Probe System) have been used to diagnose PTB and EPTB. However, these diagnostic tests are expensive and their sensitivity is often similar or even lower than that observed when using the manual in-house PCR for detection of smear-negative PTB (Yuen *et al.*, 1997; Eing *et al.*, 1998). Therefore, it is becoming obvious that the development of a molecular diagnostic assay for rapid detection of *Mycobacterium tuberculosis* complex is urgently needed.

The aim of this study was to develop and evaluate a nested PCR assay for rapid detection of *M. tuberculosis* complex in body fluids and aspirates, based on IS6110 sequence analysis of the organism as a target DNA.

## MATERIALS AND METHODS

**Bacterial strains and clinical specimens:** Nine reference strains (*Mycobacterium flavescens*, *Mycobacterium duvalii* and *Mycobacterium tuberculosis* strains were provided by the National Health Laboratory, Khartoum, Sudan. Clinical specimens were obtained from 50 patients admitted to the National Ribat University Medical Teaching, Khartoum, Sudan. All specimens were decontaminated by the Petroff (1951) sodium hydroxide (NaOH) methods. About 0.5 mL of the residual sediments was inoculated into Lowenstein Jensen (LJ) medium. After the detection of a positive culture the presence of Acid-Fast Bacilli (AFB) was confirmed by Ziehl-Neelsen (ZN).

**Preparation of DNA from bacterial culture:** A loopful culture of each strain was suspended in 500  $\mu$ L distilled water, inactivated at 80°C for 30 min and pelleted by centrifugation. The supernatant was collected in a clean eppendorf tube and The DNA concentration was determine by spectrophotometer using 260 nm wave length. Five  $\mu$ L of the supernatant was used as a template DNA in the PCR amplification (Kirsi *et al.*, 2003).

**DNA extraction from clinical samples:** Clinical samples including sputa, cerebrospinal fluid, bronchoalveolar lavage, pleural fluid, ascetic fluid, abscess, urine and were centrifuged at 5000 $\times$  g for 10 min. The supernatant was discarded and the sediment was used for DNA extraction. Extraction of DNA from clinical samples was made possible using a commercially available QIAamp tissue kit (QIAGEN Inc. Chatsworth, CA) according to the manufacturer's instructions. Briefly, 200  $\mu$ L of fluid sediment 20  $\mu$ L of proteinase K at a concentration of 20  $\mu$ g mL<sup>-1</sup> stock solution and 200  $\mu$ L of lysing buffer were pipetted into 1.5 mL eppendorf tube. The mixture was incubated at 37°C for 30 min and then at 70°C for 30 min. Two hundred micro liters of absolute ethanol was then added to the sample and mixed by vortexing. The mixture was then transferred to the QIAamp spin column and placed in a clean a 2 mL-collection tube and centrifuged at 6000 $\times$  g for 1 min at room temperature. The QIAspin column was washed twice using 500  $\mu$ L of washing buffers A and B, respectively for 1 min. The QIAamp spin column was placed in a clean 1.5 mL eppendorf tube and the DNA was eluted with 200  $\mu$ L of double distilled water preheated at 70°C. A maximum DNA yield was obtained by spinning at 12,000 $\times$  g for 2 min at room temperature. The DNA concentration was determined by spectrophotometer at 260 nm wave length. Five micro liters of the suspended nucleic acid was used in the PCR amplification.

**Selection of the primers for PCR amplification:** The PCR assay was performs as basically described by Aradaib *et al.* (2005). For the first amplification step, a pair of primers (TB1 and TB2) was selected from the published sequences of the gene encoding the insertion sequence IS6110 of the organism (4). Primer TB1 included bases 290-316 of the positive sense strand (5)- GGC GGG ACA ACG CCG AAT TGC GAA. TB2 included bases 833-856 of the complementary strand (5)- CGA GCG TAG GCG TCG GTG ACA AAG. Using primers TB1 and TB2, the primary PCR amplification will produce a 567 bp PCR product. For the second amplification step, a pair of internal (nested) primers (TB3 and TB4) was designed from the same sequence of IS6011 gene cited above. TB3 included bases 431-450 of the positive sense strand (5)- TAC TAC GAC CAC ATC AAC CG. TB4 included bases 721-740 of the complementary strand (5)- GGG CTG TGG CCG GAT CAG CG. Using primers TB3 and TB4, the nested PCR assay will produce a 310 bp PCR product.

The primers were synthesized on a DNA synthesizer (Milligen/Bioscience, a division of Millipore Burlington, MA) and purified using oligo-pak oligonucleotide purification columns (Glen Research Corporation, Sterling, VA) as per manufacturer's instructions.

**Polymerase Chain reaction:** A stock buffered solution containing (150  $\mu\text{L}$  10 $\times$  PCR buffer, 100  $\mu\text{L}$  of  $\text{MgCl}_2$  at a concentration of 25 mM and 50  $\mu\text{L}$  of each dNTPs mixture at a concentration of 10 mM) was prepared in 1.5 mL eppendorf tube. The primers were used at a concentration of 20 pg  $\mu\text{L}^{-1}$  and double distilled water was added to bring the volume of the stock buffer solution to 1.5 mL. About 2  $\mu\text{L}$  of the primers, 5.0  $\mu\text{L}$  of the target DNA and 42  $\mu\text{L}$  of the stock solution were added into 0.5 mL PCR tubes and mixed by vortexing.

About 1  $\mu\text{L}$  of Taq DNA polymerase (Perkin Elmer) at a concentration of 5.0 U  $\mu\text{L}^{-1}$  were used. All PCR amplification reactions were carried out in a final volume of 50  $\mu\text{L}$ . The thermal cycling profiles were as follows: a 5 min initial incubation at 95°C, followed by 40 cycles of 94°C for 1 min, 62°C for 30 sec and 72°C for 45 sec. The PCR amplification tubes were then subjected to a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ). Following amplification, 15  $\mu\text{L}$  from each PCR containing amplicons were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the primary 567 bp PCR products were easily identified following visualization under UV light.

**Nested polymerase Chain reaction:** For the nested PCR amplification, 2.0  $\mu\text{L}$  of the primary PCR product, produced by primers TB1 and TB2) were transferred to 0.5 mL PCR tube containing 2  $\mu\text{L}$  of nested primers (TB3 and TB4) and 45  $\mu\text{L}$  of stock PCR mix. The Taq DNA polymerase was used at a concentration of 5.0 U  $\mu\text{L}^{-1}$ . The nested pair of primers was expected to amplify a 310 bp PCR product, internal to the annealing sites of primers TB1 and TB2. Positive and negative controls were included in each run and all precautions to prevent cross-contamination were observed. All PCR amplifications were carried out in a final volume of 50  $\mu\text{L}$ . The thermal cycling profiles were as follows: a 2 min incubation at 95°C, followed by 30 cycles of 94°C for 1 min, 55°C for 30 sec and 72°C for 45 sec and a final incubation at 72°C for 10 min. Thermal profiles were performed on a Techne PHC-2 thermal cycler (Techne, Princeton, NJ). Following amplification, 15  $\mu\text{L}$  from each PCR containing amplicon were loaded onto gels of 1.0% SeaKem agarose (FMC Bioproduct, Rockland ME) and electrophoresed. The gels were stained with ethidium bromide and the bovine-specific PCR amplicons were easily identified following visualization under UV light.

## RESULTS

The described PCR-based assay provides sensitive and specific detection of *Mycobacterium tuberculosis* complex used in this study. Using the outer pair of primers (TB1 and TB2), the PCR assay detected the primary 567 bp PCR product from  $\geq 100$  fg DNA from the organism (Fig. 1). The sensitivity studies indicated that the internal pair of primers (TB3 and TB4) increased the sensitivity of the nested 310 bp PCR products were amplified from as little as 0.001 fg of bacterial DNA, equivalent to a single copy of bacteria (Fig. 2).

The specificity studies indicated that the described PCR assay did not amplify the primary or the nested PCR products from DNA extracted from other related bacteria including, *Mycobacterium flavescens*, *Mycobacterium duvalii* and *Mycobacterium paratuberculosis* (Fig. 3). Application of this nested PCR assay to DNA extracted from clinical samples resulted in direct detection of the nested 310 bp PCR product from a variety of body fluids and homogenates including; sputa, Cerebrospinal Fluid (CSF), bronchoalveolar lavage, pleural effusions, ascetic fluid, abscess and urine (Fig. 4).

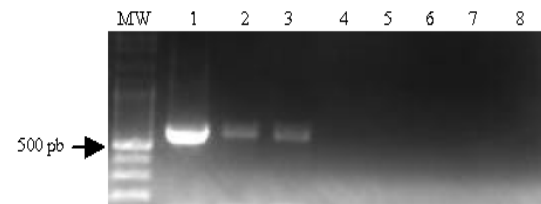


Fig. 1: Sensitivity of the PCR for detection of the primary 570 bp PCR product from *Mycobacterium tuberculosis*, using primers TB1 and TB2. Lane MW: Molecular Weight marker (100 bp NA ladder); Lane 1-7: 1.0 pg, 100 fg, 10 fg, 1.0 fg, 0.1 fg, 0.01 fg and 0.001 fg of bacterial DNA, respectively; Lane 8: Nucleic acid free sample (negative controls)

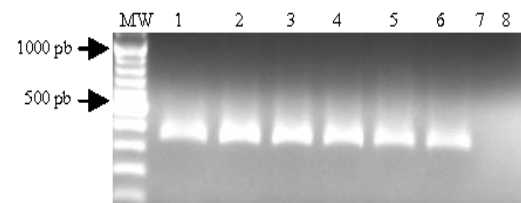


Fig. 2: Sensitivity of the nested PCR for detection of the nested 310 bp PCR product from *Mycobacterium tuberculosis*, using primers TB3 and TB4. Lane MW: Molecular Weight marker (100 bp NA ladder); Lane, 1-7: 1.0 pg, 100 fg, 10 fg, 1.0 fg, 0.1 fg, 0.01 fg and 0.001 fg of bacterial DNA, respectively; Lane 8: Nucleic acid free sample (negative controls)

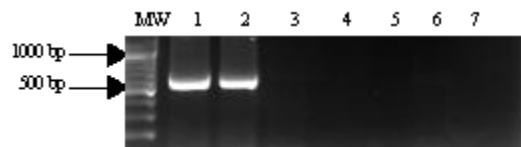


Fig. 3: Specificity of the nested polymerase Chain reaction for detection of *Mycobacterium tuberculosis*. Amplification product was detected from 1.0 pg of *Mycobacterium tuberculosis* DNA but not from other related bacteria. Lane MW: Molecular Weight marker; Lane 1-2: 1.0 DNA from *Mycobacterium tuberculosis* (positive controls). Lane 3: 1.0 pg DNA from *Mycobacterium flavescens*; Lane 4: *Mycobacterium divalii*; Lane 5: *Mycobacterium paratuberculosis*; Lane 6: 1.0 pg DNA from *E. coli*; Lane 7: Nucleic acid free sample (negative control)

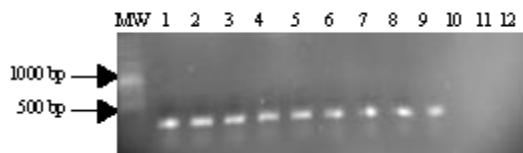


Fig. 4: Visualization of the 310 bp nested PCR product on ethidium bromide-stained agarose gel from biological fluids. Lane MW: Molecular Weight marker (100 bp DNA ladder); Lane 1: *Mycobacterium tuberculosis* (positive control); Lane 2: Sputum sample; Lane 3: Ascetic fluid; Lane 4: Cerebrospinal Fluid (CSF); Lane 5: Abscess; Lane 6: Urine sample; Lane 7: Bronchoalveolar lavage; Lane 8: Pleural fluid; Lane 9: Sputum sample; Lane 10-11: non-infected sputum and blood samples; Lane 12: Nucleic acid free water (negative control)

## DISCUSSION

Rapid and accurate diagnosis allows proper management of a disease. The current methods used to detect *Mycobacterium tuberculosis* complex are either time consuming or expensive. Therefore, a rapid, reliable, simple and cost effective method would be highly desirable, especially in developing countries where prevalence of tuberculosis is extremely high.

In a earlier study, fast plaque TB (FASTPlaqueTB) tests were reported to be potentially useful tools for the diagnosis of tuberculosis (Wilson *et al.*, 1997; Albert *et al.*, 2002; Muzaffar *et al.*, 2002; Alcaide *et al.*,

2003; Gali *et al.*, 2003; Park *et al.*, 2003). FASTPlaque TB test for detection of *M. tuberculosis* complex in body fluids and aspirates was reported to have sensitivity and specificity of 93.5 and 98.4%, respectively (Revathi, 2002). Molecular methods for detection of TB proved rapid and sensitive, but the high cost of these assays and requirement for sophisticated equipment renders the previously described assays inappropriate for routine use in developing countries with high prevalence of the disease (Sechi *et al.*, 1997; Aceti *et al.*, 1999). The laboratory diagnosis of TB in the Sudan remains problematic because of lack of laboratory facilities. In sub-Saharan Africa, the HIV epidemic has contributed to the spread of the disease (De Albuquerque Mde *et al.*, 2001). Strikingly, the high HIV seroprevalence accompanied by the often undiagnosed TB in these countries contribute to diagnostic and treatmentss delays. The delay in treatment subsequently leads to further transmission of bacilli to more susceptible patients.

The development of sensitive and rapid diagnostic tools to detect and confirm active TB infection would be advantageous in a variety of circumstances including clinical disease investigation, epidemiological surveys and treatment programs. The nested PCR described in this study is therefore, desperately needed especially among HIV-infected individuals in Sudan with a high burden of these two infectious diseases. In present study, we evaluated the performance of a nested PCR amplification in body fluid and aspirates using the IS6110 sequence of the *M. tuberculosis* complex as a target DNA to be amplified.

In the present study, some samples of the same specimen gave inconsistent results. This suggests that *M. tuberculosis* complex DNA is represented in low copy numbers or is unevenly distributed in fluid samples (Kafwabulula *et al.*, 2002; Jourveshomme *et al.*, 1998). It is therefore, suggested that the fluid samples be centrifuged to deposit the bacteria prior to DNA extraction. The specificity of the PCR was similar to that reported in other studies evaluating Sechi's method and automated direct amplification tests (Yuen *et al.*, 1997; Eing *et al.*, 1998; Kafwabulula *et al.*, 2002). Diagnosis of TB in a resource poor country, such like Sudan, is problematic. The cost and lack of suitably trained staff are the major problems.

Although, the medical teaching hospital has a well-organized laboratory with professional personnel, implementation of a molecular technique in this setting must be considered carefully. The technique may, however be useful for confirming *M. tuberculosis* in patients suspected to be microbiological-negative PTB or

EPTB when clinical and bacteriological diagnoses are not conclusive, especially in the presence of an HIV co-infection, considering that body fluids are biological sample that can be obtained by a non-invasive technique.

The negative control samples used in this study were collected from patients attending the clinics for respiratory diseases other than TB. In the present study, we were able to detect TB in a variety of clinical sample using nPCR. This is the 1st time ever a molecular technique has been used for routine diagnosis of PTB or EPTB on a practical scale in the Sudan. The nPCR will undoubtedly contribute towards improving diagnosis and treatment of tuberculosis infected patients. The time required from submission of samples to final results was consistently 8 h and hence, definitive diagnoses could be affordable within the same researching day. The nested amplification is necessary to increase the sensitivity of the PCR and to confirm the specificity of the primary amplified 567 bp PCR product. The nPCR does not require nucleic acid hybridization confirmation, which usually takes overnight. The *M. tuberculosis* infected patients participating in this study came to our University hospital from different geographical locations in the Sudan. This observation confirmed that the IS6110 has conserved nucleotide sequences among cognate genes of *M. tuberculosis* complex. The described nPCR assay is easy to perform and presents a low-cost, rapid means of detection of tuberculosis. The assay could provide an alternative to the currently available detection methods. In addition, the limited capital outlay and training required make this assay suitable for use in developing countries, such like Sudan.

### CONCLUSION

The described nPCR assay should be used as a valuable tool for rapid, sensitive and specific detection of tuberculosis in biological fluids from suspected patients. The assay should also be considered for inclusion during epidemiological surveys and control programs of tuberculosis.

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