

Rapid differentiation of *Mycobacterium tuberculosis* and *Mycobacterium leprae* from sputum by polymerase chain reaction

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ABSTRACT

Differentiation of *M. tuberculosis* and *M. leprae* by polymerase chain reaction (PCR), when acid-fast bacilli (AFB) were present in sputum from patients at Anandaban hospital, was carried out. Thirty sputum samples microscopy positive for AFB were collected and were subjected to culture. Bacterial DNA was extracted and PCR was performed using primers specific for *Mycobacterium tuberculosis* and *Mycobacterium leprae* DNA. Twenty samples were from patients with clinical TB and 10 from patients with clinical leprosy. Fifteen of the TB samples were positive in both TB PCR and culture, among the reminders four were TB PCR negative and one was positive for TB PCR. All TB samples were negative for leprosy PCR. Of the leprosy samples, five were TB PCR and culture positive, and negative for leprosy PCR. The remaining five samples were negative for both TB PCR and culture but positive in leprosy PCR. Five of ten clinical leprosy samples were positive for tuberculosis. This indicates that AFB in the sputum of leprosy patients might be *M. tuberculosis* or *M. leprae*. Thus PCR can be used for rapid differentiation of *M. tuberculosis* and *M. leprae* present in sputum where AFB microscopy is inconclusive.

Keywords: DNA, PCR, tuberculosis, leprosy, Nepal.

INTRODUCTION

Association between tuberculosis and leprosy in individuals living in an area endemic for both diseases has been little documented although it is likely to be common. One of the rare studies on this subject was done in 1895 by Armauer Hansen, who found tuberculosis to be the most common cause of death among leprosy patients in Norway.¹ Some studies suggest that tuberculosis and leprosy might interact or interfere with one another and it has been suggested that leprosy might encourage the development of tuberculosis.²

Generally, tuberculosis and leprosy are diagnosed by detection of clinical signs and symptoms, but these are not always specific or present in atypical forms of the diseases. Diagnosis is therefore normally confirmed by routine conventional laboratory tests *viz.* microscopic detection of AFB in sputum smears for tuberculosis and slit skin smears for leprosy. Although AFB microscopy is rapid, cheap, easy and is recommended by WHO for examination of smears, this procedure has advantages and limitations.

With the widespread nature of TB worldwide and occurrence of other mycobacterial infections, it has been agreed that the AFB sputum smear test alone does not offer any definitive advantage in terms of species identification and differential diagnosis.³ New diagnostic tests are needed to improve the detection of both AFB-positive and AFB-negative cases of tuberculosis. This need is most acute in those developing countries with the largest share of the world's tuberculosis cases.⁴

Sputum culture to isolate mycobacteria is a highly sensitive method which permits detection of a minimum of 10 to 100 viable bacilli in the volume of cultured material. The specificity of culture for the diagnosis of TB is greater than 99.0%. In general, sputum culture adds 20.0 to 30.0% to the total number of bacteriologically confirmed pulmonary TB cases which are diagnosed by smear microscopy.⁵

The association of tuberculosis with other related diseases such as leprosy may create confusion in managing patients. The actual problem in diagnosis arises when large numbers of *M. leprae* are present in mucosal secretions, such as sputum, in leprosy patients who have a high Bacterial Index (BI) as by skin slit smear. Thus, in these cases, if clinical signs suggestive of tuberculosis are seen, examination of sputum samples for AFB is inconclusive: the bacteria seen may be either *M. leprae* or *M. tuberculosis*. In this clinical situation, it is necessary to determine whether the bacteria detected are leprosy or tuberculosis bacilli, in order that the correct treatment is given.

Though leprosy is a chronic infectious disease, mortality is rare and it is important to rule out causes of death among leprosy patients where tuberculosis and leprosy are endemic. In this regard, the present study aimed to use of PCR as a means of differentiating between *M. leprae* and *M. tuberculosis* when these are present in the sputum. For rapid diagnosis, bacterial DNA was extracted from sputum, and PCR for IS6110 (TB specific) and RLEP (leprosy specific) were performed. Samples were examined microscopically for AFB, and cultured to detect the presence of TB bacteria in order to evaluate the sensitivity and specificity of the PCR in comparison to culture.

MATERIALS AND METHODS

Specimens: Thirty AFB microscopy positive sputum samples were collected from patients after informed consent, with clinical symptoms of either tuberculosis, leprosy or both from Anandaban hospital in Kathmandu, Nepal. Samples were collected in sterile leak proof plastic containers and processed for PCR and culture.

Concentration and culture: Petroff's method was used for concentration of the samples.⁶ The sample was taken in a graduated centrifuge tube and an equal volume of 4.0% NaOH was added and mixed well. This was incubated at 37°C for 10 minutes with frequent gentle mixing and centrifuged at 2500xg for 30 minutes. The supernatant was discarded and the remaining material was used for culture and DNA extraction.

Lowenstein-Jensen (L-J) method⁶ was followed for the culture. After concentration, the samples were streaked on culture slants and incubated at 37°C for 8-10 weeks.

Decontamination and DNA extraction: Pellets from the concentrated sputum were decontaminated by adding an equal volume of 2.0% NaOH, 0.5% N-acetyl-L-cysteine (NALC) to the concentrated sputum sample and vortexed. After being left for 20 minutes to decontaminate, the entire specimen was centrifuged at 10,000xg for 15 minutes. After discarding the supernatant, 50mM Tris HCl (pH 8.3) was added in an amount equivalent to the original volume of thinned sputum. The pellet was re-suspended and centrifuged as before. The pellet was re-suspended in 50mM Tris HCl (pH 8.3) equivalent to not less than 1/20th of the original thinned sputum. This was heat inactivated at 80°C for 20 minutes in a dry heat block. After cooling to room temperature, an equal volume of chloroform was added and mixed by vortexing and the aqueous phase stored in the refrigerator until DNA extraction.

Fifty microlitres of the above decontaminated sputum sample was taken and subjected to a series of heat and cold shocks, alternately boiling for one minute at 100°C in dry heating block and snap freezing in liquid nitrogen (-196°C) for one minute, for 5 cycles. The extracted DNA was quantified using standard spectrophotometric measurement⁷ and diluted to 10µg/ml for use in PCR.

PCR assay: The PCR targets were the IS6110 insertion sequence-like element of *M. tuberculosis* (TB PCR) and RLEP repetitive sequences of *M. leprae* (Leprosy PCR). The sequence of primers (synthesized by MWG, Germany) from 5' to 3' were CCT GCG AGC GTA GGC GTC GG (IS6110 A) and CTC GTC CAG CGC CGC TTC GG (IS6110 B) which amplified a 123-bp fragment from *M. tuberculosis* DNA and RLEP GGA CAC GAT TAG

CGC GGC GCA CGT (PS3) and TTG TGG TGG GCT GGT GGG GTG TGG (PS4) which amplified a 455-bp product from repetitive sequences of *M. leprae* DNA.

The PCR assays were performed in 50µl reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each of deoxynucleotides dATP, dCTP, dGTP and dUTP, 5 pmol of each primer, 0.2% Dimethyl sulfoxide, 1.25 U of *Taq* DNA polymerase (Roche, Germany) to amplify the *M. tuberculosis* and *M. leprae* DNA. Beside this, an extra 1.5 mM MgCl₂ was added in PCRs to amplify *M. tuberculosis* DNA.

The optimized amplifications were carried out in a thermocycler (Hybaid Omn-E, UK) as follows: For TB DNA an initial template DNA denaturation step at 94°C for 5 minutes, followed by 30 cycles of 2 minutes denaturation at 94°C, 2 minutes primer annealing at 68°C, and 2 minutes extension at 72°C and then a final extension step at 72°C for 5 minutes. For *M. leprae* DNA, an initial template DNA denaturation step at 95°C for 5 minutes was followed by 37 amplification cycles consisting of denaturation at 94°C for 1 minute, annealing of primers at 63°C for 30 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for a further 10 minutes.

Optimized PCR conditions were found to be sufficiently sensitive to consistently detect DNA concentration as low as 90 ng/ml from *M. tuberculosis* and 50 ng/ml from *M. leprae*.

The amplicons obtained were analyzed by agarose gel electrophoresis (1.5%) after staining with ethidium bromide (0.56µg/ml) and the results were interpreted in comparison with the standard molecular weight marker (VI) from Roche.

RESULTS

In this study, 30 AFB positive sputum samples were subjected to culture and PCR for specific detection of DNA from *M. tuberculosis* and *M. leprae*.

Among these 30 samples, 20 were from patients with clinical TB and 10 were from patients with clinical leprosy. Of the 20 samples from TB patients, 15 were positive in TB PCR and culture. Among the five remaining samples, all were negative for culture, four were negative for TB PCR and one was positive for TB PCR. All the 20 samples from TB patients were negative for leprosy DNA by PCR (Fig. 1 and 2).

In comparison to culture among samples from clinical TB patients, sensitivity, specificity, predictive value of positive test, predictive value of negative test, percentage of false negative and percentage of false positive of TB PCR were 100.0%, 80.0%, 94.0%, 100.0%, 0% and 20.0% respectively.

Of the 10 samples from leprosy patients, five were TB PCR and culture positive, and were negative for leprosy PCR (Fig. 1). The remaining five TB PCR negative samples were also negative in TB culture but positive in leprosy PCR (Fig. 2). This indicates that the AFB in the sputum samples of leprosy patients may be *M. tuberculosis* or *M. leprae*. These could be efficiently differentiated using PCR.

In comparison to culture among samples from leprosy patients, sensitivity, specificity and positive predictive value of leprosy PCR were all 100.0%. Comparing PCR with culture among leprosy patients, the samples which were culture negative were positive in the leprosy PCR while those which were culture positive were positive for TB PCR. No samples were found to contain both *M. tuberculosis* and *M. leprae* either by PCR or culture.

DISCUSSION

Differentiating between *M. tuberculosis* and *M. leprae* microscopically can be difficult. Due to the long delay in obtaining culture results, PCR offers an alternative tool to differentiate between these two pathogens.

The PCR assay used in this study is based on the *M. tuberculosis*-specific multi-copy insertion sequence IS6110 for detection of *M. tuberculosis* and the *M. leprae*-specific repetitive RLEP sequence for detection of *M. leprae*.

In this study, out of 30 AFB positive sputum samples, 20 were from patients with clinical TB and 10 were from patients with clinical leprosy. Among the 20 samples from TB patients, 15 were positive in both TB PCR and culture. Of the remaining five samples, four were negative in both TB PCR and culture. One sample which was culture negative was positive in TB PCR, but was negative in leprosy PCR, which indicated that the AFB organism detected in the sputum was TB bacilli. Positivity in this case may be due to the high sensitivity of PCR, or the presence of non-viable organisms, which is sufficient for PCR amplification. Culture negativity may be due to over decontamination with NaOH while processing.

The remaining four TB PCR negative samples were culture-negative, indicating that they did not contain viable *M. tuberculosis*; they were also PCR negative for RLEP, indicating that the observed AFB was not *M. leprae*. These four samples were collected from two patients. One was a random sputum collection from one patient while the other three were from another patient. The negativity of the first sample may be due to the sample being randomly collected, as subsequent early morning samples from this patient showed positivity in culture and TB PCR. The three remaining samples were from another patient. This patient had an atypical clinical history (patient was still sputum AFB positive following 8 months of Anti Tuberculosis Treatment); samples were negative on repeated TB PCR and culture. On further analysis, results were negative and showed no inhibition when amplified with the Roche system with one common primer and one specific primer for mycobacteria. Partial sequencing of the 16 S rRNA region amplified from these samples followed by a BLAST search indicated that the AFB in this specimen was most likely to be *Prevotella sp.* (Personal communication, Prof. K. Feldmann, Germany).

Of 10 sputum samples from patients with clinical leprosy, five were found to be leprosy PCR positive, while being negative by TB PCR and culture. This indicates that the AFB seen in the microscopy examination were leprosy bacilli rather than TB bacilli. The remaining five samples were found to be TB PCR and culture positive and were negative for leprosy PCR; this indicates that the organisms seen on the AFB microscopy were TB and not leprosy bacilli. This was further confirmed by culture, which is the gold standard method for TB diagnosis. Thus, the results of PCR were in accord with that of culture, emphasizing the specificity of PCR. The rapidity of microscopy can be combined with the specificity of culture in PCR. This early knowledge obtained by the PCR result will help in the type of treatment for the patient.

While comparing TB PCR among clinical TB samples with culture as gold standard, sensitivity, specificity, predictive value of a positive test, predictive value of a negative test, percentage of false negatives and percentage of false positives were found to be 100.0%, 80.0%, 94.0%, 100.0%, 0% and 20.0% respectively. This demonstrates a high sensitivity, specificity and predictive value of a negative test of PCR compared to that of culture. Percentages of false positives and false negatives are also very low indicating the higher accuracy of the test. Percentage of false positives is small, thus lowering the probability of the disease being absent in those samples with the positive PCR result.

Eisenach *et al* initially demonstrated that a PCR method using primers designed from the IS6110 sequence proved to be a highly sensitive and specific means of diagnosing pulmonary tuberculosis.⁸ Hashimoto *et al* studied a PCR assay for the rapid detection of *M. tuberculosis* in sputum samples using the target DNA of a 123-base pair (bp) fragment of IS6110.⁹ The overall sensitivity and specificity were 86.0% and 98.0%, respectively. Gunisha *et al* suggested that PCR using IS6110 to produce 123 bp fragment of DNA is 96.0% specific but only has a sensitivity of 30.0% to detect *M. tuberculosis* in clinical specimens.¹⁰ Considerable laboratory-to-laboratory variability in the sensitivity and specificity of the PCR assay for detecting *M. tuberculosis* in clinical specimens is reported by a number of authors.^{11,12}

Our observation of five of 10 sputum samples from leprosy patients being positive for tuberculosis by PCR and culture is important since one of the causes of death in leprosy patients is tuberculosis. Further, it is important to screen for tuberculosis among leprosy patients in endemic countries where both of these diseases are common. However it should be noted that the leprosy patients enrolled in the study were not randomly selected; they have been referred for sputum sampling because they showed clinical signs of tuberculosis. Clinically, all the leprosy PCR positive patients had a high slit skin smear bacterial index (BI) and were at an early stage of multi drug therapy (MDT) treatment for leprosy. One of the patients negative in leprosy PCR also had a high BI and was at an early stage of MDT.

The result of this study illustrates the potential value of PCR as a complementary tool with other clinical and laboratory tests for investigation and differentiation of dual infections with high sensitivity and specificity. This can be used effectively for the early diagnosis of suspected cases from clinical samples. Although this study tested small numbers of patients and used a selected patient group, the outcome will significantly help in confirmatory diagnosis of clinically, microscopically and culturally confusing cases, thus facilitating effective treatment and case management.

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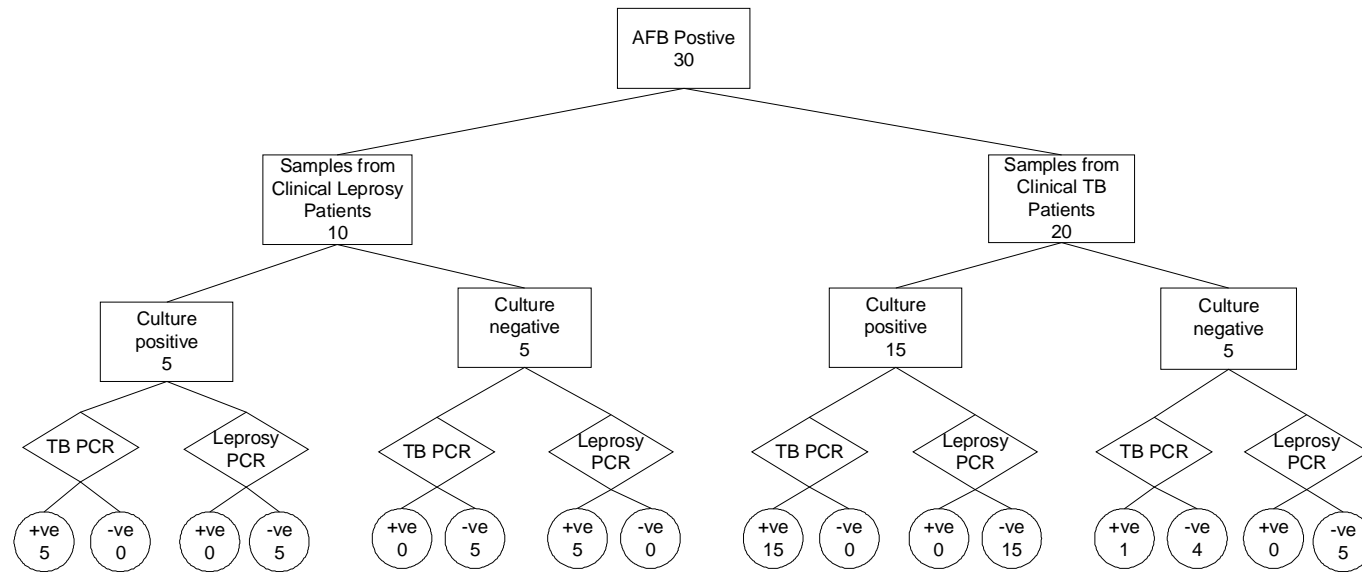


Fig. 1. Summary of clinical results of AFB positive samples in this study. Numbers indicate number of samples per group.



Fig. 2. Differentiation of *M. tuberculosis* and *M. leprae* in sputum samples. Upper and the lower portion of the gel show products for TB and leprosy PCR respectively. Lane 3, 4, 5, are positive for leprosy PCR and 7, 8, 9 are positive for TB PCR on the same samples. Lane 2 and 10 are the negative and positive controls for each PCR assay. Lane M is molecular weight marker (VI) from Roche diagnostics (Germany).