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1. Manoj Kumar, Sandeep Sharma, Anshu B. Ram and Inshad A. Khan. An efficient mycobacterial DNA extraction method from clinical samples for early diagnosis of TB using PCR. International Journal of Tuberculosis and Lung Disease. IJTLD-08-09-0486. (Accepted article in press).


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I hereby confirm that the article entitled “An efficient mycobacterial DNA extraction method from clinical samples for early diagnosis of TB using PCR”, by M. Kumar, S. Sharma, A. B. Ram and I. A. Khan, has been accepted and is scheduled for publication in a forthcoming issue of the International Journal of Tuberculosis and Lung Disease.

Clare Pierard
Managing Editor
An efficient mycobacterial DNA extraction method from clinical samples for early diagnosis of TB using PCR

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<td>Kumar, Manoj; Indian Institute of Integrative Medicine, Clinical Microbiology Unit</td>
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An efficient mycobacterial DNA extraction method from clinical samples for early diagnosis of TB using PCR

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Running title: Efficient DNA extraction method

Keywords: *M. tuberculosis*, Benzyl alcohol guanidine hydrochloride, DNA extraction, PCR, TB diagnosis,

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SUMMARY

BACKGROUND: Polymerase chain reaction (PCR) detection of *Mycobacterium tuberculosis* in the clinical samples requires the use of extraction method that can efficiently lyse the mycobacterial cells and recover small amount of DNA.

OBJECTIVE: To evaluate the use of benzyl alcohol-guanidine hydrochloride DNA extraction method (GuHClM) on blood samples.

DESIGN: GuHClM was evaluated in quantitatively spiked blood samples with *M. tuberculosis*. We assessed the IS6110 region of *M. tuberculosis* to evaluate the efficacy of the method. The method was also applied on 102 clinical samples of suspected TB individuals and compared with the smear microscopy of sputum specimens and the results of cultures.

RESULTS: This method reproducibly detected as low as 4-6 bacilli. Out of 102 clinical samples, 84 were found human immunodeficiency virus (HIV) negative, while 18 were HIV positive. In HIV negative individuals, 58.3% were found TB positive using PCR, while 47.6% and 45.2% yielded sputum and culture positive respectively. Among the HIV positive individuals 55.6% were PCR positive, whereas only 38.9% found sputum positive and 50% observed culture positive.

CONCLUSION: These results demonstrated that the identification of mycobacteria by PCR using GuHClM is very sensitive and therefore, may have wide utility in the diagnosis of TB.
INTRODUCTION

Tuberculosis (TB) is the most common cause of morbidity and mortality in developing countries, including India.\(^1\) Despite five decade of control programs and the availability of efficacious drugs, about two million people annually die due to TB, and approximately one-third of the world’s population is asymptomatically infected with *Mycobacterium tuberculosis*.\(^2\) Co-infection of HIV positive patients with TB and emergence of extensively drug resistant (XDR) TB has further worsen the situation.\(^3,4\)

Rapid diagnosis of TB is of prime importance to minimize the risk of disease transmission and for early and successful treatment. By far culturing and direct smear microscopy are considered as the gold standards and widely available diagnostic method for TB.\(^5\) Unfortunately, smear microscopy is neither specific for *Mycobacterium tuberculosis*, nor has good sensitivity. In patients with active pulmonary TB, only an estimated 45% of infections are detected by sputum microscopy.\(^6\) Moreover, in most situations in which TB is diagnosed by AFB microscopy, it should be assumed to be *M. tuberculosis* until proven by traditional culturing method, which has good sensitivity but requires weeks for results due to the slow growth of *M. tuberculosis* and delays control and treatment efforts.

The poor slow and/or time-consuming methods of TB diagnosis have stimulated the increased use of molecular techniques of mycobacterial detection. Although, the amplification of microbial specific sequences from clinical samples offers the potential for rapid detection and specific identification of pathogen, either directly or after culturing.\(^7\) but in case of mycobacteria, it hampers with two major limitations: (i) difficulty associated with breaking mycobacterial cell wall to release DNA suitable for
PCR amplification and (ii) limited sensitivity of assay for clinical samples, such as blood samples, in which the amount of mycobacterial cells or DNA may be very low. As a consequence, molecular detection of mycobacterial DNA from clinical specimens has been shown with variable sensitivities ranging between 42 to 100% and specificities ranging between 85 to 100%.\(^8\)\(^{11}\)

Mycobacterial cell is very difficult to lyse due to the presence of lipid-rich multilaminar cell envelope that provides rigidity to outer leaflet of mycobacterial cell wall. Further, mycobacterial cells or DNA are present in very low quantity in the blood of TB patients (\(<10\) CFU/ml of blood in case of extrapulmonary TB or may be lower in pulmonary TB patients\(^{12}\)). Moreover, the presence of PCR inhibitors in the clinical specimens further impedes the use of PCR assays;\(^{13}\)\(^{-18}\) therefore, the sensitivity of detection method is very critical. The DNA extraction assay must be able to recover small amount of pure and inhibitor free DNA, to enhance the use of PCR in clinical specimens.

In this study, we evaluated the use of benzyl alcohol-guanidine hydrochloride DNA extraction method (GuHClM) on the blood samples spiked with low number of mycobacterial cells for the PCR detection of \textit{M. tuberculosis}. We also applied this method on clinical samples to evaluate its potential use in TB diagnosis and compared its sensitivity with standard methods.

**MATERIALS AND METHODS**

\textit{DNA extraction from spiked samples}

Initially a set of experiment was performed to determine the detection limit of GuHClM. 10 ml of human blood was obtained from healthy volunteer, who had normal chest radiogram and no past TB history. The absence of acid-fast bacilli (AFB) was confirmed...
by AFB staining of the blood smear. The turbidity of logarithmic-phase *M. tuberculosis* culture was adjusted to a McFarland no.1 (~ $1 \times 10^7$ cfu/ml) in a sterile normal saline and was further serially diluted to 1:1 in duplicate sets, in sterile normal saline. One set of dilutions was used to estimate the CFU count by spotting on Middlebrook 7H10 plates supplemented with 10% OADC (oleic acid-albumin-dextrose-catalase). The dilutions from set 2 were used to quantitatively spike 1 ml of blood aliquots. The unspiked blood sample was also taken as negative control for DNA isolation and subsequently for PCR analysis.

The DNA was isolated by taking 400 µl of spiked blood samples in 1.5 ml screw cap microcentrifuge tube, containing 100 µl of lysis buffer {5.0 M guanidine hydrochloride-100 mM Tris-Cl (pH 8.0)} and was gently mixed on a vortex mixer. 100 µl of sterile water was added to the tube, followed by the addition of 800 µl of 99% benzyl alcohol (Rankem, India). The content was mixed again by vortexing. The tubes were incubated in a water-bath at 37°C for one hour and centrifuged at 7,000 × g at 4°C for 7 min. The supernatant, containing DNA was precipitated with equal volume of isopropanol and kept at −20°C for overnight. The precipitated DNA was collected by centrifugation at 13,000 × g for 15 min at 4°C, washed twice with 500 µl of 70% ethanol and air-dried. The DNA was resuspended in 10 µl of 10 mM Tris-Cl (pH 8.0) and used for the PCR assay.

PCR for *M. tuberculosis* was performed by using IS6110 specific, forward primer- 5’-CCT GCG AGC GTA GGC GTC GG-3' and reverse primer- 5’-CTC GTC CAG CGC CGC TTC GG-3' (19). The 50 µl of reaction mix contained 5 µl of buffer {200 mM Tris-Cl (pH 8.0), 500 mM KCl and 25 mM MgCl₂}, 0.25 µl of 25 mM MgCl₂, 1.25 µl of 2.5 mM dNTPs, 1.5 µl of each primer (10 pmol/µl), 0.5 µl (1.5 U) of Taq DNA polymerase
The PCR assay was carried out in a Thermal cycler (Bio-Rad DNA Engine\textsuperscript{R}, NSW, Australia), using the following amplification conditions: 5 min at 94°C, followed by 35 cycles each of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR products were electrophoresed in 1.5% agarose gel and visualized under UV light.

Reagent, sample preparation, PCR amplification, and product detection were performed in separate room using dedicated equipment, aerosol-resistant filter guard pipette tips, in an unidirectional work flow, to minimize the possibility of any false positive result due to carryover amplicon contamination. Positive and negative control for both preparation and PCR assay were taken in every experiment.

Clinical specimen collection and processing

Blood and sputum samples are collected as a part of the routine diagnostic examination of suspected TB patients attending TB clinic of C.D. Hospital. Blood samples were used for HIV testing and DNA isolation, while their sputum samples were tested for acid-fast bacilli smear and for mycobacterial culture, identification, and drug-susceptibility testing. Additionally, as part of the routine clinical examination at the C.D. hospital, information on current and previous history of \textit{M. tuberculosis} infection, history of contact with a TB case and a history of receiving previous anti-tuberculosis treatment was taken from the patients. Samples collection and handing were carried out, as per the standard protocols and all safety and precaution necessary for the handling of the samples, were followed.

We did not intervene to modify routine clinical practice. At these sites, treatment for TB
and HIV were freely available from government providers using standard WHO-
recommended regimens.

This study was approved by the Council of Scientific and Industrial Research (Reference
No. SMM0003/2003), India and the research review committee of Indian Institute of
Integrative Medicine (Formally known as Regional Research Laboratory), Jammu, India.

Verbal informed consent was sought from all suspected cases of TB included in this
study.

Between November 2Q 0^m and December 2004, 102 blood and sputum samples were
collected from the hospital, which were diagnosed (clinically or radiologically) or
suspected of having TB. All the individuals included in the study were suffering from
fever malaise, chest pain, and chronic coughs with or without sputum production for
more than one month. Blood samples were collected in sterile tubes containing 100 µl of
5% (wt/v) EDTA solution as an anti-coagulant and were immediately shifted to BSL-3
lab Indian Institute of Integrative Medicine, Jammu for processing.

Immunodeficiency virus (HIV) test was carried out by using commercially available TRI-
DOT Rapid HIV flow-through test (Mitra & Co., New Delhi, India). The sputum samples
were digested and decontaminated using equal volume of 4% NaOH-NALC (N-acetyl-L-
cysteine) solution and used for smear preparation and inoculation of two L-J slants for
each specimen. All the smear slides were subjected to Ziehl-Nelsen (ZN) staining. Slide
reading was not limited to 300 fields, but rather 400 tp 500 fields were read to ensure
maximum smear coverage. The L-J slants were incubated at 37°C for 4-5 weeks in a 5%
CO₂ incubator and were examined weekly for growth. Bacterial colonies were identified
as M. tuberculosis by conventional identification methods.19
DNA isolation PCR amplification

DNA was isolated from the blood samples using GuHClM described above. A total of 400 µl of blood sample was taken for DNA isolation. Finally air-dried DNA pellet was dissolved in 10 µl of 10 mM Tris-Cl (pH 8.0) and directly used for PCR as described above.

RESULTS AND DISCUSSION

The GuHClM was extensively evaluated for DNA extraction from spiked blood samples and subsequent PCR sensitivity. Overall efficacy was determined by DNA quality and specific PCR assay. Although, the quantities of *M. tuberculosis* DNA, even at the highest inoculums level, were negligible compared with amount of human DNA from the whole blood, even than the blood specimens containing 4 to 6 bacilli per sample were reproducibly detected by this method, while mycobacterial DNA was not detected in extraction control (uninoculated blood) (Figure 1). This limit of detection therefore equated to 72 copies of target/ml of blood (assuming ~18 IS6110 gene copies per mycobacterial cell), which is showing maximum sensitivity that the assay could be expected to achieve in testing of clinical specimens. This value compares favorably to results for the diagnosis of TB generally obtained by conventional method such as sputum microscopy (requires >10^3 to 10^4 mycobacteria/ml).^20^ Since the number of bacilli in adult blood is usually ≤ 10 CFU/ml, in case of extrapulmonary TB,^12^ which can be easily detected using this method. Moreover, the use of PCR assay for detection of bacteria in blood samples has been limited due to the presence of inhibitory substances in the blood,^14,21^ while GuHClM was found efficient in removing PCR inhibitors, thereby leading to reproducible DNA amplification.
The GuHClM was extensively employed on the clinical samples of the patients. The PCR results thus obtained were compared with the conventional methods. A total of 102 blood and their sputum samples respectively from suspected TB individuals were included in this study. Of the total 102 individuals, 84 were found HIV negative, while 18 were HIV positive. Of the 84 HIV negative individuals; 49 (58.3%) were found positive for TB by PCR assay, in comparison with sputum analysis that were positive in 40 (47.6%), while only 38 (45.3%) yielded culture positive (Table 1 and 2). Among the HIV positive individuals, we found a substantially higher number of PCR positive 10 (55.6%), whereas 9 (50%) were culture positive and only 7 (38.9%) of them were diagnosed for TB by sputum AFB analysis, because smears are often less sensitive in non-cavity disease or in HIV co-infection patients. Of the 10 patients found PCR positive and co-infected with HIV, two were without clinical confirmation of TB. Further analysis of these patients showed that one was diagnosed for TB earlier and had received anti-TB drugs for ~20 days, while other patient was first time diagnosed for TB and he had no past history of TB. Such findings are really worrying for resource-limited settings, and where HIV-infection rate is very high. In our study we found 2 PCR negative patients but were either sputum smear positive or cultural positive for TB. Possible explanations for these patients are, either they were having low level infection or infected with \textit{M. tuberculosi}s strain, which do not carry IS6110. During the clinically evaluation of this study, we found around 80% new cases having no past history of TB; while 10% of patients were treatment defaulter; an additional one health-care worker was also found TB positive. Our results do not reflect an outbreak of extrapulmonary TB or over burden of bacterial load in patients, because only one patient showed a military pattern on chest
radiograph and the susceptibility profile of the isolates shows that TB patients included in this study were infected with different *M. tuberculosis* strains (data not shown).

A recent study also has reported the significance of PCR in the detection of *M. tuberculosis* DNA in the blood of pulmonary TB patients, whereas blood cultures only yielded a positive result in 4% of patients. Although, the presence of Mycobacterial DNA in the blood, in the absence of viable bacteria, suggested the effective role of phagocytosis mediated damage of mycobacterial cells, but in such cases blood PCR could be an effective diagnostic tool for the detection of infection. For this reason, we used whole blood inoculated with low numbers of *M. tuberculosis* cells to simulate the low-level of infection present in blood from patients with pulmonary TB. The data generated from this study suggests that guanidine hydrochloride DNA extraction method is probably a superior to other traditional methods for the diagnosis of TB. Furthermore, this DNA extraction method is technically simple, less expensive with overall very good sensitivity. Moreover, the data of our study suggests that guanidine hydrochloride DNA extraction method may be optimal for extraction of DNA from whole blood for the diagnosis of TB.
ACKNOWLEDGEMENTS

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REFERENCES


Fig. 1. 1.5% agarose gel electrophoresis of PCR products amplified with *IS6110* (123 bp) gene primers of *M. tuberculosis*. Lane No. 1. PCR positive control of *M. tuberculosis*, lane No. 2-7, PCR amplification of *IS6110* fragment from blood sample spiked with 125, 62, 31, 16, 8, and 4 CFU/sample, lane No. 8, performed with sterile blood sample (negative control). Arrow indicates the PCR fragments specific for *M. tuberculosis*.

PC, Positive control; NC, Negative control.
Fig. 2. Agarose gel electrophoresis of PCR products amplified with IS6110 (123 bp) gene primers of *M. tuberculosis*. Lane 1, 3-kb DNA ladder; lane 2, PCR amplification of IS6110 fragment from clinical blood sample; lane 3, positive control of *M. tuberculosis*; lane 4, performed with sterile blood sample (negative control). Arrow indicates the PCR fragments specific for *M. tuberculosis*. 
Table 1 Patient’s characteristics (n=102).

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<th>Characteristics</th>
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<td>Male</td>
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<td>Overall</td>
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<tr>
<td>&lt; 10</td>
<td>3 (2.9%)</td>
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<tr>
<td>≥ 60</td>
<td>14 (13.7%)</td>
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<tr>
<td>Clinical (sputum) and/or radiological suggestion of active TB</td>
<td>40 (39.2%)</td>
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<tr>
<td>PCR positive for TB</td>
<td>49 (48%)</td>
</tr>
<tr>
<td>HIV-infected</td>
<td>18 (17.7%)</td>
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<td>HIV-infected and PCR positive for TB</td>
<td>10 (9.8%)</td>
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<td>Clinical (sputum) positive for TB and HIV co-infected</td>
<td>7 (6.9%)</td>
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Table 2 Concordance of PCR detection for *M. tuberculosis* with sputum microscopy for AFB and cultural analysis.

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