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Original Research Paper

# Peripheral Blood and Diagnosis of Tuberculosis of Spine, Bone and Joint

Microbiology

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With the decline of pulmonary tuberculosis, there is significant decline in the incidence of bone and joint tuberculosis (TB) in the western world. However, it still remains a serious problem in underdeveloped countries including India (1, 2). In 2012, a total of 8.6 million people fell ill with TB and 1.3 million died from it, including 3, 20,000 HIV infected individuals. In 2012, an estimated 4,50,000 were new cases of multidrug-resistant TB globally.(3) Bone and joint tuberculosis, a cause of deformity and lifelong disability is usually neglected due to emphasis on open cases of pulmonary tuberculosis in almost all the developing countries. However, it needs to be detected and treated early. Approximately 10% of tuberculous infections are extrapulmonary, and 10% of such infections involve the musculoskeletal system. Skeletal tuberculosis is an indolent disease, and delay in diagnosis has been reported to be as long as 16 to 19 months (4). Definite diagnosis of the disease requires isolation of the bacterium, for which we generally need specimens from the affected site. However, it is not feasible to collect affected tissue of bone and joint tuberculosis due to ethical issue and technical difficulty in the early stage. Even if such difficult specimens are collected, acid fast staining fails to detect TB and the cultures are not positive in >50% of the cases due to their paucibacillary nature (5). In recent years, protocols based on nested polymerase chain reaction (PCR) have raised hopes for a reliable and fast diagnosis of such extrapulmonary tuberculosis (6,7). Nested PCR provides an easier and more accurate method to detect low number of infecting pathogens because it takes care of PCR inhibitors present in the biological specimens. In the recent past, we could detect *M.tuberculosis* by amplifying specific target using nested PCR in peripheral blood collected from pulmonary tuberculosis cases. Encouraged with this finding, we planned to see the presence *M. tuberculosis* bacteria in peripheral blood from clinically suspected cases of spine, bone and joint tuberculosis in the present study.

This study was carried out during the period of 2012-2014 in Department of Microbiology in collaboration with Department of Orthopaedics of the University Hospital of Banaras Hindu University, Varanasi, India. A total of 117 cases were selected depending on the clinical and radiological suspicion of tubercular involvement of spine, bone and/or joint presenting with fever, low backache, tibial, knee or elbow swelling not responding to routine antibiotics. The study design was approved by the Institutional Ethics Committee and informed consent was taken from each of the patients.

The samples were collected under strict aseptic precautions in the operation theatre. The specimens were synovial fluid, disc, bone marrow aspirate and pus from the diseased part and tissues, such as synovial or vertebral bone (closed or open biopsy) and blood. All the samples were subjected to nested PCR after DNA extraction.

DNA was extracted from the specimens following the methods described by Sambrook *et al*. (8) Heat shock protein gene *(hsp65)* of *M. tuberculosis* a conserved gene targeting which in house designed already tested primers hsp TB**F1** and hsp TB**R1** were selected for amplification of a 494 bp nucleotide sequence for primary PCR; hspTB**F2** and hspTB**R2** were selected for amplification of a 218 bp nucleotide sequence for nested PCR (9).

The oligo sequences (5′-3′) were as follows:

**For Primary PCR:**

hspTB*F1*: AAA AGC CGG ATG GCA ATT CG

hspTB*R1*: ATT ACC GGC TTG GAC CCC CTG

**For Nested PCR:**

hspTB*F2*: ATG CGG CGC ACA CCG AAG ACA AG

hspTB*R2*: TCA ACG CGC TGT CTA GCT TGT C

PCR reaction was carried out in 25 µl volume. Reaction mix contained 10 x reaction buffer (5 µl/sample), dNTPs (2.5 mmol each base), forward and reverse primers (concentration 10 ϱmol/ µl) and *Taq polymerase* enzyme (1U). Amplification was carried out on Bio Rad, Thermo Cycler, with a heated lid. The hot start method was employedby heating at $94^0$C for 5 min initially. Thereafter, amplification was carried out for 35 cycles at $92^0$Cfor 1min (denaturation), $65^0$C for 1min (annealing) and $72^0$C for 1min (extension). Extra extension was carried out at $72^0$C for 7 min. The amplification products of primary PCR was again amplified with nested primers following same protocol. The final amplification products were analyzed on 2 % agarose gel stained with ethidium bromide under UV light. Positive (DNA extracted from *M.tuberculosis* growth) and negative (double distilled water) controls were run with each batch of samples analysed. In our study, we used the reference strain H37Rv *M. tuberculosis* as positive control and a reagent blank (no target DNA) as negative control.

None of the specimens were found positive for acid fast bacteria by Ziehl Neelsen staining. Of the 117 specimens examined; 47 (40%) were found to be PCR positive for *M. tuberculosis*. Nested PCR done in the blood samples from these 47 cases showed a positivity of (14/47, 30%) (Table). In the light of great difficulty in specimen collection from clinically suspected cases of osteo-articular tuberculosis and inadvertent delay in the start of antitubercular therapy due

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to diagnostic dilemma, the present, although small study indicates that mycobacterial DNA do circulate in peripheral blood in cases of paucibacillary tuberculosis also. There are a few studies which have shown the presence of the bacterium in peripheral blood circulation. Miyazaki *et al* (1993) by using nested PCR was able to show the presence of M. tuberculosis DNA in 16.6% (4/24) of the military tuberculosis cases (10). A study from Brazil carried out on blood collected from clinically suspected cases of tuberculosis of paediatric age group showed significantly higher detection of *M. tuberculosis* by nested PCR in extrapulmonary than that obtained in the cases of pulmonary TB. However in this study the authors have not examined the specimens from affected part of the body (11). Another study from Taiwan; targeting mpt64 gene has shown that the circulation of bacilli in the peripheral blood is more likely in cases of the extrapulmonary form of the disease than the pulmonary(12). Another group of authors have indicated the difficulty in finding *M. tuberculosis* in extrapulmonary specimens, in which there are few bacilli, leading to low sensitivity of smear microscopy and of culture (13). The authors have also implicated the presence of inhibiting factors that can interfere in the DNA amplification using PCR. However, in our study also using the blood of paucibacillary patients, we found the sensitivity of PCR to be quite good in extrapulmonary cases. Although, no explanation has been given by the previous workers why the TB PCR positivity was low in pulmonary cases, it seems to be an advantage to the paucibacillary cases. These DNA can be detected by using sensitive and specific nested PCR. Positivity of the *M. tuberculosis* nucleic acid marker in 1/3$^{rd}$ of those cases whose affected tissues were also positive for HSP specific amplification of the bacterium, indicates the possibility of better sensitivity if we would have examined multiple blood specimens collected at different sittings or large volume of blood is collected or nested PCR is carried out in DNA isolated from Buffy coat probably carrying the bacterium or mRNA is isolated from the peripheral blood and either nested or real time PCR is performed. Detection of m RNA further will confirm the viability of bacteria along with higher sensitivity because high copy number of them in comparison to chromosomal target. This is a preliminary study looking into a paucibacillary tuberculous infection of bone joints and discs etc. The encouraging results suggest for study involving large number of subjects will definitely provide some clue in peripheral blood samples before the development of serious morbidity and mortality.

**Table: showing positivity for specific heat shock protein (hsp) gene of M. tuberculosis in different samples collected from suspected cases of tuberculosis**

| Specimens | Synovial Fluid | Pus | Disc specimen | Bone marrow aspirate | Total positivity in non blood samples | Blood from patients positive for amplification |
|---|---|---|---|---|---|---|
| PCR positive/ Total number of the sample | 9/21 (43%) | 16/37 (43.24%) | 21/58 (36.2%) | 1/1 (100%) | **47/117 (40.1)** | **14/47 (30%)** |

Figures in parenthesis are percentages

**References:**


1. Shah AA, Ahmed S, Shah H, Raziq F. Bone tuberculosis in Abbottabad. J Pakistan Med Assoc 1992 ; 42 : 180-181.
2. Shannon FB, Moore M, Houkom JA, Waecker NJ. Multifocal cystic tuberculosis of bone : Report of a case. J Bone Joint Surg 1990 ; 72-A : 1089-1092.
3. Global strategy and targets for tuberculosis prevention, care and control after 2015 World Health Organization 2013 EB 134/12 Provisional Agenda Item 6.1
4. Williams KD. Tuberculosis and other unusual infections. Tuberculosis. Diagnosis. In : Terry Canale S (ed). Campbell's Operative orthopaedics. Mosby-Year Book, St Louis, 1998, pp 626-627.
5. Hopewell PC. Overview of clinical tuberculosis. In :Bloom BR (ed). Tuberculosis. Pathogenesis, protection and control. American Society for Microbiology,Washington DC, 1994, pp 25-46.
6. Eisenach KD, Cave MD , Crawford JT. PCR detection of Mycobacterium tuberculosis. In : Persing DH,Smith TF, Tenover FC, White TJ (eds). Diagnostic molecular microbiology. Principles and applications. American Society for Microbiology, Washington DC, 1993, pp 191-196.
7. Iseman MD. Tuberculosis. Epidemiology In : Bennet JC (ed). Cecil Textbook of Medicine. Benett & Plum, Philadelphia,1996, pp 1683-1684.
8. Sambrook, J, et al. Molecular Cloning: A Laboratory Manual, Third Edition, Cold Spring Harbor Laboratory Press, New York (2000)
9. Sareen A, Khare GN, Nath G, Singh S. Role of polymerase chain reaction in osteoarticular tuberculosis. Indian J Ortho 2006;40:264-266.
10. Miyajaki, Y. Koga, H., Kohno, S. and Kaku M. Nested polymerase chain reaction in clinical samples. J. Clin. Microbiol. 1993, 31, 2228
11. Honore S, Vincensini JP, Hocqueloux L, Noguera ME, Farge D, Lagrange P, et al. Diagnostic value of a nested polymerase chain reaction assay on peripheral blood mononuclear cells from patients with pulmonary and extra-pulmonary tuberculosis. Int J Tuberc Lung Dis. 2001;5(8):754-62.
12. Shin-Hwar Wu, Cheng-Mao Ho and Jang-Jih Lu. Diagnosis of Tuberculosis by PCR-based Amplification of mpt64 Gene from Peripheral Blood. International Journal of Biomedical Laboratory Science (IJBLS) 2013 Vo1. 2, No. 1:25-30
13. Honoré-Bouakline S, Vincensini JP, Giacuzzo V, Lagrange PH, Herrmann JL. Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. J Clin Microbiol. 2003;41(6):2323-9.