Extra Pulmonary Tuberculosis: A Diagnostic Dilemma

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Abstract Tuberculosis remains a major public health problem globally, with India being one of the high burden countries. The common causative agent is *Mycobacterium tuberculosis* but in developing countries *M. bovis* is reported as a potential human pathogen. Almost 20% of all reported cases of tuberculosis are of extra pulmonary form of disease. Diagnosis of extra pulmonary tuberculosis (EPTB) is not always possible with conventional methods, due to the long time required and the paucibacillary nature of samples; hence the need of rapid molecular methods. A prospective study was conducted on 300 patients of EPTB over a period of 5 years. These patients were suspected cases of tubercular meningitis, tubercular ascites and tubercular lymphadenitis. Samples analyzed were cerebrospinal fluid, ascitic fluid and lymph node fine needle aspirate. A two step PCR targeting *hup B* gene was used. Clinical response to anti tubercular therapy (ATT) was taken as positive (gold standard). PCR for *hup B* gene was positive in 147 samples out of 155 ATT responders. Of these 85.71% were infected with *M. tuberculosis*, 9.52% with *M. bovis* alone and 4.76% showed co infection with both *M.tb* and *M. bovis*. The sensitivity and specificity of PCR was 90.32 and 94.48% respectively.

Keywords *Mycobacterium tuberculosis* · *Mycobacterium bovis* · *hup B* gene · Extra pulmonary tuberculosis

Introduction

Tuberculosis, a disease still to be conquered in developing countries has staged resurgence in developed world due to HIV infection and multi drug resistance acquired by the infectious agent. This has prompted world health organization (WHO) to declare tuberculosis as a global emergency [1].

According to the Global Tuberculosis Control WHO report 2009, there were 9.27 million new cases of tuberculosis in 2007 with South East Asia Region bearing 55% of the global burden. There were 13.7 million prevalent cases and 1.78 million deaths due to tuberculosis in 2007. Total number of global cases are still increasing in absolute terms as a result of population growth. India ranks top in being the highest TB burden country in the world [2].

The last decade has witnessed shifting trends in tubercular infection, with extra pulmonary tuberculosis (EPTB) emerging as an important entity. EPTB constitutes about 15–20% of all cases of tuberculosis in immunocompetent patients and accounts for more than 50% of cases in HIV positive individuals [3, 4]. It is a protean disease that can virtually affect any organ. The common sites are lymph nodes, genito-urinary tract, bone and joints, and meninges.

Tuberculosis in human population is mainly caused by *Mycobacterium tuberculosis* (*M.tb*), an acid fast, aerobic and slow growing bacillus. However *Mycobacterium bovis* (*M.bo*), which is primarily a bovine pathogen, has been reported to cause tuberculosis in humans [5], mainly extra pulmonary form. The clinical manifestations of both are indistinguishable. A study on extra pulmonary clinical samples obtained from cattle and humans showed *M. tuberculosis* infection in 15.7% of humans while *M. bovis* was seen in 26.8% cattle [6]. Mixed infection with both the species was seen in 8.7 and 35.7% of human and cattle

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samples respectively. This denotes the prospect of potential transmission of these pathogens from cattle to human (zoonosis) and vice versa (humanosis).

Prabhakar et al. (1997) discovered a histone like protein of *M. tuberculosis* [7]. The gene coding for it was named as *hup-B* (*Rv* 2986) by Cole et al. [8]. It has a 74% bias for G/C in the third position of codons, which is similar to other mycobacterial genes. The gene has 645 bp in *M. tuberculosis* but in *M. bovis* it consists of only 618 bp. This is due to a deletion of 27 bp (9 amino acids) after codon 128 in the C-terminal portion of the *hup-B* gene in *M. bovis*. Based on this 27 bp deletion it is possible to differentiate between the two species, using a two step nested PCR [9].

Diagnosis of EPTB poses a challenge to the clinician due to its variety of presentations and insidious onset which does not bring the patient to the physician at an early stage of disease. Absence of typical clinical features and often negative conventional diagnostic tests (smear microscopy and culture) due to paucibacillary nature of samples also contribute to delay in diagnosis. Thus rapid molecular diagnostic methods are the need of the day.

Our study focuses on using a two-step nested PCR targeting *hup-B* gene to identify *M.tb* complex and differentiate between *M.tb* and *M. bovis* infection in clinical samples of EPTB.

Materials and methods

The prospective study was conducted in the Departments of Biochemistry, Medicine, Pediatrics, Microbiology & Pathology, Lady Hardinge Medical College and associated hospitals over a period of 5 years (2003–2007) after institutional ethical clearance.

Patients

A total of 300 patients were enrolled in our study after written informed consent. They were 100 cases of suspected tubercular meningitis (TBM) in children selected on basis of Modified Ahuja criteria [10]. One hundred patients of suspected tubercular ascites (TBA) were enrolled from Medical wards. These presented with ascites where other causes like cirrhosis and malignancy were ruled out. 100 Fine needle aspirates positive by cytology/smear for tuberculosis in suspected tubercular lymphadenitis (TBL) cases were also included in the study who presented with lymphadenopathy that did not respond to 2 weeks of routine antibiotic therapy. Patients were of all ages and both sexes. All patients were put on antitubercular therapy (ATT) Category I (2HRZE + 4 HR). They were followed

up for at least till the end of intensive phase of therapy to see the clinical response to ATT which was taken as the gold standard for positivity. The patients who responded to ATT reported a subjective sense of well being, increase in appetite and loss of malaise. They became afebrile and gained weight.

Samples

The samples analyzed were CSF-100, ascitic fluid-100, lymph node fine needle aspirates-100, Routine blood biochemical tests (Liver and Kidney function) and complete hemogram were done. Mantoux test and X-ray chest were done for all patients. X-ray skull, abdominal pelvic ultrasound and CT scan were performed where indicated.

All samples of CSF, Ascitic fluid, and lymph node fine needle aspirates were collected using aseptic precautions in sterile containers and split into four aliquots. They were subjected to (i) Cytology, (ii) Smear microscopy by Zeihl Neelson/Geimsa stain for acid fast bacilli (AFB), (iii) Culture for Mycobacteria in Lowenstein-Jensen medium, (iv) Polymerase Chain Reaction (PCR) targeting *hup B* gene.

Samples of biological fluids/biopsy were processed by Universal Sample Processing (USP) method [11] in tuberculosis laboratory, department of microbiology to extract Mycobacterial DNA for PCR. This was stored at –20°C until further use.

A two step PCR was done targeting *hup B* gene, using 40 µl reaction mix. Two sets of primers were used for the assay: N–S and C-terminal F–R. Positive and negative controls were set up with each reaction. The first amplification reaction employed primers N-(5'-GAGGGTTGG GATGAACAAAGCAG-3') and S-(5'-TATCCGTGTCTT GACCTATTTG-3'). The PCR program for N1S1 included initial denaturation (95°C for 10 min) cycle denaturation (94°C for 1 min 30 s) annealing (60°C for 1 min 30 s) extension (72°C for 1 min 50 s) for 35 cycles and final extension 72°C for 30 min. The size of amplicons obtained was 645 bp for *M.tb* and 618 bp for *M. bovis*. These products were used as templates for nested PCR to amplify the C-terminal region of *hup B* gene. The primers employed were C terminal F-(5'-CCAAGAAGGCGA CAAAGC-3') and C terminal R-(5'-GACAGCTTCTTGG CGGG-3'). The PCR program for CTFR included initial denaturation (95°C for 10 min), Cycle denaturation (94°C for 1 min), annealing (60°C for 30 s), extension (60°C for 30 s) for 35 cycles and final extension at 72°C for 7 min. The size of amplicons obtained was 116 bp for *M.tb* and 89 bp for *M. bovis*. They were resolved on 8% native PAGE and stained with ethidium bromide. The gels were viewed under ultraviolet light in gel documentation system.

Results

A total of 300 patients of clinically suspected tuberculosis were enrolled in the study and put on category I ATT for a period of 6 months. Of these 299 patients could be followed up for 2 months till the end of intensive phase of therapy. A total of 155 patients showed clinical response to anti tubercular therapy. They became afebrile, showed improvement in anorexia and general sense of well being and hence were considered positive for tuberculosis infection.

The clinical presentation of patients varied with the tissue involved in the disease process. The common complaints were fever of 2 weeks to 3 months duration in 90% of TBM cases, in 53% of ascites and 48% of TBL cases. Anorexia and weight loss were other common complaints in 30–40% of patients. History of contact with a diagnosed case of tuberculosis was positive in 34% cases of TBM and TBL and 18% in TBA cases. ESR was raised in 82–95% of patients. Changes suggestive of tuberculosis in Chest X-ray were seen in 31.2% of cases. Intradermal tuberculin test (Mantoux test) with 1 unit of PPD of more than 10 mm indurations after 48–72 h is considered positive was found in 54.6% of all cases of EPTB (Fig. 1). Some patients of TBM also had vomiting and convulsion and 40% of patients showed abnormal CT findings like hydrocephalous/infarct/exudates. The most common lymph nodes involved were cervical followed by axillary lymph nodes. Abdominal pain was complained by 69% of suspected TBA cases. Some also complained of diarrhea/constipation. Abdominal ultrasound in 20% cases was suggestive of tuberculosis.

Smear microscopy for AFB was positive in one case of ascitic fluid. For tuberculous lymphadenitis Cytology/smear positive samples ($n = 95$) were taken to evaluate

PCR in such cases. Hence a total of 96 cases were positive for AFB smear (Fig. 1).

Mycobacterial growth was seen in 8 cases of tubercular lymphadenitis. No growth was seen in any sample of CSF or ascitic fluid (Fig. 1).

PCR for *hup B* gene was positive in 147 samples out of 155 ATT responders (94.83%) (Fig. 1). Of these 85.71% were infected with *M. tuberculosis*, 9.52% with *M. bovis* alone and 4.76% showed co infection with both *M.tb* and *M. bovis* (Table 1).

The sensitivity and specificity of PCR was 90.32 and 94.48% respectively. The positive and negative predictive values are 95.23 and 88.88% respectively.

Discussion

Diagnosis of EPTB is challenging in a clinical set up. This is because the conventional methods like AFB smear and culture are not always positive, as most samples of EPTB like CSF, Urine, and aspirates are paucibacillary in nature which also contributes to the milder form of infection. *M.tb* growth on Lowenstein-Jensen culture medium is also rarely positive besides a long time required for confirmation. Hence in our study clinical response to ATT is considered as positive for tuberculosis.

300 cases of clinically suspected extra pulmonary tuberculosis were enrolled after informed written consent and put on ATT. They were followed up at least till the 2 months of intensive phase of therapy to see its response. The patients were of all age groups and both sexes. Constitutional signs and symptoms like low grade fever, anorexia, malaise, weakness is seen in majority of cases that improved with therapy. Besides this most had lymphocytosis and raised ESR in peripheral blood examination

Fig. 1 Investigation profile in ATT responsive cases

Table 1 Mycobacterial infection in PCR positive cases

Type	Percentage
<i>M. tuberculosis</i>	85.71
<i>M. bovis</i>	9.52
Coinfection (<i>M.tb</i> and <i>M. bovis</i>)	4.76

but these are non-specific. Mantoux test was positive in 54.6% of EPTB cases. It has also reported about 50% positivity by Mantoux test. X-ray chest showed changes positive for tuberculosis in 31.2% of all cases suggesting that EPTB may be secondary to pulmonary origin in such cases.

Smear stained by Zeihl-Neelson stain for AFB was positive in 31.2% EPTB samples. It has been reported that a positive smear requires about 10^4 bacilli/ml [13]. Thwaites et al. reported that a minimum of 6 ml of CSF is required for smears to be AFB positive [14] which is not possible in clinical practice more so in children, which explains no positive TBM case. In TBL cases we have enrolled smear/cytology positive FNAC aspirates, yet we got 5 cytology positive AFB smear negative cases who responded to ATT clinically giving a sensitivity of 95% and specificity of 100%. In TBA cases also due to low bacillary count only one positive smear was obtained.

Culture for Mycobacterial growth was positive in 8 cases of TBL and negative in all cases of TBA and TBM due to paucibacillary nature and disparity in distribution of bacilli in sample aliquots. Singh et al. [15] has reported 14% positive culture in TBL. A study by Sinha et al. [16] has found culture positivity in <20% in TBA.

PCR targeting *hup B* gene was positive in 94.83% cases of ATT responders. The overall sensitivity and specificity of PCR was 90.32 and 94.48% respectively. The positive and negative predictive values are 95.23 and 88.88% respectively.

Various workers using different targets for amplification in extra pulmonary samples have reported a wide range of sensitivity and specificity. De Wit et al. [17] report 22–90% and 77–100% sensitivity and specificity respectively in pleural effusion cases. A study by Chakravorty et al. [11] on extra pulmonary samples showed a sensitivity and specificity of 46.7–83% and 67–100% respectively using *dev R*, *dev S* gene as target. A study by Shah et al. [18] on CSF has reported a sensitivity and specificity of 60 and 88.6% respectively. Another study by Nambam et al. on TBM reports sensitivity and specificity of 92 and 98.7% respectively and PPV of 98.8% [19]. A study by Negi et al. showed a sensitivity of 77.3% using IS 6110 as gene target [20].

Of the total PCR positive cases, 85.71% were infected with *M. tuberculosis* 9.52% with *M. bovis* alone and 4.76% showed co infection with both *M.tb* and *M. bovis*. Study by

Prasad et al. report that of the 34.7% of PCR positive cases, *M.tb* was 15.7%, *M. bovis* was seen in 10.3% and mixed infection in 8.7% of samples [6]. Various studies by Kidane et al., Wei et al., and Cosivi et al. showed presence of *M. bovis* infection in humans [21–23]. In India there is little epidemiological data about zoonotic tuberculosis caused by *M. bovis*. It is important to identify species within the *M.tb* complex because of the zoonotic implications of bovine tuberculosis in developing countries. An assay that differentiates *M.tb* and *M. bovis* would prove useful in developing new vaccines against tuberculosis. Further *M. bovis* is intrinsically resistant to pyrazinamide, a 1st line ATT, which necessitates its replacement with another drug.

The good sensitivity and specificity of PCR targeting *hup B* gene advocates its use in routine diagnosis of extra pulmonary tuberculosis where the conventional methods are limiting.

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