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Original Work

Evaluation of nested Polymerase Chain Reaction targeting *hup B* gene in the diagnosis of tubercular ascites

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ABSTRACT: Abdominal tuberculosis usually has nonspecific presentation, frequently mimicking other diseases. Because of the limitations of the conventional methods of diagnosis of extra pulmonary tuberculosis, focus is shifted to molecular methods. The objectives of this study are to evaluate the role of nested Polymerase Chain Reaction (PCR) targeting hup B gene as a rapid diagnostic modality of tubercular ascites and also to detect the infecting species (*Mycobacterium tuberculosis* and *Mycobacterium bovis*). 100 suspected tubercular ascites patients were enrolled in the study. Ascitic fluid was processed by Universal Sample Processing (USP) method and two steps nested PCR was performed targeting hup B gene. Patients were put on Anti Tubercular Therapy [Category I, (2 HRZE + 4 HR) 3, RNTCP, India]. A positive response to therapy was considered as gold standard and PCR assay was compared to determine sensitivity, specificity, positive and negative predictive value in diagnosis of tubercular ascites. 79 patients could be followed up to see the response to therapy. Of these, 39 were PCR positive and 35 responded to Anti Tubercular therapy. The sensitivity, specificity, positive and negative predictive value were found to be 97.1%, 88.6%, 87.2% and 97.5% respectively. The good sensitivity and specificity obtained in the study suggests the use of PCR targeting hup B gene as a routine diagnostic tool for tubercular ascites. Another added advantage is the ability to identify between *M. tuberculosis* and *M. bovis* which otherwise have similar clinical presentation.

KEY WORDS: ***Hup B gene; M. tuberculosis; M. bovis; Nested Polymerase Chain Reaction***

INTRODUCTION

Tuberculosis continues to be the leading cause of death due to a single infectious agent, despite the availability of preventive and therapeutic measures. Globally, around two billion people are infected and eight million new cases are added per year. In India, the prevalence of tuberculosis is around 380 million[1]. The situation has aggravated with the almost pandemiclike situation of human immunodeficiency virus (HIV) - acquired immunodeficiency syndrome (AIDS) where tuberculosis is a major opportunistic infection.

The causative agent in mammals is mycobacteria belonging to the "Mycobacterium tuberculosis complex"[2] which comprises of *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium caprae* and *Mycobacterium canetti*. *Mycobacterium tuberculosis* is the major human pathogen, although the other members of the complex especially *Mycobacterium bovis* has been recognized as a potential human pathogen.

The most frequent site of tubercular infection is lung but extra pulmonary tuberculosis (EPTB) may

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be seen in 10-15% cases. Tuberculosis of gastrointestinal tract is the sixth most frequent form of extra pulmonary tuberculosis after lymph nodes, genitourinary, bones, joints and meningeal tuberculosis[3]. In India, abdominal tuberculosis accounts for 0.08% of all hospital admissions and the incidence varies from 2-11% of the tuberculosis patients. Abdominal tuberculosis generally includes the tubercular lesions of gastrointestinal tract (GIT), peritoneum, lymph nodes of mesentery, retroperitoneum[3].

Abdominal tuberculosis can have a varied presentation, frequently mimicking other diseases. Initial symptoms of abdominal tuberculosis like fever, pain, diarrhea, constipation, weight loss, anorexia and malaise are non-specific and non-alarming[4]. Thus the primary disease progresses to the advanced stages leading to complications like ascites, obstruction, fistulas and peritonitis. This increases the morbidity and worsens the prognosis. Hence early diagnosis of tubercular ascites is crucial to prevent the morbidity and mortality related to it.

The conventional methods of diagnosis employ the microscopic identification of acid-fast bacilli (AFB) in smears stained by Ziehl -Neelsen technique and culturing of organisms in Lowenstein-Jensen medium. Culture is still considered as "gold standard" for diagnosis of tuberculosis but it requires 4-6 weeks for getting the confirmed diagnosis. Added to this is the paucibacillary nature of the extra pulmonary ascitic sample. This highlights the need for diagnostic techniques that are both rapid and sensitive.

In this scenario, the molecular methods like Polymerase Chain Reaction (PCR) plays vital role in early and rapid diagnosis of extra pulmonary paucibacillary tubercular ascites.

Various researchers like Prabhakar et al[5] reported the identification of an immunogenic histone like protein of M. tuberculosis (hlp Mt). The gene for this protein, named hup B[5,6] consists of 645bp in M. tuberculosis and 618bp in *M. bovis*. The difference in the base pairs has been attributed to a 27bp (9 amino acids) deletion after codon 128 at the C-terminal portion of the hup B gene in M. bovis[5-7]. It is not present in other mycobacterial strains[5].

Nested PCR targeting C-terminal of hup B gene can be used for early and rapid diagnosis of extra pulmonary paucibacillary tubercular ascites and it has an added advantage of differentiating the two infecting species (*M. tuberculosis* and *M. bovis*). This differentiation is of clinical significance as *M. bovis* is intrinsically resistant to pyrazinamide, a first line drug in anti-tubercular therapy, which necessitates its replacement with another drug[8].

Hence we evaluated the role of nested Polymerase Chain Reaction (PCR) targeting hup B gene in diagnosis of tubercular ascites and also to detect the infecting species (*M. tuberculosis* and *M. bovis*).

METHODOLOGY

The study was conducted jointly in the Departments of Biochemistry, Microbiology and Medicine, Lady Hardinge Medical College and Associated Hospitals, New Delhi, India after institutional ethical clearance.

A total of 100 patients meeting the selection criteria were enrolled in the study after informed written consent. Approximately 4 ml of ascitic fluid samples were taken under aseptic condition and collected in sterile vials from patients admitted in medicine wards of SSK Hospital after informed written consent.

Selection criteria

Any patients presenting with unexplained ascites were selected for study initially. They underwent routine cytological analysis (Inflammatory cell count like total leukocyte count, polymorphonuclear neutrophils and differential counts) and biochemical analysis for ascitic fluid (Total protein, albumin).

Of the above patients, all patients with exudative ascites (Total protein >2.5 gm/dl)[9] were taken up for the study. Patients with cardiac and chronic liver disease were included in the study only if the ascitic fluid indicated an increased inflammatory cell count. Diagnosed cases of cancer, which could present with ascites were excluded from the study.

The patients were subjected to detailed history and clinical examination. Routine biochemical investigations (glucose; liver function tests like total and direct bilirubin, alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase; kidney function tests like urea, creatinine and uric acid; electrolytes like sodium, potassium, calcium and phosphate; total protein and albumin; lipid profile like cholesterol, triglyceride) were done with serum samples.

The ascitic fluid samples were stored at -200C in aliquots till further processing. It was subjected to:

- Biochemical analysis [Adenosine Deaminase (ADA),Lactate, Total protein, Albumin, Serum Ascitic Albumin Gradient (SAAG)]
- AFB smear microscopy and culture in Lowenstein-Jensen medium (LJ medium)
- Polymerase Chain Reaction (PCR)

The ascitic fluid samples were processed in the Mycobacteriology laboratory, Department of Microbiology, Lady Hardinge Medical College, New Delhi. Self protection practices such as use of overalls, double mask, double gloves, footwear and the hood with UV laminar airflow were followed. Mycobacterial DNA were extracted by using Universal Sample Processing (USP) method[10,11].

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Nested PCR assay for *hup B* gene

The two step nested PCR assay of the processed clinical samples, targeting the hup B gene, was done using a 40µl reaction. The preparation of this reaction mixture was carried out in a hood with a laminar airflow taking all necessary precautions to prevent contamination and cross infection. Two sets of primers were used for the assay – N1S1 and CTFR.

N1 -- (5′ – GAGGG TTGGGATGAACAAAGCAG - 3′)
S1 -- (5′ - TATCCGTGTGTCTTGACCTATTTG - 3′)
CTF -- (5′ - CCAAGAAGGCGACAAAGG - 3′)
CTR -- (5′ - TTAGGGGACACCAAGCCCTCAGGAAGAGCA - 3′)

PCR assay targeting hup B gene was done using a 40µl reaction mixture. The composition of the master mixture were water 27µL, buffer 4µL (1X), magnesium chloride 2 µL (125 mM), dNTPs 0.8µL (200µM), forward and reverse primer 1µL (0.5µM) each and Taq polymerase 0.2µL (1 U). DNA template is added to this 36 µl master mixture as following to make a 40 µl reaction mixture:

- For clinical samples - 36µl of the master mixture + 4µl of the DNA template
- For positive controls - 36µl of the master mixture + 2µl of the template DNA of *M. Tuberculosis / M. Bovis* + 2µl of sterile water
- For negative controls - 36µl of the master mixture + 4µl of sterile water

The eppendorf tubes were vortexed and then centrifuged. After this they were put in the thermal cycler (PTC BIO RAD) for DNA amplification.
The program for N1S1 PCR assay includes initial denaturation (95°C x 10'), cycle denaturation (94°C x 1.30'), cycle annealing (60°C x 1.30'), cycle extension (72°C x 1.50') and final extension (72°C x 30') with 35 numbers of cycles. Two 645 and 618bp products were obtained for *M. tuberculosis* and *M. bovis* respectively. The amplicons were electrophoresed on 1.5% agarose gel containing ethidium bromide and viewed under ultraviolet light in a gel documentation system (Alpha DigiDoc, Alpha Innotech Corporation). Using the amplified products of NISI PCR assay, a nested PCR for amplification of the C – terminal of the hup B gene was done.
The program for CTFR-PCR assay includes initial denaturation (95°C x 10'), cycle denaturation (94°C x 1'), cycle annealing and extension (60°C x 0.30') and final extension (72°C x 7') with 35 numbers of cycles. The size of the amplicons obtained after the second amplification was 118bp and 89bp for *M. tuberculosis* and *M. bovis* respectively. The products were resolved in 8% PAGE, stained with ethidium bromide and viewed in a gel documentation system (Alpha DigiDoc, Alpha Innotech Corporation).

Follow up

All patients were put on therapeutic trial of Category I ATT and were followed up for 3 months. The response to therapy was considered as the gold standard in our study.

Statistical analysis

Taking response to therapy as gold standard, sensitivity, specificity, positive predictive value and negative predictive value of different assay methods were calculated. The observed data were analysed by SPSS version 12.

RESULT

Of the hundred patients enrolled in our study, forty eight patients showed positivity for the hup B gene PCR assay. Of these 48 patients, 45 were positive for *M. tuberculosis*, 1 patient was infected with *M. bovis* and co-infection with both species was seen in two patients. (**Figure 1**)

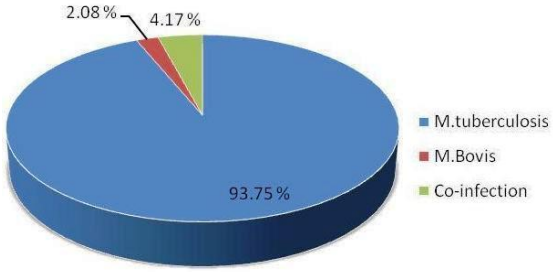

Figure 1: Type of Mycobacterial Infection in ascitic fluid

A highly significant association was found with pH, ADA, IgG, cholesterol and lactate. (**Table 1**)

Follow up of patients

One hundred patients were enrolled in the study on the basis of clinical suspicion of tubercular ascites. All were put on therapeutic trial of anti tubercular therapy (ATT) [Category I, 2 (HRZE)3 + 4 HR as per Revised National Tuberculosis Control Programme (RNTCP), India]. Patients were followed up after 3 months to see the response to therapy. 21 patients were lost to follow up. 79 patients were left in the study to see the response to therapy and out of these79 patients, only 39 were PCR positive and 35 responded to Anti Tubercular therapy. (**Table 2**)

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Table I .Comparative analysis of Ascitic fluid parameters in PCR positive patients (n=48)

Parameters	Positive	%	P value
pH (> 7)	30	62.5	0.0001
SAAG (<1.1 gm/dl)	18	37.5	0.042
ZN stain	1	2.1	0.954
Culture	0	-	-
ADA (> 30 U/L)	48	100	0.0001
IgG (> 400 U/ml)	46	95.8	0.0001
AF / Serum glu. ratio (<0.96)	31	64.6	0.907
Cholesterol (>55 mg/dl)	33	68.8	0.0001
Lactate (<25mg/dl)	43	89.6	0.0001

Table 2: Comparison between response to ATT and PCR

		Response to ATT		
		Positive	**Negative**	**Total**
PCR assay	**Positive**	34	5	39
	Negative	1	39	40
Total		35	44	79

Taking ATT response as gold standard, PCR assay had sensitivity, specificity, positive and negative predictive value are 97.1%, 88.6%, 87.2% and 97.5% respectively.

DISCUSSION

Tuberculosis (TB), though curable, still remains a major killer disease worldwide and twenty one percent of the world's TB-infected population is in India[1]. Tuberculosis in humans is mainly caused by *Mycobacterium tuberculosis* but *Mycobacterium bovis* has also been recognized as a potential human pathogen.

Conventional methods like AFB smear microscopy and Culture are time consuming, thus they cannot be used for rapid diagnosis of tuberculosis. Polymerase chain reaction (PCR), targeting various genes for amplification has been tried as a rapid diagnostic technique.

Our results show that the PCR assay targeting hup B gene was positive in 48 patients including forty five patients positive for M. tuberculosis, one for *M. bovis* and two for both *M .tuberculosis* and *M. bovis*. In last few years, various researchers like Grange et al[12], Cosivi et al[13] and Cole et al[14] reported *M. bovis* infection in humans. Earlier studies on hup B gene by Mishra et al[7], Prasad et al[6] and Shah et al[15], also reported *M. bovis* infection in human extra pulmonary samples.

Of the 100 patients enrolled in our study, all were put on therapeutic trial of anti tubercular therapy (ATT) [Category I, 2 (HRZE)3+ 4 HR as per Revised National Tuberculosis Control Programme, India (RNTCP)]. Patients were followed up after 3 months to see the response to therapy. Twenty one patients were lost to follow up. 79 patients were left in the study to see the response to therapy and out of these 79 patients, only 39 were PCR positive and 35 responded to Anti Tubercular therapy.

Of the 35 patients that responded to ATT, only 34 were PCR positive. The false negative result found in the study could be attributed to the paucibacillary nature of the disease and disparity in the distribution of the bacilli in the sample aliquots[9].

Five false positive cases were obtained in the study. These cases did not improve after 3 months of therapeutic trials with ATT (Category I, RNTCP, India)[1] and were later diagnosed as carcinoma liver, carcinoma ovary, 2 cases of polymicrobial peritonitis and bacterial peritonitis respectively. In the 2 patients in whom malignancy was diagnosed, the false positivity could be attributed to the fact that in India where prevalence of tuberculosis is very high, the immunosuppressive nature of a malignant lesion is capable of reactivating a latent tubercular infection; hence malignancy and tuberculosis may coexist.

The PCR assay in our study showed a sensitivity of 97.1% and specificity of 88.6%. Various researchers using different targets in extra pulmonary samples have shown sensitivity and specificity ranging from 22-90% and 77-100% respectively[2,16]. A study by Chakravorty et al[10] on extra pulmonary samples showed a sensitivity and specificity of 46.7-83% and 67-100% respectively. The positive predictive value 87.2% and negative predictive value 97.5% obtained in our study also correlated with the work by Chakravorty et al[10,11].

CONCLUSION

The good sensitivity and specificity obtained in the study advocates the use of PCR in future use as a routine diagnostic tool for tubercular ascites. Another added advantage is the ability to identify between *M. tuberculosis* and *M. bovis* which otherwise have similar clinical presentation.

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