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EARLY DIAGNOSIS AND SPECIES DIFFERENTIATION IN MULTI DRUG RESISTANT-TUBERCULOSIS BY NESTED POLYMERASE CHAIN REACTION TARGETING *HUP B* GENE

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Abstract

Introduction - Early diagnosis and prompt treatment of tuberculosis is the need of the time to prevent the morbidity and mortality related to it. The diagnosis of tuberculosis by conventional methods(culture) are time consuming.
Objectives - Early diagnosis of tuberculosis by using molecular method (PCR) targeting *hup B* gene and to find out whether *M.bovis* could be a cause of MDR-TB or not .
Materials and Methods - 50 MDR-TB patients were enrolled in our study after informed written consent. After processing the samples by Universal Sample Processing (USP) method, two step nested PCR was performed using two sets of primers (N1S1 & CTFR) of *hupB gene*. The amplicons were resolved on 8 % Polyacrylamide gel electrophoresis (PAGE), stained with ethidium bromide and viewed in a gel documentation system.
Results - In our study, we observed that 86 % of patients have positive PCR assay for *hup B* gene. Of the total 43 patients those were positive in PCR assay for *hup B* gene; all (43) were infected with *Mycobacterium tuberculosis.* None of the patients were infected with *Mycobacterium bovis.*
Conclusion - PCR targeting *hup B* gene can be used for early diagnosis of tuberculosis and it has the added advantages of differentiating between *Mycobacterium tuberculosis* and *Mycobacterium bovis.*

KEY WORDS - PCR, Mycobacterium tuberculosis, Mycobacterium bovis, *hup B* gene

INTRODUCTION

Drug resistant tuberculosis has been reported since the early days of introduction of anti-tuberculosis therapy but recently multi drug resistant tuberculosis (MDR-TB) has been an area of growing concern and is posing threat to the global efforts of tuberculosis control. Prevalence of MDR-TB in a community mirrors the functional state and efficacy of tuberculosis control programme and realistic attitude of the community towards implementation of such programmes [1]. Multi-drug resistant tuberculosis (MDR-TB) is defined by WHO as disease due to *M.tuberculosis* that is resistant to Isoniazid (H) and Rifampicin (R).XDR-TB is a subset of MDR-TB with additional resistance to any fluoroquinolone (Ciprofloxacin,Ofloxacin etc) and one of the second line injectables namely Kanamycin, Capreomycin and Amikacin [2].

This MDR-TB has been shown to be almost twice as common in tuberculosis patient co-infected with Human Immunodeficiency Virus (HIV) compared to tuberculosis patient without HIV [3].The mean survival time for patients who are co-infected with HIV and MDR-TB is about 2 months from the time of diagnosis, with 1 year mortality rate of 60 % as compared to 30 % in non-HIV infected persons [4]. Of late, there has been a special concern for conversion of poorly managed MDR-TB to XDR-TB (Extensively drug resistant-tuberculosis).XDR-TB is the untreatable form of tuberculosis [5].The combination of XDR-TB and HIV is virtually the death sentence for the patient. There are an estimated 490,000 new MDR-TB cases every year and 30,000 new XDR-TB cases every year [6].

Thus early diagnosis and prompt treatment is the need of the time to prevent the morbidity and mortality related to multi drug resistance tuberculosis.

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

Thus, the principal objective of our research project is early diagnosis of tuberculosis targeting *hup B* gene and to find out whether *M.bovis* could be a cause of MDR-TB or not.

MATERIALS AND METHODS

The study was conducted jointly in the Departments of Biochemistry and Microbiology, Lady Hardinge Medical college and Associated Hospitals, New Delhi and Department of Chest clinic, Lok Nayak Hospital, New Delhi.

A total of 50 cases of MDR-pulmonary TB were enrolled in our study after informed written consent. Early morning sputum samples were collected from the patients admitted in MDR-TB ward in Lok Nayak Hospital in sterile plastic container and immediately recapped.

Selection criteria

Patients of any age group or sex who were diagnosed to be MDR-TB by Culture (LJ medium) and drug sensitivity testing (DST).

The sputum samples were stored at - 20 ^{0} C until processed. They 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].

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 40 µl 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´ - GAGGGTTGGGATGAACAAAGCAG – 3´)

S1 -- (5´ - TATCCGTGTGTCTTGACCTATTTG - 3´)

CTF -- (5´ - CCAAGAAGGCGACAAAGG - 3´)

CTR-- (5´ –TTAGGGGACACCAAGCCCTCAGGAAGAGCA - 3´)

Table I

Preparation Of Master Mixture For N1S1 And CTFR PCR assay

PCR assay targeting *hup B* gene was done using a 40µl reaction mixture.

	Volume (µL)	Concentration
Nuclease free Water (H_2O)	27	
Buffer	4.0	1 X
$MgCl_2$	2.0	125 mM
dNTPs	0.8	200 µ M
Forward primers	1.0	0.5 µ M
Reverse primers	1.0	0.5 µ M
Taq Polymerase	0.2	1.0 U
Total	36	

DNA template is added to this 36 µl master mixture as following to make a 40 µl reaction mixture.

For clinical samples

36 µl of the master mixture + 4 µl of the DNA template

For positive controls

36 µl of the master mixture + 2 µl of the template DNA of *M.tuberculosis* / *M. bovis* + 2 µl of sterile water.

For negative controls

36 µl of the master mixture + 4 µl 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.

A 645 and 618 bp product 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 118 bp and 89 bp 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).

RESULTS

In our study,we observed that 86 % of patients have positive PCR assay for *hup B* gene.

Table II

PCR assay for *hup B* gene

PCR assay (*hup B* gene)	No of patients (n=50)	Frequency(%)
Positive	43	86
Negative	7	14
Total	50	100

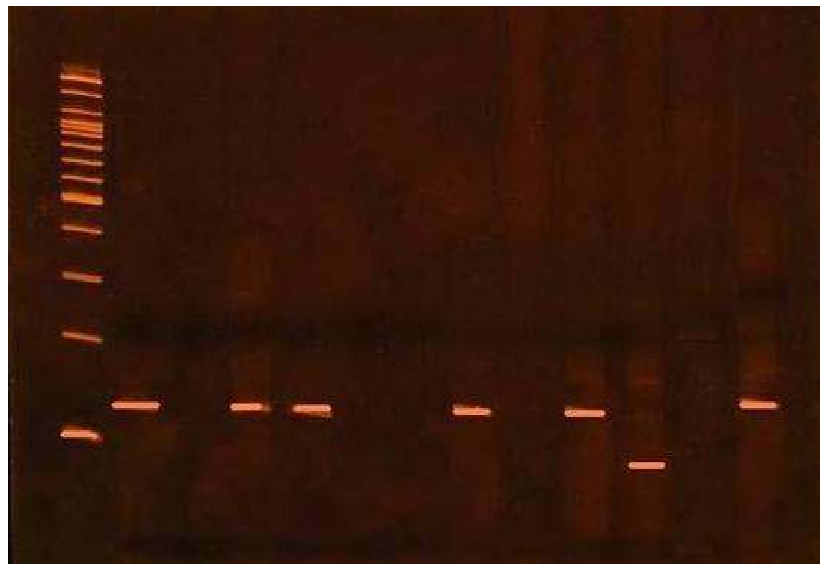
Of the total 43 patients those were positive in PCR assay for *hup B* gene; all (43) were infected with *Mycobacterium tuberculosis.* None of the patients were infected with *Mycobacterium bovis.*

Figure I

Type of infection

PCR PRODUCTS FOR hupB GENE USING PRIMERS CTF/R

RESOLVED ON 8% POLYACRYLAMIDE GEL



L A B C D E F G H I J K L

L: 100 bp DNA ladder
A: *M. tuberculosis* positive control (116 bp)
J: M. *bovis* positive control (89 bp)
B: Negative control
C, D, G, I, L: Sputum samples positive for *M. tuberculosis* (116 bp)
E, F, H, K: Negative sputum sample

The gel picture showing the PCR products for *hup B* gene using CTF/R primer and resolved on 8% Polyacrylamide gel electrophoresis is shown below.

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[2]. The enormity of the problem has increased with the emergence of multidrug resistant (MDR) strains of *Mycobacterium tuberculosis* .Dual infection with human immunodeficiency virus (HIV) and MDR-TB is a virtual death sentence in this era.

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.

Amplification of *hup B* gene and its further characterization indicates the presence of infection by Mycobacterium species. Ours is an effort to identify the species of *M.tuberculosis* complex in cases of MDR-TB targeting *hup B* gene.

In our study, we observed that (43/50) 86 % of patients were positive in PCR assay for *hup B* gene .

The sensitivity limit of the N-S and nested C-terminal F-R PCR assays were 0.1 ng of DNA (10^4 bacilli) and 50 fg of DNA (5 bacilli) respectively[12]. Contamination of samples with PCR inhibitors may occur even after best possible conditions. The presence of inhibitors not detected by the control amplification. Small volume of sample may be another limiting factor in some patients. These may be the reasons for the false negative results obtained in (7/50) 14 % of cases.

The *hup B* gene shows a 27 bp deletion at C-terminal in *M.bovis* as compared to *M.tuberculosis*. Based on this, by the PCR amplification, we can differentiate the two species.

All 43 patients positive for PCR assay targeting *hup B* gene were infected with *Mycobacterium tuberculosis.* None of the patients showed infection with *Mycobacterium bovis* (Table II).

However, previous studies done by Nambam B[12] et al 2006 in CSF samples reported *M.bovis* to be a cause of tuberculosis. But our study (pulmonary sputum samples) did not show any *M.bovis* infection. Nambam B[12] et al 2006 studied in extra pulmonary(CSF) samples. This may suggest that *M.bovis* may be a cause of extra pulmonary tuberculosis.

Incidence of *M.bovis* infection as such is reported to be low and mostly reported in extra pulmonary tuberculosis (EPTB) and no study so far has reported MDR/XDR tuberculosis to be due to *M.bovis*. Thus our study was undertaken to ascertain whether *M.bovis* could be a cause for MDR tuberculosis or not.

Hence, the conclusion of our present study is that PCR targeting the *hupB* gene can be used for diagnose tuberculosis and it also differentiate between *M. tuberculosis and M. bovis* infection. This differentiation is of clinical significance as *M.bovis* is intrinsically resistant to pyrazinamide[12], a first line drug in anti-tubercular therapy, which necessitates its replacement with another drug.

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