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Rapid Detection of Pulmonary and Extra Pulmonary *Mycobacterium Tuberculosis* (MTB) In Clinical Samples of M.P. Region

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ABSTRACT: India is facing a very high burden of *Mycobacterium tuberculosis* (M.TB) cases and MDR *Mycobacterium tuberculosis* cases epidemiological studies have indicated an urgent need for early detection and prevention strategies from M.TB related infections. Molecular biology provides highly accurate fast and fast detection of M.TB and its use in confirmation of MDR TB and other complex clinical features of M.TB has increased tremendously. Resulting in setting up molecular biology lab across India for TB detection in the present study a novel protocol for extraction of M.TB DNA was used and silica columns were used which showed low inhibition and no contamination properties it was coupled with nested PCR targeting a specific conserved region of IS6110. The clinical samples showed a diverse range of pulmonary and extra pulmonary. The research work showed excellent results with the new M.TB DNA isolation protocol and also was successful in reduction of reporting time period. The paper discusses extraction, treatment and interpretation and results for M.TB. The work can be used DNA based for development of highly accurate M.TB and MDR M.TB detection program and policy. In present study detection of M.TB from different types of pulmonary and extra pulmonary samples for this nucleic acid extraction method was developed using lysis buffer and pre lysis buffer and wash buffer I and binding buffer and elution buffer by using silica columns. For detection of MTB samples were showed better sensitivity in nested PCR targeting IS6110.

I. INTRODUCTION

TUBERCULOSIS (TB) is a major threat to public health worldwide, infecting more than one third of the world's population. India has the world's highest burden of TB, accounting for approximately one global incidence an estimated 2 million cases annually and ranks seventeenth among the 22 high burden countries in terms of TB incidence. TB is one of the leading causes of mortality in India, killing two persons every 3 min, i.e., nearly 1000 per day. Rapid diagnosis followed by immediate initiation of treatment is essential to arrest the progression of this fatal disease, not only at the individual level but also in the community. This problem has to be taken into account when devising TB control the causative agent of Tuberculosis. First discovered in 1882 by Robert Koch, *M. Tuberculosis* has an unusual, waxy coating on its cell surface primarily due to the presence of mycolic acid. Tuberculosis (TB) is a major threat to public health worldwide, infecting more than one third of the world's population. India has the world's highest burden of TB, accounting for approximately one fifth of global incidence an estimated 2 million cases annually and ranks seventeenth among the 22 high-burden countries in terms of TB incidence. Initial diagnosis of TB is usually based on clinical grounds, but a definitive diagnosis requires the isolation and identification of the infecting organism. Sputum smear microscopy with Ziehl-Neelsen (ZN) staining is the most widely used rapid method in most laboratories for diagnosing TB under India's Revised National Tuberculosis Control Programme. However, the ZN smear technique suffers from several limitations, such as low sensitivity and specificity, especially for extra-pulmonary TB.

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Microscopic examination requires $>10^3$ to 10^4 mycobacteria/ ml, and the diagnostic laboratory must either process the specimen shortly after collection or store it at 4°C to inhibit the growth of contaminating micro-organisms. Culture of *Mycobacterium Tuberculosis* in Löwenstein-Jensen medium, the gold standard for laboratory diagnosis, although sensitive, is laborious, takes 4–8 weeks and should be followed by identification tests such as the niacin test. Such methods do not sufficiently enhance the speed of diagnosis, which delays treatment, leading to mortality and further transmission.

II. MATERIAL AND METHODS

CLINICAL'S SAMPLES- In the scope of an open prospective study conducted from January 2015 to December 2016 the specimens were collected from patients with suspected M. tuberculosis infection on the basis of clinical criteria. A total of 1000 samples from suspected TB patients were collected from different regions of Bhopal M.P. The respiratory specimens were Sputum ;100 , Bronchoalveolar Lavage (BAL); 94, Pleural Fluid; 91, Non-respiratory samples included Cerebrospinal Fluid (CSF); 61, Urine;15, Blood ;70, Endometrial Tissue;191, Menstrual Blood ;113, Pus ;130, Ascitic Fluid ;24, Tissue ;19, Core- Biopsy ;4, ET Aspirate ;46, Potts Spine;7, 18 other body fluid , Peritoneal Fluid; 4, Synovial Fluid; 5 , and 3 samples were of kidney fluid .

SAMPLE PROCESSING AND DNA EXTRACTION METHOD- Different clinical samples such as Pulmonary and Extra Pulmonary were taken from suspected TB patients and the extraction of MTB was done using silica column based DNA extraction protocol with the help of lysis buffer ,wash buffer I, Wash buffer II and elution Buffer. The extraction method was based on the lysis of M.TB cell wall and the binding of released nucleic acid with the help of silica column in presence of certain chaotropic salts (GuSCN, GuHCl) and removal of the impurities and contamination from the preparation. Clinical samples from TB patients were processed for DNA isolation. Briefly 200 μl of sample was taken in 1.5 ml Micro centrifuge tubes and centrifuge for 5 min. at 12000 rpm. The supernatant was Discarded .The pellet was obtained. The pellet method was subjected to DNA extraction protocol using the c column based MTB DNA extraction kit. The pellet (sample) was mixed with 180 μl of buffer BT1(Pre Lysis Buffer) and 20 μl lysozyme and sample was vortexed for 1minute and incubated at 37°C for 20 minute for inactivation of Mycobacteria .Sample was vortexed again after 20 minute incubation then 25 μl proteinase k was added and kept on water bath at 56°C for 30 minute in the presence of 200 μl lysis buffer. After then procedure the samples were kept for incubation 70°C for 10 minute and the DNA released was precipitated using 210 μl chilled ethanol for immobilization on the silica column. After then two washing steps were performed with 500 μl of wash BBW was added and centrifuge for 1 minute at 10,000. Flow through was discarded and then secondary washing was done by 500 μl buffer BB5 centrifuge for 1min at 10,000. Flow through was again discarded and after washing drying silica column was done. The absorbed DNA samples in silica column was free of inhibitors and further eluted out in elution buffer with the help of 40 μL TE Buffer. The DNA sample was taken for PCR analysis.

AMPLIFICATION OF DNA–Amplification of M.TB DNA was done by nested PCR using M.TB Polymerase chain reaction Kit Targeting IS6110. In the first step DNA segment was amplified by external primer. All reactions were taken to a final volume of 30 μl containing 15.5 μl amplification premix (reaction buffer with magnesium chloride $[\text{MgCl}_2]$, dNTPs and Taq DNA polymerase), 6 μl primer mixes and 4 μl of 8-methoxypsoralen (8-MOP). First PCR the total reaction volume was 25.5 μl the volume of extracted M.TB DNA was added 4.5 to get final volume of 30 μl . Then the PCR mix was properly mixed by mini spine centrifuge. In negative control tube 4.5 μl of sterile water was added along with the PCR mix. In positive control tube 4.5 μl of positive control was added along with PCR mix The Thermo Cycler program involves incubation of the mixture an internal control was introduced each amplification reaction and was co-amplified with the target DNA from the clinical specimen to check for the presence of inhibitors in clinical samples. In addition, the 8-MOP system was used to extinguish the template activity of contaminated DNA. The thermo cycler program involves incubation of the mixture, first at 94°C for 15 minute to activate the Taq polymerase, followed by 30 subsequent cycles at 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 30 seconds and 72°C for 5 minutes.

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TARGET SEQUENCE DETECTION BY SECOND PCR USING FIRST PCR AMPLIFIED PCR PRODUCT -

After complete amplification of the DNA segment, the amplified product was used as DNA template for the amplification of 190-bp segments; 15.5µl of the amplification premix, 7.0µl primer mixes, 6µl 8-MOP and 28.5 µl of above the reaction mixture was added to 1.5µl of the first PCR product to the PCR tube to get final volume of 30 µl then the PCR mix was properly mixed by mini spine centrifuge. After an initial denaturation at 94°C for 15 min, 35 amplification cycles were performed. Each cycle consisted of denaturation at 94°C for 30 s, annealing of primers at 62°C for 30 seconds and primer extension at 72°C for 30 seconds, 72°C for 5 minutes.

Positive and Negative controls were inoculated in different Room having temperature control and all precaution to prevent cross contamination was observed. The amplified PCR products were electrophoresed on a 2 % Agarose gel with 0.5mg/ml of Ethidium bromide concentration. Detection of Mycobacterium tuberculosis (MTB) from diverse clinical samples were detected along with 219 bp ladder under UV light in UV transilluminator.

III. RESULT

STUDY OF THE POSITIVITY RATE OF THE SAMPLES IN NESTED PCR - In this study a total 1000 different type of clinical samples were collected from hospital and labs of Bhopal area the statically analysis revealed that out of 1000 cases 809 cases were of extra pulmonary type of samples and 191 samples were reported samples pulmonary . The study revealed that in 809 extra pulmonary cases sample 167 found to be positive and in case of 191 pulmonary samples 78 cases were found to be positive. High positive rate with sample it revealed conform the distribution of statically correlation to different samples type. Revealing 53.84%in case of extrapulmonary pus samples to 14.42% of Whole Blood extra pulmonary samples. The detection rate found to be high and fairly aureate as compare to normal technique for Mycobacterium tuberculosis (M.TB) detection the extracted DNA was amplified with the help of nested PCR using **PCR M.TB Kit** in which **IS6110** region targeting pair of primers was taken.

Table -1 Total Number of clinical samples processed and the positive sample found in that clinical samples.

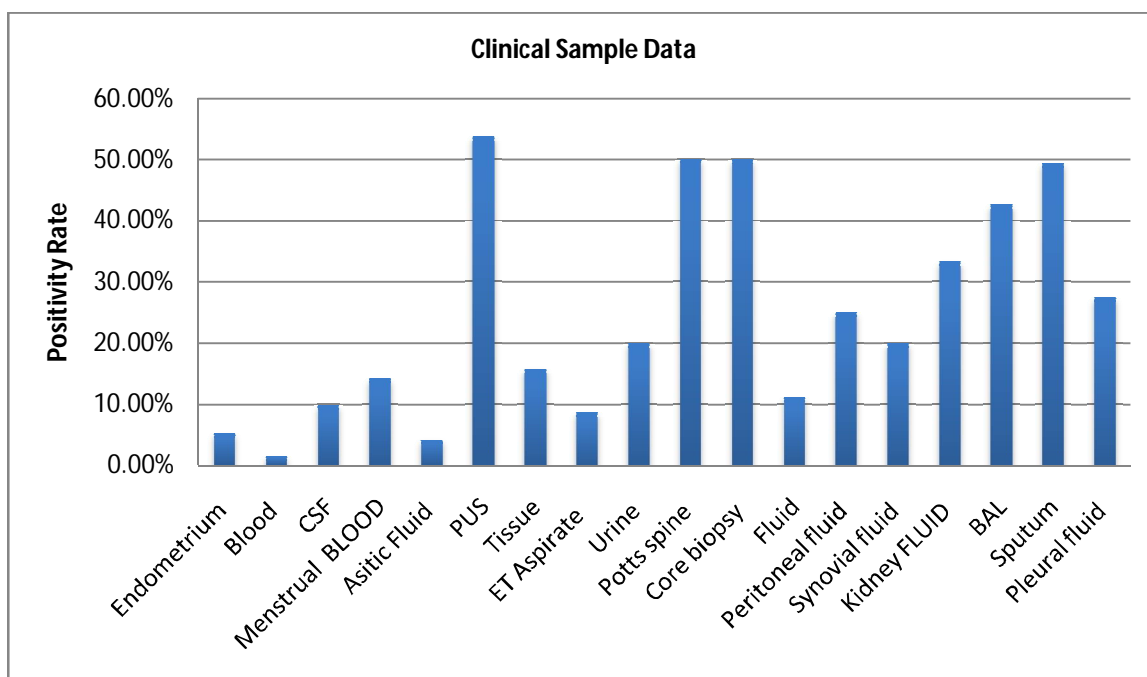
S. No.	Sample	Type of sample	Total no of sample	Found Positive	Positivity Rate
1.	Endometrium	Extra Pulmonary	191	10	5.23 %
2.	Whool Blood	Extra Pulmonary	70	1	1.42 %
3.	CSF	Extra Pulmonary	61	6	9.83 %
4.	Menstrual Blood	Extra Pulmonary	113	16	14.15 %
5.	Ascitic Fluid	Extra Pulmonary	24	1	4.16 %
6.	PUS	Extra Pulmonary	130	70	53.84 %
7.	Body Tissue	Extra Pulmonary	19	3	15.78 %
8.	ET Aspirate	Extra Pulmonary	46	4	8.69 %
9.	Urine	Extra Pulmonary	15	3	20 %
10.	Potts spine	Extra Pulmonary	7	4	50.14 %
11.	Core biopsy	Extra Pulmonary	4	2	50 %
12.	Body Fluid	Extra Pulmonary	18	2	11.11%
13.	Peritoneal fluid	Extra Pulmonary	4	1	25 %
14.	Synovial fluid	Extra Pulmonary	5	1	20 %
15.	Kidney fluid	Extra Pulmonary	3	1	33.33 %
16.	BAL	Extra Pulmonary	94	42	44.68%
17.	Pericardial Fluid	Extra Pulmonary	5	0	0
18.	Sputum	Pulmonary	100	53	53 %
19.	Pleural fluid	Pulmonary	91	25	27.47 %

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Graph 1- Clinical sample positivity data from different type of sample

RESULT ANALYSIS OF PCR PRODUCT-Band appeared on the gel documentation system were analyzed samples containing only internal control IC band 571 were negative while samples containing both bands IC and M.TB band were Positive and 219 bp fragments were positive considered as a positive result. It was found that the silica based columns method the maximum sensitivity as well as more distinct band it also showed better sensitivity giving good results.

IV. DISCUSSION

M.TB occurs worldwide and cause wide spread mobility and morality pulmonary and extra pulmonary site is known to be associated with M.TB Infection. In fact it is well known that pulmonary Tuberculosis patients go on to develop extra pulmonary Tuberculosis. PCR was used as a tool for detection M.TB DNA in both types of samples pulmonary and extra pulmonary from suspected TB Patients. 1000 samples were taken from suspected TB patient belonging to Bhopal region were checked for the presence of mycobacterium by targeting the IS6110. DNA sequences of 1000 samples were taken out of 245 samples from to be positive. PCR has proven to be accurate short time period toll for early detection of M. TB. Its results can help doctor for early diagnosis of M.TB and it has also proved to be helpful in clinically complex cases. The error rate due to its high specificity is reduce making it a promising a reliable alternative toll for rapid detection of M.TB from pulmonary and extra pulmonary samples.

V. CONCLUSION

We conclude the PCR assay is highly sensitive and specific toll available for the diagnosis M.TB in all types specimens obtained TB patients. Our research indicated that extra pulmonary samples can also be very accuquality detected by nested PCR and our result show high degree of correlation with detection of TB in extra pulmonary samples in our state Madhya Pradesh. M.TB burden is increasing as indicated by various studies and extra pulmonary detection by traditional method is time consuming and difficult interns of sample processing but by using PCR, extraction technique

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not only detection time period is reduce but very high degree of accuracy of M.TB detection is also achieved in cases of pulmonary TB PCR related detection have achieved very high degree of clinical co-relation with M.TB infection and nested PCR data is now common useful feature. If early detection of M.TB in pulmonary related cases as Molecular Biology detection and diagnosis has proved .Their importance in early detection of TB and drug resistant property exhibited by diverse pathogen PCR method will now be more applicable and accurate of many disease in near future. India requires Molecular Biology detection for reducing and eliminating M.TB infection making a country healthy and diseases free. Thus trying to spared the awareness about Tuberculosis, we collected 1000 samples 191 samples were pulmonary TB cases and 814 samples were extra pulmonary The study revealed that in 814 extra pulmonary cases sample 161 found to be positive and in case of 191 pulmonary samples 78 cases were found to be positive and also develop a M.TB Nested PCR Kit which require less expenditure of money in diagnosing. The disease of M.TB as compare to the diagnostics kits available in market. We conclude that PCR assay is highly sensitive and specific tool available to date for the diagnosis of M. tuberculosis in all types of specimens obtained from patients with a clinical suspicion of tuberculosis whether pulmonary or extra-pulmonary and can be reliably used for rapid identification and diagnosis of TB.

ABBREVIATIONS- **TB:** Tuberculosis, **PCR:** Polymerase Chain Reaction, **MTB:** Mycobacterium Tuberculosis, **CSF:** Cerebro Spinal Fluid, **BAL:** Bronchoalveolar lavage, **MDR:** Multiple Drug Resistance, **WHO:** World Health Organization.

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