International Journal of Drug Development & Research
| October-December 2012 | Vol. 4 | Issue 4 | ISSN 0975-9344 |
Available online http://www.ijddr.in
Covered in Official Product of Elsevier, The Netherlands
SJR Impact Value 0.03 & H index 2

Full Length Research Paper
Covered in Index Copernicus with IC Value 4.68 for 2010

Conventional PCR usage for the detection of Mycobacterium Tuberculosis complex in Cerebrospinal Fluid by MPB64-Target PCR

Sharma Narotam [1]*, Sharma Veena[**], Nautiyal Satish Chandra[1], Singh Prem Raj[1], Rajeev S Kushwaha[1], Sailwal Shivani[1], Ghosh Shayan[1], Naushad Ahmer[1], Singh R.K[1]

[1*]Molecular Research Laboratory, Department of Biochemistry SGRRIM & HS Patel, Nagar, Dehradun (U.K) 248001.

[**]Department of Bioscience and Biotechnology, Banasthali University Banasthali – 304022, Rajasthan.

Abstract

Tuberculous meningitis is a severe and potentially fatal form of Tuberculosis. Diagnosis of Tuberculous meningitis (TBM) is challenging. Thus rapid, accurate and confirmatory diagnosis is necessary to initiate required therapy. The diagnostic workup involves detection of acid-fast bacilli (AFB) in the cerebrospinal fluid (CSF) by microscopy or culture, however, the difficulty in detecting the organism poses a challenge to diagnosis. The use of the polymerase chain reaction (PCR) in the diagnostic approach to *Mycobacterium tuberculosis* (MTB) meningitis has been reported as a fast and accurate method using in- house protocols and several commercial kits available. We analysed the performance of PCR for the diagnosis of MTB meningitis in 64 consecutive patients, using MTB culture as gold standard.We used PCR as a tool for detecting mycobacterial *mpb64* gene in CSF samples from suspected TB patient. 64 clinical specimens were taken from the patients attending SMI Hospital, Dehradun. 45 were PCR positive, 32 were culture positive and 2 were Zn smear positive.The sensitivity of PCR, culture and microscopy for CSF samples was, thus, 70%, 50% and 3% respectively. Therefore, compared to the conventional culture assay, the PCR can be considered a more useful and advanced technique for the rapid and accurate diagnosis of TBM. Thus PCR is better tool for the diagnosis of Tuberculous meningitis combined with the other concerned tests.

*Corresponding author, Mailing address:
Sharma Narotam
Email: sharmanarotam5@gmail.com

Key words:
Tuberculous meningitis; polymerase chain reaction; Central Nervous System.

***How to Cite this Paper*:**
Sharma Narotam*, Sharma Veena, Nautiyal Satish Chandra, Singh Prem Raj, Rajeev S Kushwaha, Sailwal Shivani, Ghosh Shayan, Naushad Ahmer, Singh R. K "Conventional PCR usage for the detection of Mycobacterium Tuberculosis complex in Cerebrospinal Fluid by MPB64-Target PCR" Int. J. Drug Dev. & Res., October-December 2012, 4(4): 206-210.

Article History:----------
Date of Submission: 03-09-2012
Date of Acceptance: 21-09-2012
Conflict of Interest: NIL
Source of Support: NONE

Introduction

In 2009 the global burden of Tuberculosis (TB) in the given WHO report says that there are 9.4 million incident cases (range, 8.9 million–9.9 million), 14 million prevalent cases (range, 12 million–16 million), 1.3 million deaths among HIV-negative people (range, 1.2 million–1.5 million) and 0.38 million deaths among HIV-positive people (range, 0.32 million–0.45 million). HIV-positives had an estimated 11-13% of these cases, where 80% of these cases accounting in Africa [1]. In India 500,000 people die per year from TB with one person dying every minute [2]. Once TB was thought to be a major contributor to the burden of disease in poor countries, and was nearly completely eradicated in the Western world but now it has resurged worldwide and become a global issue. It is the second leading cause of death from infectious diseases (4% of all deaths) and 88 million new cases of TB have occurred throughout the world during the decade gone by (1990-1999) [3]. The focal point in minimizing the risk of TB transmission is rapid diagnosis of the disease, especially in the wake of the emergence of drug-resistant TB and its severe implications for human immunodeficiency virus-infected patients. Early diagnosis plays a vital role in diagnosis of TB [4, 5]. Although acid fast bacilli (AFB) microscopy and Lowenstein–Jensen medium (LJ) culture remain the accepted "gold standard" and most popular among all the methods currently employed worldwide for TB diagnosis, however these traditional methods are either slow or have low sensitivity, especially with the samples that contain low bacterial load which can affect treatment by either delaying or causing inappropriate empiric therapy for TB [6, 7]. In recent times maximum attention has been devoted to developing nucleic acid amplification (NAA) diagnostic technologies owing to their rapidity and sensitivity. Numerous gene targets and several methods for isolating mycobacterium DNA from clinical specimens have been reported with encouraging results [8]. The present study was carried out to selectively target the *mpb64* gene in Mycobacterium tuberculosis complex (MTB complex) with 240 bp product for the detection of meningitis tuberculosis. 240bp mannose binding protein64 gene may be the most specific sequence for the diagnoses of the MTB complex by PCR, because the lowest number of false positive result was obtained with the mpb64 protein coding gene (10%) in comparison to IS6110 (62%) and heat shock protein (38%) [9]. The *mpb64* gene codes for the MPB64 protein and is a 240 bp region (nucleotides 460–700). The sequence of MPB64 gene primers used was MPB64F 5'TCCGCTGCCAGTCGTCTTCC 3' and MPB64 R-5'GTCCTCGCGAGTCTAGGCA3'.

Processing of clinical specimen:

Cerebrospinal fluid is used as a clinical sample as it typically lack common inhibitors of PCR assay such as haeme, endonucleases and exonucleases that can lead to false negative PCR result. For the proposed study sixty four cerebrospinal fluid were collected from different departments of SMI Hospital, Patel Nagar, Dehradun and were transported properly at 4°C to the Molecular Research Laboratory for further processing. Acid fast staining and culture of MTB was also performed for all the samples. For DNA isolation from the 64 CSF clinical specimens, 200µl of CSF was taken as starting volume. 20µl of ProteinaseK (20mg/ml) were added in all the clinical specimen with the addition of 200 µl of extraction buffer (Lysis buffer 1 and 2). The mixture was vortexed (for 15-20 sec) and incubated at 60°C for 20 minutes thereafter was transferred to 90°C for 10 minutes. 200µl of chilled absolute ethanol was added to each specimen in order to precipitate the DNA. All the contents were vortexed properly and then transferred into prearranged silica columns and centrifuged at 10,000 rpm for 3 minutes at 4°C. Now the columns were transferred to fresh collection tubes. 500µl wash buffer [Reconstituted by 60 ml 98% absolute ethanol

(Mol. Grade)] and centrifuged at 10,000 rpm for 3 minutes at 4°C after which the contents were transferred to fresh collection tubes; the procedure was repeated again followed by dry wash at 13000 rpm for 2 minutes. The columns were transferred to autoclaved microfuge tubes and 60 µl of pre heated milli Q water was added to each column which was then centrifuged at 13000rpm at 4°C for 5min.The column was discarded and eluted DNA was collected in micro centrifuge tube.

PCR Set up and Amplification

PCR was carried out for all the CSF specimens including controls. PCR was performed for the amplification of gene *mpb64* using primers Forward primer P1-5'-TCCGCTGCCAGTCGTCTTCC and Reverse primer P2 5'-GTCCTCGCGAGTCTAGGCCA. A reaction mixture of 50 µl containing 10X PCR buffer (250 mM Tris Hcl, 500 mM KCl), 0.2 mM dNTPS, 25µM primers, *Taq polymerase* (3 units) and Mg^{2+} ions (25mM) as $MgSO_4$ was prepared. 25µl of DNA template was added in the 25µl of master mix and amplification was done in thermal cycler (Veriti, Applied Biosystems) for 40 cycles. Cycling conditions include; initial denaturation at 94°C for 4 min, denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and primer extension at 72° C for 30 sec were provided with final extension of PCR products at 72° C for 7 minutes.

Results

All the sixty four CSF samples were subjected for PCR, acid fast staining and culture. Acid fast staining showed only two positive while 32 samples were culture positive and 45 samples were PCR positive. PCR proved to be a more sensitive method, detecting 45 out of 64 cases (70%), whereas only 32 were culture positive (50%) and for AFB staining two were found positive (3%). The sensitivity of PCR, culture and microscopy was, thus, 70%, 50% and 3% respectively. Tuberculous meningitis (TBM) is a medical emergency that requires urgent treatment because early intervention reduces the risk of mortality.

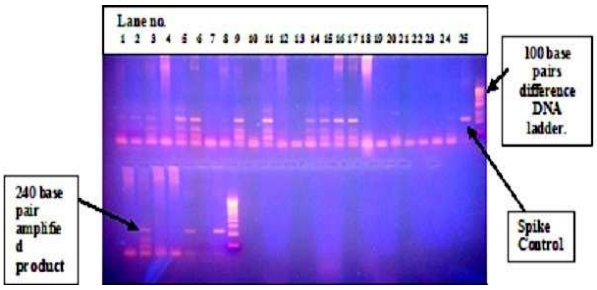

Figure 1: PCR amplification of 240 bp of mpb64 gene of M. *tuberculosis* on 1.5% agarose gel.

Discussion

Diagnostic techniques based on amplification have the potential to increase the sensitivity for detecting mycobacterium as well as to dramatically reduce the time usually necessary to detect and identify these organisms in clinical specimens [10, 11]. Early confirmatory diagnosis of TBM is difficult to establish because of its pleomorphic clinical presentation[12, 13]. Delayed diagnosis and treatment may be associated with many severe CNS complications. Most studies have used IS6110 as a target for PCR-based diagnosis of TBM with variable degrees of success [14, 15, 16, 17, 18, 19, 20]. However, this insertion element is absent in a proportion of M.tuberculosis isolates from Indian strain of mycobacterium tuberculosis which reasons against its utility as a sole target for gene amplification for the detection of tuberculosis in India [21, 22]. In contrast, MPB 64 sequences were universally detected in M. tuberculosis strains in India because 240bp Mpb64 protein gene may be the most specific sequence for the diagnoses of the TB meningitis by PCR, because the lowest number of false positive result was obtained with the mpb64 protein coding gene (10%) in comparison to IS6110 (62%) and heat shock protein (HSP) (38%) [23, 24]. .

Conclusion:

The mortality and morditiy rate of Tuberculosis meningitis is high in recent year. Early diagnosis of tuberculosis meningitis is very important taking CSF as starting specimen in the treatment of TB meningitis. Direct smear and culture method is most simple method use to diagnosis of Tuberculosis, but is time taking and sometime not give acquarate result [25, 26]. Detection of tuberculosis by PCR is rapid, novel method and it gives result in 2-3 hours and its result is most probably accurate [27]. A molecular method including PCR is thus a specific, sensitive and rapid technique for the detection of tuberculosis meningitis [28]. We here propose that for timely detection and management of Tuberculosis meningitis PCR method can be employed where ever possible as it will cut the time required for detection of the disease and it also help in making the physicians take timely decisions for patient management.

Acknowledgment

The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support and guidance.

Conflict of Interest: None

REFERENCES

1) World Health Organization. Global Tuberculosis Control: A Short Update to the 2009 Report; World Health Organization: Geneva, Switzerland, 2009.
2) Tuberculosis Control India. http://www.tbcindia.org.
3) T. W. P. Chow, B. K. Lim, and S. Vallipuram, "The masquerades of female pelvic tuberculosis: case reports and review of literature on clinical presentations and diagnosis," *Journal of Obstetrics and Gynaecology Research*, 2002. vol. 28, no. 4, pp. 203– 210.
4) Khan MH. Tuberculosis: need to revitalize its control programme in Pakistan (editorial). J Coll Physicians Surg Pak 1996; 6:3
5) Corbett EL, Marston B, Churchyard CJ, De Cock KM Tuberculosis in sub-Saharan Africa: Opportunities, challenges, and change in the era of antiretroviral treatment. Lancet 2006; 367: 926–937.
6) Jonas V,Alden MJ, Curry JI, Kamisango K, Knott CA, Lankford R,Wolfe JM,Moore DF. Detection and identification of mycobacterium tuberculosis directly from sputum sediments by amplification of rRNA. J Clin Microbio 1993; 31: 2410-2416.
7) Noel AB, Lecossier D,Nassf X, Birgite Gicquel, Frebault VL, Hance AJ.Rapid diagnosis of tubercuosis by amplification of Mycobacterial DNA in clinical samples. Lancet 1989; 2(8671):1069-1071
8) Thornton, C. G., K. M. MacLellan, T. L. Brink, Jr., D. E. Lockwood, M. Romagnoli, J. Turner, W. G. Merz, R. S. Schwalbe, M. Moody, Y. Lue, and S. Passen. Novel method for processing respiratory specimens for detection of mycobacteria by using C18-carboxypropylbetaine: blinded study. J. Clin. Microbiol. 1998; 36:1996–2003.
9) WHO report 2010 Global tuberculosis control.
10) Horner PJ, Moss FM: Tuberculosis in HIV infection. *Int J STD AIDS* 1991, 2:162-7.
11) Selwyn PA: Tuberculosis in the AIDS era: A new threat from an old disease. *State J Med* 1991, 91:233-35.
12) Kashyap RS, Biswas SK, Purohit HJ, Chandak N, Agarwal N, Taori GM, Daginawala HF: Application of Mancini technique as a diagnostic test in CSF of tuberculous meningitis patients. *Med Sci Monit* 2002, 8:95-98.
13) Katti MK: Assessment of antibody responses to antigens of *Mycobacterium tuberculosis* and *Cysticercus cellulosae* in cerebrospinal fluid of chronic meningitis patients for definitive diagnosis as TBM/NCC by passive hemagglutination and immunoblot assays. *FEMS Immunol Med Microbiol* 2002, 33:57-61.
14) Miorner, H, Sjobring, U., Nayak, P. & Chandramuki, A. Diagnosis of tuberculous meningitis: a comparative analysis of 3 immunoassays, an

immune complex assay and the polymerase chain reaction. Tuber Lung Diseases. 1995; 76, 381–386.

15) Kox, L. F., Kuijper, S. & Kolk, A. H. Early diagnosis of Tuberculous meningitis by polymerase chain reaction. Neurology 1995; 45, 2228–2232.

16) Nguyen, L. N., Kox, L. F., Pham, L. D., Kuijper, S. & Kolk, A. H. The potential contribution of the polymerase chain reaction to the diagnosis of tuberculous meningitis. Arch Neurol. 1996;53, 771–776.

17) Jatana, S. K., Nair, M. N., Lahiri, K. K. & Sarin, N. P. Polymerase chain reaction in the diagnosis of tuberculosis. Indian Pediatr. 2000; 37, 375–382.

18) Narayanan, S., Parandaman, V., Narayanan, P. R., Venkatesan, P., Girish, C., Mahadevan, S. & Rajajee, S. Evaluation of PCR using TRC4 and IS6110 primers in detection of tuberculous meningitis. J Clin Microbiol . 2001. 39, 2006–2008.

19) Bhigjee, A. I., Padayachee, R., Paruk, H., Hallwirth-Pillay, K. D., Marais, S. & Connoly, C. Diagnosis of tuberculous meningitis: clinical and laboratory parameters. Int J Infect Dis.2007;11, 348–354.

20) Rafi, W., Venkataswamy, M. M, Nagarathna, S, Satishchandra, P. & Chandramuki, A. Role of IS6110 uniplex PCR in the diagnosis of tuberculous meningitis: experience at a tertiary neurocentre. Int J Tuberc Lung .2007;11, 209–214.

21) Narayanan, Parandaman, V, Narayanan, P. R., Venkatesan, Girish, Mahadevan, S. & Rajajee, S. Evaluation of PCR using TRC4 and IS6110 primers in detection of tuberculous meningitis. J Clin Microbiol. 2001; 39, 2006–2008.

22) Radhakrishnan, I., K, M. Y., Kumar, R. A. & Mundayoor, S. Implications of low frequency of IS6110 in fingerprinting field isolates of Mycobacterium tuberculosis from Kerala, India. J ClinMicrobiol. 2001 ; 39, 1683.

23) Lee, B. W, J. A. Tan, S. C. Wong, C. B. Tan, H. K. Yap, P. S. Low, J. N. Chia, and J. S. Tay. Comparison of protocols involving three mycobacterial DNA sequences, IS*6110*, 65 kDa antigen, and MPB64. J. Neurol. Sci. 1994; 123:173–179.

24) Liu, P. Y, Z. Y. Shi, Y. J. Lau, and B. S. Hu . Rapid diagnosis of tuberculous meningitis by a simplified nested amplification protocol. Neurology. 1994; 44:1161–1164.

25) Nakajima, H., K. Ashida, H. Yamasaki, K. Shinoda, and N. Ohsawa. Intracranial tuberculoma with spontaneous recovery. Rinsho Shinkeigaku. 1995; 35:521–525.

26) Scarpellini, P., S. Racca, P. Cinque, F. Delfanti, N. Gianotti, M. R. Terreni, L. Vago, and A. Lazzarin. Nested polymerase chain reaction for diagnosis and monitoring treatment response in AIDS patients with tuberculous meningitis. 1995; 9:895–900.

27) Shankar, P., N. Manjunath, K. K. Mohan, K. Prasad, M. Behari, Shriniwas, and G. K. Ahuja. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. Lancet. 1991;337:5–7.

28) Wolfe, et al.Toward a microchip-based solid extraction method for isolation of nucleic acids. Electrophoresis 27, 2003;727-733.