Research Article [Parabia *et al.*, 3(5): May, 2012]
CODEN (USA): IJPLCP **ISSN: 0976-7126**

INTERNATIONAL JOURNAL OF PHARMACY & LIFE SCIENCES

Evaluation of diagnostic potential of real-time PCR (Q-PCR) for pulmonary tuberculosis

Ankita S. Bhardvaj, Farzin M. Parabia*, Kuldeep J. Patel, Mukund Chandra Thakur and Arif Khan

Ashok & Rita Patel Institute of Integrated Study and Research in Biotechnology and Allied Sciences (ARIBAS), New Vallabh Vidyanagar, (Gujarat) - India

Abstract

Q-PCR is a rapid confirmative tool which requires support of traditional techniques to confirm the diagnosis of pulmonary tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB). In present study the Q-PCR results were compared with the traditional techniques of diagnosis smear, culture, histological and cytological methods. Total 68 infected samples were evaluated. Out of which 49 were of body fluids and 19 were of tissue samples. Out of 49 body fluid samples, Q-PCR gave 2 false negative results (4.08% error). The smear and culture had 51.02% and 59.37% positive results with confirmed Q-PCR. The results indicated that the sensitivity of Q-PCR is significant and higher than the other traditional methods. But however the confirmed diagnosis required the essential opinion of traditional tests, any of the single evaluation method had only 29% chances to diagnose TB.

Key-Words: Pulmonary tuberculosis (TB), *Mycobacterium tuberculosis*, diagnosis, Real-Time PCR, Q-PCR

Introduction

Mycobacterium tuberculosis (MTB) is the causal organism for pulmonary tuberculosis (TB). There are three major infectious diseases threatening to the world; TB, AIDS and malaria. TB kills 1.8 million people annually (WHO, 2010). TB is one of the secondary infections in HIV infected person and has serious issue of transmission (Harries et al., 2010). However TB can be treated efficiently, therefore fast and confirmed diagnosis help to employ specific treatment combinations. The recent techniques based on PCR, aid in diagnose TB more efficiently (Helb et al., 2010; Rachow et al., 2011; Wallis et al., 2010). The conventional diagnosis involved mainly Acid Fast Bacilli (AFB) smear and culture method. The results of these tests are ambiguous. Even culturing takes about 3 to 6 weeks and usually requires at least three samples. Therefore, PCR based method that target the specific sequences present in heterogeneous mass of DNA serve as an excellent tool for fast and confirmed diagnosis (Soini and Musser, 2001; Woods, 2001). This technique is extremely helpful for the patient of AIDS suspected for TB (Sechi et al., 1997). Q-PCR has advantage to confirm the amplification of target DNA, where agarose gel electrophoresis is not required.

*** Corresponding Author**
E.mail: farzin_parabia@yahoo.co.in

The principal methodology is same in PCR and Q-PCR but there is greater potential of Q-PCR for the diagnosis of pulmonary tuberculosis in terms of sensitivity and specificity (Miller at al., 2002; Ortu et al., 2006; Papaparaskevas et al., 2008; Soini and Musser, 2001). The reported sequences used for Q-PCR are listed in Table 1.

In present investigation the suspected samples were collected from 68 cases classified into body fluid and tissue samples. There are various available diagnostic methods for TB (Cernoch et al., 1994; Forbes et al., 2007). The diagnostic parameters used for body fluid samples were smear stained with Zheil-Neelsen method and culture detected by BACTEC™ MGIT™ 960 Mycobacterial Detection System. The tissue samples were confirmed by histological and cytological evaluations. All these traditional approach of TB diagnosis were compared with the efficiency of modern and rapid diagnostic tool Q-PCR.

Material and Methods

Collection of pathological Samples

Body fluids (sputum, pus, pleural fluid, cerebral spinal fluid (CSF), urine and synovial fluid) or the tissue samples (endometrial curetting, bronchio alveolar lavage (BAL), FNAC's and histological biopsies) were taken as test materials. The samples collected from particular patient were based on the availability of the specimen. The presence of *M. tuberculosis* in fluid and

tissue samples was evaluated by Q-PCR as well as smear, culture and histo-cytological methods.

Sample digestion and DNA isolation

Prior to isolate DNA, the samples were digested and decontaminated as per the method described by Cernoch et al. (1994) and Isenberg (1992). After digestion the obtain pellets were re-suspended in 1ml phosphate buffer and further processed for DNA isolation. The DNA was isolated by DNA isolation kit (Qiagen, Germany) as per manufacturer instructions. The obtained DNA samples were stored at -20°C until use.

Preparation of reaction mixture for Q-PCR

PCR Mastermix with TaqMan® probe specific to *M. tuberculosis* (MTB) was used for Q-PCR. As exogenous positive and negative control, genomic DNA of *M. tuberculosis* H37RV (ATCC 27294) (Bifani et al., 2000) and *M. fortuitum* (ATCC 6841) were respectively used. The specific customized primers MTB-F: 5'-CTCGGTGAGAAGACCGTCA -3' and MTB-R: 5'- GTCCTCGATGCCCCAGAT-3' with MTB-Probe 5'-[FAM]-AGCTCGAGGCCGAACTGTTCAC- [TAMRA]-3' were used. The probe was labeled with fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' terminal and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' terminal. The cycle was run according to the program, stage-1 at 50°C for 2 min, stage-2 at 95°C for 10 min, stage 3 having 50 cycles at 95°C for 15 sec to 60°C for 1 min.

Conventional method used for detection of MTB

Smear and culture method were used to detect MTB. Mycobacteria are Acid Fast Bacilli (AFB) and best detection method is Zheil-Neelsen staining method (Selvakumar et al., 2005). The body fluid samples were spread on the slides for smear preparation. Presence of MTB in tissue was studied by histological approach. Biopsy tissue samples were fixed and its paraffin sections were processed by Ziehl Neelsen method (Hematoxylin and Eosin, 1992). Similar approach was adopted for cytological study, where tissue smear were used (Hematoxylin and Eosin, 1992).

Fully automated BACTEC™ MGIT™ 960 Mycobacterial Detection System was used for culturing MTB. The confirmation of *M. tuberculosis* was done by the emitted fluorescent signals. A fluorescent compound (Tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate) was sensitive to the presence of oxygen dissolved in the broth. Initially Oxygen quenched the emissions but later on actively respiring microorganisms consumed the oxygen and allowed the fluorescence. Culture vials were incubated for minimum 6 weeks and were considered as negatives after maximum incubation period of 56 days.

Results and Discussion

Total 68 patients (Table 2&3) were enrolled for diagnosis of tuberculosis (TB) through Real time PCR IS6110 from Indian population. Each sample was evaluated by multiple testing methods coupled with Q-PCR to judge the reliability of the instrument for diagnosis. However all tests were rarely positive for a same case, therefore confirmed diagnosis was done by the positive results of at least two tests.

Out of which 49 were body fluid samples and confirmed through Q-PCR, smear and culture (Table 2). The two false negative samples were detected by Q-PCR indicated 4.08% error and diagnosed positive by smear (Table 2) from body fluids. Out of 49 body fluid samples, 22 showed positive results in both Q-PCR as well as smear test (Table 2). However, remaining 25 samples were positive in Q-PCR and negative in smear test indicated 51.02% failure of smear test in body fluid samples. Out of 49 samples, 16 samples were positive for Q-PCR as well as culture. Whereas, 36 were negative for culture and positive for Q-PCR indicated 59.37% failure of culture method. Altogether only 9 cases were positive for all the tests i.e. 18.36% sensitivity of getting positive diagnosis from all the techniques. This indicated the significance of multiple testing for confirmed diagnosis of TB.

The 19 tissue samples out of 68 total samples were diagnosed by Q-PCR, culture, histology and cytology (Table 3). Out of 19 samples, 16 samples (84.21%) gave false positive tests in Q-PCR. PCR is higher sensitivity and hence could have shown false positive results. Even the culture was also negative for 3 positive tissue samples diagnosed by Q-PCR and histo/cytological evaluation (Table 3). This indicated that even culture technique has somehow not confirmed diagnostic potential and needs confirmation of additional tests. The 16 false positive samples in Q-PCR were confirmed by cytological and histopathological tests. This false positive Q-PCR results and negative cultural and clinical findings could be due to contamination or early disease with low number of bacilli or may be latent infection which was picked up by Q-PCR when patients were still asymptomatic and before the structural damage to the tissue had taken place.

Microscopic examination and culture were the classical approach to diagnose TB though less sensitive. Later on the diagnosis was done by the faster method based on PCR (Bennedsen et al., 1996). However PCR results were also variable and required proper standardization. Several reports are available to confirm the reliability

of PCR for TB diagnosis (Rattan, 2000, Bennedsen et al., 1996). The reported sensitivity for PCR was 91.4% for smear-positive specimens by Bennedsen et al. (1996). The detection through Q-PCR than PCR will save more time to diagnose. The earlier reported Q-PCR sensitivity for TB positive samples was 92% (Drosten et al., 2003), 92.3% (Beqaj et al., 2007) and with 100% specificity for multiple species diagnosis than the smear and culture tests. PCR efficiency by Ortu et al. (2006) was 10% and 100%, respectively, compared to different conventional methods. The response is also dependent on the protocol preferred to isolate DNA. The report is even available to confirm the sensitivity of Q-PCR against applied protocols for DNA isolation (Thakur et al., 2011). Therefore Qiagen kit (Germany), the most referred technique for DNA isolation from *M. tuberculosis* was selected for DNA isolation in the present study. In the present work the similar approach was carried out to compare the Q-PCR efficiency with smear, culture, histological and pathological techniques and find the much higher efficiency and specificity to diagnose compared to conventional methods. The results obtained from the present study were true to 95.91% of Q-PCR for the confirmed diagnosis of TB when the smear was positive.

A total of 68 samples were studied and Q-PCR was found to be most sensitive (95.91%) method for the diagnoses of TB. However the 100% confirmed diagnosis was achieved with the coupling of other conventional techniques like smear, culture, histological and cytological analysis. These data justifies the importance of traditional techniques with the modern approach of diagnostic tools. The initial test may be done by Q-PCR but the results have to be confirmed through traditional approach before confirmed diagnosis and treatment. Therefore Q-PCR can serve as fast and reliable diagnostic tool but still can't replace the conventional techniques of diagnosis.

Acknowledgement

The authors are gratefully acknowledged Mr.Rajeev Sharma for his technical support during the research and Prof. Minoo H. Parabia (Rasiklal Manikchand Dhariwal Ayurvedic Hospital and College, Vagaldhara, Valsad-396 375, India) for his valuable suggestions. The authors are also acknowledged the encouragement and support of Prof. Pradip Patel, Head, ARIBAS and Charutar Vidya Mandal (CVM), Vallabh Vidyanagar, Gujarat, India.

References

1. Bennedsen J., Thomsen V.O., Pfyffer G.E., Funke G., Feldmann K., Beneke A., Jenkins P.A., Hegginbothom M., Fahr A., Hengstler M., Cleator G., Klapper P. and Wilkins E.G. L. (1996). Utility of PCR in Diagnosing Pulmonary Tuberculosis, *Journal of Clinical Microbiology, 34(June):1407–1411.*
2. Beqaj S.H., Flesher R., Walker G.R. and Smith S.A., (2007). Use of the Real-time PCR assay in conjunction with MagNA Pure for the detection of Mycobacterial DNA from fixed Specimens, *Diagn Mol Pathol, 16(Sep): 169-173.*
3. Bifani P., Moghazeh S., Shopsin B., Driscoll J., Ravikovitch A. and Kreiswirth B.N. (2000). Molecular characterization of *Mycobacterium tuberculosis* H37Rv/Ra variants: distinguishing the mycobacterial laboratory strain, *J Clin Microbiol. 38(Sept):3200-4.*
4. Cernoch P.L., Enns R.K., Saubolle M.A. and Wallace R.J. (Jr.) with Coordinating Editor: Weissfeld A.S. (1994). Cumitech 16A: Laboratory diagnosis of the Mycobacterioses. ASM Press. Washington, DC.
5. DesJardin L. E., Chen Y., Perkins M. D., Teixeira L., Cave M. D., and Eisenach K. D. (1998). Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS6110 DNA in sputum during treatment of tuberculosis, *J. Clin. Microbiol. 36:(July):1964–1968.*
6. Drosten C., Panning M. and Kramme S. (2003). Detection of *Mycobacterium tuberculosis* by Real-Time PCR Using Pan-Mycobacterial Primers and a Pair of Fluorescence Resonance Energy Transfer Probes Specific for the *M. tuberculosis* Complex, *Clinical Chemistry, 49(10): 1659-1661.*
7. Forbes B.A., Banaiee N., Beavis K.G., Brown-Elliott B.A., Laata P.D., Elliott L.B., Hall G.S., Hanna B., Perkins M.D., Siddiqi S.H., Wallace R.J. and Warren N.G. (2007). Laboratory detection and identification of mycobacteria; Proposed guideline. CLSI document M48-P. Clinical and Laboratory Standards Institute, Wayne, PA.
8. Harries A.D., Zachariah R., Corbett E.L., Lawn S.D., Santos-Filho E.T., Chimzizi R., Harrington M., Maher D., Williams B.G and De Cock K.M. (2010). The HIV-associated tuberculosis epidemic–when will we act? *Lancet 375:(May): 1906–1919.*
9. Helb D., Jones M., Story E., Boehme C., Wallace E., Ho K., Kop J., Owens M.R., Rodgers R., Banada P., Safi H., Blakemore R., Ngoc Lan N. T., EJones-López. C., Levi M., Burday M., Ayakaka I., Mugerwa R. D., McMillan B., Winn-Deen E., Lee C., Dailey P., Perkins M.D., Persing D.H. and Alland D. (2010). Rapid detection of *Mycobacterium tuberculosis* and Rifampin resistance by use of on-demand, near-patient technology, *J Clin Microbiol 48(Jan): 229–237.*
10. Hematoxylin Allen T.C. and Eosin (1992). Bacteria, Fungi and other microorganisms. In: Armed Forces Institute of Pathology, Laboratory methods in Histotechnology. Prophet EB, Mills B, Arrington JB and Sobin LH, ed. Washington D.C. American Registry of Pathology, *203-231, 53-58 & 219.*
11. Isenberg, H.D. (ed). (1992). Clinical microbiology procedures handbook, vol. 1. American Society for Microbiology, Washington, D.C.

12. Lachnik J., Ackermann B., Bohrssen A. Maass S., Diephaus C., Puncken A., Stermann M. and Bange F.C. (2002). Rapid-Cycle PCR and Fluorimetry for Detection of Mycobacteria. *J. Clin. Microbiol., 40(Sep):3364*.
13. Miller N., Cleary T., Kraus G., Young A.K., Spruill G. and Hnatyszyn H. J (2002). Rapid and Specific Detection of *Mycobacterium tuberculosis* from Acid-Fast Bacillus Smear-Positive Respiratory Specimens and BacT/ALERT MP Culture Bottles by Using Fluorogenic Probes and Real-Time PCR, *Journal of Clinical Microbiology, 40(Nov): 4143–4147.*
14. Ortu S., Paola M., Sechi L.A., Pirina1P., Saba F., Vertuccio C., Deriu A., Maida I., Mura M.S. and Zanetti S. (2006). Rapid detection and identification of *Mycobacterium tuberculosis* by Real Time PCR and Bactec 960 MIGT, *The New Microbiologica, 29: (Oct):75-80*.
15. Papaparaskevas J., Houhoula D.P., Siatelis A., Tsakris A. (2008). Molecular-Beacon-Based Real-Time PCR for Detection and Quantification of *Mycobacterium tuberculosis* DNA in Clinical Samples, *Journal of Clinical Microbiology, 46(Sep):3177–3178.*
16. Rachow A., Zumla A., Heinrich N., Rojas-Ponce G., Mtafya B., Reither K., Ntinginya N.E., Grady J., Huggett J. Dheda K., Boehme C., Perkins M., Saathoff E. and Hoelscher M. (2011). Rapid and Accurate Detection of *Mycobacterium tuberculosis* in Sputum Samples by Cepheid Xpert MTB/RIF Assay—A Clinical Validation Study, *PLoS ONE. 6 (June): e20458*.
17. Rattan A., (2000). PCR for diagnosis of tuberculosis: Where are we now? *Ind. J Tub., 47:79-82.*
18. Sechi L.A., Pinna M.P., Sanna A., Pirina P., Ginesu F., Saba F., Aceti A., Turrini F., Zanetti S., Fadda G. (1997). Detection of *Mycobacterium tuberculosis* by PCR analysis of urine and other clinical samples from AIDS and non-infected patients, *Moll. Cell. Probes, 11:(Aug): 281-285.*
19. Selvakumar N., Gomathi S.M., Rahman F., Syamsunder A., Duraipandian M., Wares F., Narayanan P.R. (2005). Comparison of variants of carbol-fuchsin solution in Ziehl-Neelsen for detection of acid-fast bacilli, *Int J Tuberc Lung Dis 9(June):226–229.*
20. Soini H. and Musser J.M. (2001). Molecular diagnosis of *Mycobacteria*. *Clinical Chemistry, 47 :(Feb): 809-814*.
21. Tevere V.J., Hewitt P.L., Dare A., Hocknell P., Keen A., Spadoro J.P. and Young K.Y. (1996). Detection of *Mycobacterium tuberculosis* by PCR amplification with pan Mycobacterium primers and hybridization to an *M. tuberculosis*-specific probe, *J Clin Microbiol; 34: (Apr):918–23*.
22. Thakur R., Sarma S. and Goyal R., (2011). Comparison of DNA Extraction Protocols for *Mycobacterium Tuberculosis* in Diagnosis of Tuberculous Meningitis by Real-time Polymerase Chain Reaction, *J Glob Infect Dis., 3(Oct-Dec): 353–356*.
23. Wallis R.S., Pai M., Menzies D., Doherty T.M., Walzl G., Perkins M.D. and Zumla A. (2010). Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice, *Lancet 375: (May):1920–1937*.
24. WHO World Health Organization Report (2010). M/XDR-TB Surveillance and Control: Global Update. Geneva, Switzerland.
25. Woods G.L. (2001). Molecular techniques in Mycobacterial detection. *Arch. Pathol. Lab. Med., 125(Jan): 122-126*.

Table 1: Reported primers and probes for Q-PCR used to diagnose MTB

No.	Specific Gene	Amplified Product	Primers	Probe	Reference
1.	IS6110	163-bp	IS6(5'GGCTGTGGGTAGCAGACC3') IS7(5'CGGGTCCAGATGGCTTGC-3')	5'TGTCGACCTGGGCAGGGTTCG3'	Desjardin et al., 1998
2.	16S rRNA	--	KY18 (5'CACATGCAAGTCGAACGGAAAGG3') KY75 (5'GCCCGTATCGCCCGCACGCTCACA3')	KY172-T3 (5'GGTGGAAAGCGCTTTAGCGGT-3')	Tevere et al., 1996
3.	16S rRNA	100-bp	LC 5 (5'GGC GGA GCA TGT GGA TTA3') LC 4 (5'TGC ACA CAG GCC ACA AGG GA3')	anchor probe LC 11 (5'CGCGGGCTCATC CCACACCG3') and sensor probe LC 12 (5'TAAAGCGCT TTC	Lachnik et al., 2002
4.	16S rRNA	300-bp	LC 7 (5'GAT AAG CCT GGG AAA		

			CTG3') LC 8 (5'CTA CCG TCA ATC CGA GAG3')	CACCACAAG A3')	
5.	16S rRNA	1,000-bp	LC 1 (5'GAG TTT GAT CCT GGC TCA GGA3') LC 4 (see 100-bp fragment)		
6.	ITS	220-bp	Sp1 (5'ACCTCCTTTCTAAGGAGCACC 3') Sp2 (5'GATGCTCG CAACCACTATCCA3')	5' anchor probe 4602 (5'GTGGGGCGTAGGCC GTGAGGGG3') and 3' detection probe 4600 (5'GTCTGTAGTGGGCG AGAGCCGGGTGC3')	Miller et al., (2002)

Table 2: The body fluid samples were confirmed for TB through Q-PCR, smear and culture

No of samples	Q-PCR	SMEAR	CULTURE
9	+	+	+
5	+	+	–
3	+	+	–
4	+	–	+
13	+	–	–
8	+	–	–
1	+	–	–
4	+	–	–
2	–	+	–
Total:49			
Confirmed diagnosis	95.91%	38.77%	26.53%
Diagnosis error	4.08%	51.02%	59.37%

'+' indicates positive results; '–'indicates negative results.

Table 3: The tissue samples were confirmed for TB through Q-PCR, culture, histological and cytological evaluation

Total	Q-PCR	CULTURE	HISTO/CYTO
3	+	–	+
9	+	–	–
7	+	–	–
Total: 19			

'+' indicates positive results; '–'indicates negative results.