

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

PCR and Elisa Methods (IgG and IgM): Their Comparison with Conventional Techniques for Diagnosis of Mycobacterium Tuberculosis

¹MirDavood Omrani, ²Mohammad Hassan Khadem Ansari and ²Davood Agaverdizadae

¹Department of Genetic, Motahary Hospital, Kashani AVE, Urmia, Iran

²Department of Clinical Biochemistry, Urmia School of Medicine, Urmia, Iran

Abstract: In order to establish a rapid and stable method for diagnosis of Mycobacterium tuberculosis infection and minimize the side effects of delayed diagnosis on patients and health system, a cross sectional study was carried out. Since, the infection rate with this bacteria increasing and one of the reasons for this increase is long process of laboratory identification, therefore establishing new diagnosis methods could decrease disease rate. To achieve this aim, collected sputum and blood specimens from 50 patients with clinical suspicion of pulmonary tuberculosis were studied with both traditional, acid-fast stain (AFB) and culture method compare to Enzyme-linked immunosorbent assay (Elisa) (IgG and IgM) and Polymerase Chain Reaction (PCR) methods. The sensitivity and specificity of all methods were determined by using the PCR results as the gold standard. The overall sensitivity, specificity, positive predictive value and negative predictive value of AFB were 17.64, 100, 100 and 70.12%. These values for culture method was 29.41, 100, 100 and 73.33% and for IgG antibody were 66.7, 81.81, 64.7 and 81.81% and IgM antibody were 70.58, 90.9, 80 and 85.71%, respectively. It was concluded that maximum sensitivity and specificity can be achieved by PCR method.

Key words: Pulmonary diseases, sputum, AFB method, molecular method

INTRODUCTION

It was estimated that about one-third of the world's population is infected with Mycobacterium tuberculosis with approximately 9 million new cases each year and it remains one of the deadliest diseases in the world (Palomino, 2005; Anderson *et al.*, 2008). In order to control the disease before entering to the active stage, rapid diagnosis of the affected cases is very critical. Traditional laboratory diagnosis of tuberculosis is based on the methods of Ziehl Neelsen acid-fast stain (AFB) and on laboratory culture of the causative organism Mycobacterium tuberculosis (Nagpaul, 1967). The Ziehl Neelsen staining method, although rapid and inexpensive, lacks sensitivity in clinical specimens varying between 30 and 70%, especially when disease occurs in children or co infected with Human Immunodeficiency Virus (HIV) (Long, 2001). On the other hand culture of the organism as the definitive tool for the diagnosis of tuberculosis in many health systems, can take as long as 10 weeks (Cousins *et al.*, 1992). Also some microscopy-positive specimens fail to yield mycobacteria on culture. This may be due to the harsh chemical treatment which is used to decontaminate specimens, or because of contamination with other bacteria. A report estimates that the sensitivity of culture method is about 50% (Daniel, 1990). But the

data differs between investigators. Long (2001) reported the sensitivity of culture about 80-85 and specificity 98%. But this report also emphasis on slow growth of microbes. Since, the accurate and on time diagnosis of tuberculosis in adults and children in many cases is very critical and conventional microscopy or culture methods are unable to respond to this demand, finding a replacement for these methods clearly needed (Kafwabulula *et al.*, 2002). ELISA and Polymerase Chain Reaction (PCR) Methods as alternative methods were recommended (Okuda *et al.*, 2004; El-Dawi *et al.*, 2004; Caws *et al.*, 2000; Kolk *et al.*, 1998; Cohen *et al.*, 1998; Garg *et al.*, 2003). Serological identification for Mycobacterium tuberculosis antibody is now an accepted technique and is widely used in clinical practice. ELISA for detection of antibodies: against Mycobacterium tuberculosis was introduced by Charpin *et al.* (1990). ELISA is an attractive simple and cheap technique and many studies have shown good results with a high sensitivity and specificity especially for antigen detection of Mycobacterium tuberculosis in Cerebro-Spinal Fluid (CSF) and blood serum. Satisfactory characteristics of diagnostic accuracy have been reported by Cocito (1991), Gupta *et al.* (1995) and Mitchison (2005). In addition to Elisa Methods, the polymerase chain reaction (PCR) has also the potential to overcome the

limitations of sensitivity and specificity of the traditional staining and culture techniques (Cousins *et al.*, 1992; Daniel, 1990; Kafwabolula *et al.*, 2002). PCR has been shown to be sensitive and specific for the identification of mycobacterial DNA using sputum and other body fluids; the potential for its use in urine samples has also been demonstrated (Kafwabolula *et al.*, 2002). Therefore, it was tried in this report by comparing the results of conventional identification methods such as AFB staining and culture methods with Elisa and PCR techniques in detection of *Mycobacterium tuberculosis* microbe encourage the health system manager for more application of these techniques in their routine *Mycobacterium tuberculosis* diagnosis.

MATERIALS AND METHODS

Fifty Sputum and also 50 blood specimens from 50 patients (mean age 46 year) with clinical suspicion of pulmonary tuberculosis who referred to Imam educational university hospital at uremia, Iran during the period of 2003-2004 were collected. Sputum was decontaminated using incubation with equal volume of 4% NaOH at 37°C for 60 min with intermittent vortexing. Samples were centrifuged at 10000 g at room temperature for 15 min and the pellet was divided to three portions, one for AFB staining, the second for culture and the third part for PCR. Serum was also separated for *Mycobacterium tuberculosis* IgG and IgM assays to detect antibodies. Smears were prepared for acid-fast microscopy as recommended (Abate *et al.*, 2004). Slide after staining with the Ziehl Neelsen method were examined under microscope. At least 300 fields were checked for detection of *Mycobacterium tuberculosis* (Abu-Amero and Halablab, 2004). If there were not any Acid fast bacilli then it was reported as negative (Abate *et al.*, 2004). About 200 µL sample were then inoculated onto 9 slants of media (Löwenstein-Jensen). The cultures were examined weekly (4-8) for the growth. Bacterial colonies were identified as *Mycobacterium tuberculosis* by conventional identification methods (Abate *et al.*, 2004). *Mycobacterium tuberculosis* DNA assay was performed using Sinagen diagnostic kit (Sinagen Company, Tehran, Iran). Bacterial DNA amplified in Eppendorf PCR machine (5860 Gradient Eppendorof Master Cyclyer, Germany). Final product of PCR was extruded on 2% agarose gel with proper markers (50bp DNA ladder, Roche Company, Germany). Samples were determined to be PCR-positive when the 163bp *Mycobacterium tuberculosis* DNA fragments was present on gel and were declared PCR-negative when this fragment was absent. All the tests carried out in duplicate. Patient's serums were tested

semi-quantitatively for IgG and IgM antibodies detection at laboratory conditions using Anda TB ELISA test, by antigen 60 kits (Strasbourg Cedex France). Following instruction of company, Elisa IgG was considered positive when titer was >225 Elisa units and Elisa IgM was considered positive when Elisa titer was >1 Elisa unit. The sensitivity and specificity of all methods were determined by using the PCR results as the gold standard.

RESULTS

This blind study included 100 clinical specimens of 50 patients to compare the different diagnostic modalities for the detection of tuberculosis and to evaluate the efficacy of PCR and Elisa Methods. Our results showed that the AFB staining method sensitivity, as the first line screening method is very low (17.64%) and we miss more than 80% of our true positive cases. By using culture method as confirmatory test, this sensitivity is slightly increased (29.41%). Using new methods such as ELISA antibody this sensitivity increased to about 70% and by applying PCR method this sensitivity reached to 100% (Fig. 1). The overall sensitivity, specificity, positive predictive value and negative predictive value of AFB staining, culture method, IgG antibody and IgM antibody were summarized in Table 1 and 2.

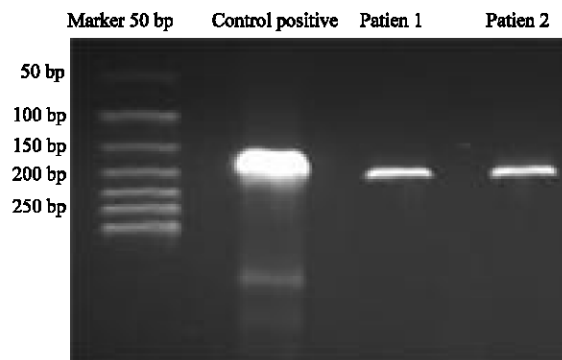


Fig. 1: PCR product of amplified *Mycobacterium tuberculosis* was run on 2% agarose gel. Patients as well as positive control showed expected 163 bp fragment. 50 bp size marker was run for control

Table 1: The result of 50 samples showed true and false positive and negative values

Samples (N)	AFB	Culture	Elisa IgG	Elisa IgM	PCR
True positive	3	5	11	12	17
False positive	0	0	6	3	0
True negative	33	33	27	30	33
False negative	14	12	6	5	0
Total	50	50	50	50	50

Table 2: The overall percent of sensitivity, specificity, positive predictive value and negative predictive values of studied samples with different methods

Parameters (%)	AFB	Culture	Elisa IgG	Elisa IgM
Sensitivity	17.64	29.41	66.70	70.58
Specificity	100.00	100.00	81.81	90.90
PVP	100.00	100.00	64.70	80.00
PVN	70.12	73.33	81.81	85.71
False positive	0.00	0.00	18.18	9.00
False negative	82.35	70.58	35.29	41.66

PVP: Predictive Value Positive, PVN: Predictive Value Negative

DISCUSSION

The accurate diagnosis of Tuberculosis remains difficult and in many cases the ethological agent TB is not detected by conventional microscopy or culture (Kafwabulula *et al.*, 2002). Therefore finding definitive and rapid diagnosis methods in TB in routine clinical work or improving the quality of the used methods is in priority for health centers of many countries. The sensitivity and specificity of these methods has been under compact surveillance for many years. For example diagnosis of AFB positivity in smears depends on the bacillary load of the specimen, sputum quality and quantity, health centers equipments and experience of microscopist (Long, 2001). Different studies have reported a wide range of AFB positivity ranging from as low as 30% to as high as 70% (Long, 2001). Rafi and Naghily (2003) and Abu-Amero and Halablab (2004) reported the sensitivity of AFB smear 3.4% and specificity of 100%. In Long study sensitivity of AFB smear ranged between 30-70% and specificity 98% (Long, 2001). In this study the sensitivity of AFB smear was 17.64% and specificity was 100% which is in accordance with above studies. There are several reasons for low sensitivity and specificity of AFB smears and error encounter during smear reading (Long, 2001; Rafi and Naghily, 2003). Therefore it should be noted; that sputum smear performing as a quick and easy to activate method for preliminary confirmation of diagnosis is necessary but is not enough and other methods should be applied as complementary methods for decision making. Traditionally culture of the *Mycobacterium tuberculosis* is one of the oldest methods used in many health centers units as a confirmatory test. But present study showed that the sensitivity of the culture was also low (29.41%). Culture procedure takes about 4-8 weeks in average (Long, 2001; Cousins *et al.*, 1992). Culture of clinical specimens could confirm the diagnosis in smear-positive cases. But the limitation of this method is slow growth of microbes, even with selective liquid media (Long, 2001). Enzyme-linked immunosorbent assay (ELISA)-based serological tests to detect antibodies to *Mycobacterium tuberculosis* are simple and inexpensive and are a potentially practical tool for the diagnosis of active

pulmonary TB (Okuda *et al.*, 2004; Charpin *et al.*, 1990; Cocito, 1991; Gupta *et al.*, 1995; Mitchison, 2005). Wang *et al.* (1993) reported the sensitivity of Elisa method for IgG antibody detection of *Mycobacterium tuberculosis* were between 61.9-81.4% and specificity 85.4%. Kaustova (1996) found the sensitivity of the Elisa methods differs in various groups but the specificity was between 80-98.5%. Gevaudan *et al.* (1992) calculated the sensitivity of Elisa IgG 98.6% and specificity 86.7%. Charpin *et al.* (1990) colleagues found sensitivity of Elisa IgG 48% and IgM 76% and specificity for IgG 71% and for IgM 98%. As we see from the above studies, almost all of the assays are limited by sensitivity, especially in smear-negative TB patients. An additional limitation is the variability in sensitivity, depending on both the investigator and the geographic origin of the survey participants (Cousins *et al.*, 1992; Garg *et al.*, 2003). However, the reported specificity of 90% is acceptable for a serodiagnostic test (Okuda *et al.*, 2004). Present study with ELISA test (ANDA-Tb) for the serological diagnosis of tuberculosis in serum samples in 50 individuals with respiratory Tuberculosis showed sensitivity for Elisa IgG was 66.7% and IgM 70.58% and specificity were 81.81 and 90.9%, respectively. This results as Okuda *et al.* (2004) reported are acceptable and it is possible for us to conclude that this particular ELISA method is very useful as a complementary test for the diagnosis of tuberculosis. While immunodiagnostic techniques may show promise, but generally they lack sufficient sensitivity and often the necessary specificity (Caws *et al.*, 2000). Therefore PCR is the most widely applied alternative rapid diagnostic technique for *Mycobacterium tuberculosis* detection. PCR has enhanced the diagnostic predictability of the disease especially in the extrapulmonary, paucibacillary samples. High specificity and sensitivity have been reported in different samples. The technique is capable of picking as few as ten to fifty tubercle bacilli. When PCR technique is performed under quality controlled conditions, false negatives (due to underfined polymerase inhibitors) and false positives (due to cross contamination during sample collection or in the laboratory) can easily be avoided (Singh *et al.*, 2002). PCR generally was based on the amplification of the IS6110 insertion sequence, which belongs to the IS3 family and is found in almost all members of the *Mycobacterium tuberculosis* complex. Most strains of *Mycobacterium tuberculosis* carry 10-15 copies, which are present in a wide variety of chromosomal sites (Caws *et al.*, 2000). In a study conducted in Sudan at 2004 a comparison between conventional method AFB smear and PCR technique for

detecting TB showed: Microscope sensitivity was 65.4% and the specificity was 90.5%, whereas sensitivity of the IS6110 was 88.5% and specificity was 98.6%. They concluded that though IS6110 sensitivity was 13.1% higher than smear method, it provided a significant improvement in specificity for the diagnosis of pulmonary tuberculosis (El-Dawi *et al.*, 2004). The sensitivity of the PCR for patients suspected of TB as Kolk *et al.* (1998) reported was 86%, which is dramatically higher than the 31% for microscopy. In another study conducted by Cohen and colleagues, they found the sensitivity of the in-house PCR detection method for TB diagnosis 100% and Roche detection method sensitivity 95%. They concluded that PCR may be a useful tool to evaluate patients for tuberculosis within the first hospital day (Cohen *et al.*, 1998). The sensitivity and specificity of PCR for the IS6110 target obtained 100% in comparison with the culture method in Ogusku on TB (Morand *et al.*, 2006). The results confirmed the effectiveness of PCR methodology using primers for the IS6110 gene sequence and permit the PCR method to be applied to samples sent by services that do not identify the Mycobacterium tuberculosis by other methods (Morand *et al.*, 2006). By using this technique it was possible to detect all the affected cases of TB in the referred patients. The overall sensitivity, specificity values for PCR method were 100 and 100%, respectively. Therefore it is possible to reach to this conclusion that PCR has a potentially important role in improving the diagnostic accuracy in clinical specimens. PCR test is a reliable method for rapid diagnosis of pulmonary tuberculosis regardless of the AFB smear and culture results.

ACKNOWLEDGMENT

This research was supported by a grant (No. 1382/p) from Deputy of Uremia Medical Science Research Unit.

REFERENCES

- Abate, G., A. Aseffa, A. Selassie, S. Goshu, B. Fekade, D. WoldeMeskal, D. and H. Miorner, 2004. Direct colorimetric assay for rapid detection of rifampin-resistant mycobacterium tuberculosis. *J. Clin. Microbiol.*, 42: 871-873.
- Abu-Amero, K. and M.A. Halablab, 2004. Evaluation of the Cobas Amplicor MTB test for the detection of mycobacterium tuberculosis complex. *East Mediter. Health J.*, 10: 329-335.
- Anderson, B.L., R.J. Welch and C.M. Litwin, 2008. An assessment of the ability of mycobacterium tuberculosis serology to detect active TB disease. *Clin. Vaccine Immunol.*, 15: 644-649.
- Caws, M., S.M. Wilson, C. Clough and F. Drobniewski, 2000. Role of *IS6110* targeted PCR, biochemical, clinical and immunological criteria for diagnosis of *Tuberculous meningitis*. *J. Clin. Microbiol.*, 38: 3150-3155.
- Charpin, D., H. Herbault, M.J. Gevaudan, M. Saadjian, P. De-Micco, A. Arnaud, D. Vervloet and J. Charpin, 1990. Value of ELISA using A60 antigen in the diagnosis of active pulmonary tuberculosis. *Am. Rev. Respir. Dis.*, 142: 380-384.
- Cocito, C.G., 1991. Properties of the mycobacterial antigen complex A60 and its applications to the diagnosis and prognosis of tuberculosis. *Chest*, 100: 1687-1693.
- Cohen, R.A., S. Muzaffar, D. Schwartz, S. Bashir, S. Luke, L.P. McGartland and K. Kaul, 1998. Diagnosis of pulmonary tuberculosis using PCR assays on sputum collected within 24 hours of hospital admission. *Am. J. Respir. Crit. Care. Med.*, 157: 156-161.
- Cousins, D.V., S.D. Wilton, B.R. Francis and B.L. Gow, 1992. Use of polymerase chain reaction for rapid diagnosis of tuberculosis. *J. Clin. Microbiol.*, 30: 255-258.
- Daniel, T.M., 1990. The rapid diagnosis of tuberculosis: A selective review. *J. Lab. Clin. Med.*, 116: 277-282.
- El-Dawi, T.G., N.S. Saeed and M.E. Hamid, 2004. Evaluation of a PCR-amplified IS6110 insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan. *Saudi Med. J.*, 25: 1644-1647.
- Garg, S.K., R.P. Tiwari, D. Tiwari, R. Singh, D. Malhotra, V.K. Ramnani, G.B. Prasad, R. Chandra, M. Fraziano, V. Colizzi and P.S. Bisen, 2003. Diagnosis of tuberculosis: Available technologies, limitations and possibilities. *J. Clin. Lab. Anal.*, 17: 155-163.
- Gevaudan, M.J., C. Bollet, D. Charpin, M.N. Mallet and P. De-Micco, 1992. Serological response of tuberculosis patients to antigen 60 of BCG. *Eur. J. Epidemiol.*, 8: 666-676.
- Gupta, S., S. Kumari, J.N. Banwalikar and S.K. Gupta, 1995. Diagnostic utility of the estimation of mycobacterial antigen A60 specific immunoglobulins IgM, IgA and IgG in the sera of cases of adult human tuberculosis. *Tuber. Lung Dis.*, 76: 418-424.
- Kafwabulula, M.K.A., T. Nagatake, J. Gotoh, S. Mitarai and K. Oizumi-Zumla, 2002. An evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. *Int. J. Tuberc. Lung Dis.*, 6: 732-737.
- Kaustova, J., 1996. Serological IgG, IgM and IgA diagnosis and prognosis of mycobacterial diseases in routine practice. *Eur. J. Med. Res.*, 1: 393-403.

- Kolk, A.H., L.F. Kox, J. Van-Leeuwen, J.S. Kuijper and H.M. Jansen, 1998. Clinical utility of the polymerase chain reaction in the diagnosis of extrapulmonary tuberculosis. *Eur. Respir. J.*, 11: 1222-1226.
- Long, R., 2001. Smear-negative pulmonary tuberculosis in industrialized countries. *Chest*, 120: 330-334.
- Mitchison, D., 2005. The diagnosis and therapy of tuberculosis during the past 100 years. *Rev. Am. J. Respir Crit. Care Med.*, 171: 699-706.
- Morand, J.J., E. Garnotel, F. Simon and E. Lightburn, 2006. Cutaneous tuberculosis overview. *Med. Trop.*, 66: 229-236.
- Nagpaul, D.R., 1967. District tuberculosis control programme in concept and outline. *Ind. J. Tubercle.*, 14: 186-186.
- Okuda, Y., R. Maekura, A. Hirota, S. Kitada, K. Yoshimura, T. Hiraga, Y. Yamamoto, M. Itou, T. Ogura and T. Ogihara, 2004. Rapid sero-diagnosis of active pulmonary mycobacterium tuberculosis by analysis of results from multiple antigen-specific tests. *J. Clin. Microbiol.*, 3: 1136-1141.
- Palomino, J.C., 2005. Nonconventional and new methods in the diagnosis of tuberculosis: Feasibility and applicability in the field. *J. Eur. Respir. Rev.*, 26: 339-350.
- Rafi, A. and B. Naghily, 2003. Efficiency of polymerase chain reaction for the diagnosis of tuberculous meningitis. *Southeast Asian J. Trop. Med. Public Health*, 34: 357-360.
- Singh, U.B. and P. Seth, 2002. PCR diagnosis of tuberculosis-experience in India. *Indian J. Pediatr.*, 69: S20-4.
- Wang, H., J.Y. Lu, L. Liang and H.L. Liu, 1993. Evaluation of the potential role of enzyme-linked immunosorbent assay in serodiagnosis of tuberculosis. *Zhonghua Yi. Xue. Za. Zhi.*, 52: 9-14.