EDITORIAL

APPLICATION OF PCR TECHNIQUES FOR TB DIAGNOSIS AT THE INSTITUTE OF NUCLEAR MEDICINE, MOLECULAR BIOLOGY & ONCOLOGY (INMO) - UNIVERSITY OF GEZIRA, SUDAN

Nagla Gasmelseed [1], **Adil Mergani**[1], **Mutaz Abass Elimam**[1], **Mazin Elamin Mohamed**[1], **Ameer Mohamed Dafalla**[1], **Ahmed Eltahir** [2] & **Nasreldin Elwali** [1]

1. Department of Molecular Biology, National Cancer Institute (NCI), University of Gezira, Wad Medani Sudan.
2. Faculty of Medicine King Fahad Medical City, Kingdom of Saudi Arabia

Correspondence: Nagla Gasmlseed, Department of Molecular Biology, National Cancer Institute (NCI, University of Gezira, Sudan. Email: Nag_la@yahoo.com

ABSTRACT

Traditional methods for laboratory diagnosis of tuberculosis (TB) may require weeks, and delays can impede treatment and control efforts. Nucleic acid amplification (NAA) tests, such as polymerase chain reaction (PCR) and other methods for amplifying DNA and RNA, may facilitate rapid detection of microorganisms. This study emphasized our three years experience in the diagnosis of TB on clinical samples using the PCR method. Among 531 patients with suspected TB, 112 (21.3%) were positive by PCR, while 419 (78.7 %) were negative. The specimens collected from TB suspected patients were sputum, blood, ascitic fluid, pleural fluid, and gastric wash. Blood samples showed high positive results 15/41 (34%) in comparison with sputum samples which is the most frequent sample 64/ 285 (22.3%). Gastric wash samples reported low rate of
positive result 1/30 (3.3%). This rapid and sensitive test compared with the other cultural and microscopic tests have now been incorporated into our laboratory practice allowing the physicians to manage proper diagnosis and drug regimens.

Keywords: Miliary TB, Pulmonary TB, Molecular diagnosis, Gezira State

EDITORIAL

INTRODUCTION

The human pathogenic bacterium Mycobacterium Tuberculosis (TB), is the causative agent of tuberculosis, infects an estimated one-third of the world's population, resulting in 8 million tuberculosis cases and 2 million deaths each year (Pai, Kalantri et al. 2006). Tuberculosis (TB) continues to be a major public health challenge worldwide. HIV-TB coinfection is especially important and worrying as it accelerates progression of infection to active disease and amplifies spread of TB including drug resistant organism or bacilli (Glassroth 2005). The currently accepted ''gold standard'' laboratory method for detecting and identifying Mycobacterium tuberculosis is a combination of ZN-stained acid-fast bacillus (AFB) smear for initial screening and culture for bacterial isolation and identification (Hall et al 1994). Smears lack both sensitivity and specificity for Mycobacterium tuberculosis, and cultures usually require weeks before the species is definitively identified (Daniel, 1990; Kent and Kubica, 1985). The acid-fast smear test also is unable to distinguish among mycobacterial species. Ongut, et al (2006) evaluated the validity of an immunochromatographic assay, ICT Tuberculosis test for the serologic diagnosis of TB in Antalya, Turkey. They found that smear-positive pulmonary TB patients showed a higher positivity rate for antibodies than smear-negative patients, but the difference was not statistically significant. They suggest that ICT Tuberculosis test can be used to aid TB
diagnosis in smear-positive patients until the culture results are available (Ongut, Ogunc et al. 2006)
Therefore, the top priority for TB diagnostic development worldwide is a low-cost, rapid, sensitive, and specific test that could replace the acid-fast smear examination (Cho and Brennan 2007). In addition, only half of all cases of active TB worldwide are smear-positive. Most smear-negative TB cases are ignored and remain undiagnosed. Therefore, improved diagnostic methods for smear-negative TB are also needed. Microscopy and culture are still the major backbone for laboratory diagnosis of tuberculosis (TB); new methods including molecular diagnostic tests have evolved over the last two decades (Cho and Brennan 2007). PCR amplification can be rapidly performed by using primers for a highly conserved sequence within a gene common to all mycobacterial species to amplify minute amounts of DNA (Watson et al 1992); specificity for *M. tuberculosis* is achieved during the detection step. PCR allows the amplification of a targeted DNA sequence over 10^6-fold through cycles of primer annealing and nucleotide extension of target sequences by DNA polymerase at specific temperatures (Persing, 1993, Piper, and Unger1998). Research developments with PCR for the direct detection of *M. tuberculosis* in clinical specimens have demonstrated the possible use of PCR in providing a rapid diagnosis of tuberculosis (Stahl, and Urbance. 1990; Sibata and Togashi, 1992; Narita, et al., 1992; Victor et al., 1992). The Department of Molecular Biology, Institute of Nuclear Medicine Molecular Biology and Oncology (INMO) introduced PCR molecular technique to perform a molecular diagnostic service for patients and their physicians in the infectious diseases such as TB, HBV, HIV, and Chlamydia. The aims of this paper is to evaluate the molecular diagnostic services in the department and to emphasize our three years experience in the molecular diagnosis of tuberculosis and identify the best sample for miliary TB diagnosis.

MATERIALS AND METHOD

Between 2004 and 2007, 513 TB suspects (304 males and 227 females) were enrolled in the Molecular Diagnosis Unit at the Department of Molecular Biology at (INMO). The mean age of the patients was 36.65 ± 23.17 (min 1year - max 96 years old). This service serves different parts of Gezira state in addition to other surrounding states such as Khartoum, Sinnar and Gadarif states. The molecular test focused on detection of nucleic acids DNA specific to *Mycobacterium tuberculosis*, using polymerase chain reaction (PCR); amplification techniques**.**

DNA Extraction

DNA was extracted using DNP™ kit (cinnaGen Inc, Cat. No.DN115C form Iran) which was designed to isolate double stranded DNA form human and animal sources. Sputum samples were treated by Sodium hydroxide, 10 □l of protease K was added to 200 □l of homogenized sputum, and placed at 55°C for 3 hrs,

EDITORIAL

and then transferred to water bath at 95^{0}C for 20 min. , The CSF, ascitic fluid, pleural fluid were centrifuged and the supernatants were discarded, the rest were used for DNA extraction.

The lysis solution was pre warmed by placing in 37°C for 20 min and softly shaken. 200 µl of the sample (Sputum, blood, CSF ascetic fluid) was added to 400 µl of lysis solution and vortex for 15-20 sec. After complete monogenesis to the samples, 300 µl of precipitation solution was added by mixing and vortex for 3-5 sec and placed in -20°C for 20 min then centrifuged at 12,000g for 10 min. The supernatant was decanted gently and placed the tube in tissue paper for 2-3

sec. 1ml of wash buffer was added to the pellet and mixed by 3-5 sec vortexing and centrifuge at 12000 g for 5 min. The wash buffer was poured completely and the pellet was dried at 65°C for 5 min. The pellet suspended on 50 µl of solvent buffer by gentle shaking and placed at 65°C for 5 min. The solvent was precipitated by centrifugation for 30 sec at 12000g. The supernatant contain purified DNA.

Polymerase chain reaction (PCR)

Mycobacterium tuberculosis PCR detection kit (CinnaGen Inc Iran (cat.No. PR7935C)) was used for diagnosis in addition to negative and positive controls were provided by the company. The total volume of the reaction 25µl, 20µl was 1x PCR Mix, 5µl purified DNA and 0.3 µl Taq-DNA polymerase. The mixture was spun for 3-5 sec, then the tubes were transferred to preheated thermocycler machine, the program started as followed, initial denaturation at 94°C for 10 min, the following temperature profile: 94°C for 1 min for denaturation., 72°C for 2 min for annealing, 72°C for 3 min for extension followed by 30 cycles and end with 4°C min. 10 µl of the amplification samples were loaded directly in 2% agrose gel for documentation.

RESULTS

The presence of 163 bp fragments of PCR product comparing with DNA size ladder indicates positive result as in shown figure (1). There were about sixteen different types of samples as shown in figure (2).

The molecular diagnostic test shows that 112 (21.3%) had a positive result by PCR while 419 (78.7 %) had a negative PCR results as shown in figure (3). The mean age of positive samples was 36.21 ± 21.07 (min2-max 95 years old), the sex distribution of the positive samples is shown on figure (4). Most of the samples, (n=87) came from patients more than 60 years old (16.4%), then (n=54) from 36-40 years old (10.2%) as in figure (5). The comparison between the total samples and the positive ones showed higher positive samples are from (n=16) patients between 21-25 years old (14.3 %), of whom ten (62.5%) were females while only six were males (37.5%). The Types of TB in this age group were nine pulmonary tuberculosis, six were abdominal tuberculosis and one bone TB. The types of samples were sputum, ascitic pleural fluid as well as blood.

The analysis of the positive PCR result according to the type of samples showed that blood samples had a high positive result (34.1%) compared with the other samples, while the gastric wash showed very low positive results (3.3%) , pleural fluid sputum and ascitic fluid were in the same range 23.1%, 22.3% and 18.1 % respectively as shown in table 1.

All sputum samples are diagnosed clinically as pulmonary TB. The clinical diagnosis for ascitic fluid, ten were abdominal TB and two were unspecified while only one pulmonary tuberculosis. As PCR the clinical diagnosis for the blood samples, six were diagnosed as bone tuberculosis, while one case diagnosed for each cancer larynx, pulmonary tuberculosi, pericardial TB, lip TB and cryptogenic tuberculosis. Three did not have a clear clinical diagnosis. The clinical diagnoses of the positive pleural fluid were seven as pulmonary TB, one pleural effusion, abdominal TB, pleural TB and chronic renal failure.

EDITORIAL

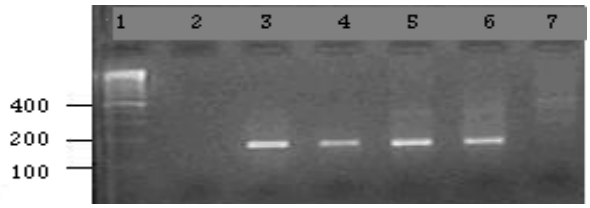

Figure (1) Electrophoresis result for PCR product

Lane 1 ladder, lane 2 negative control, lane 3 positive control, lane 4,5,6 positive samples and lane 7 negative

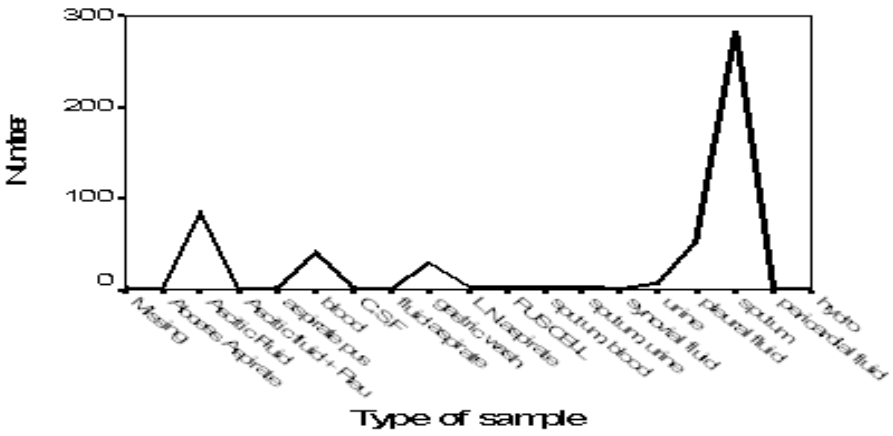

Figure (2): shows the different types of sample for TB diagnosis (N=531)

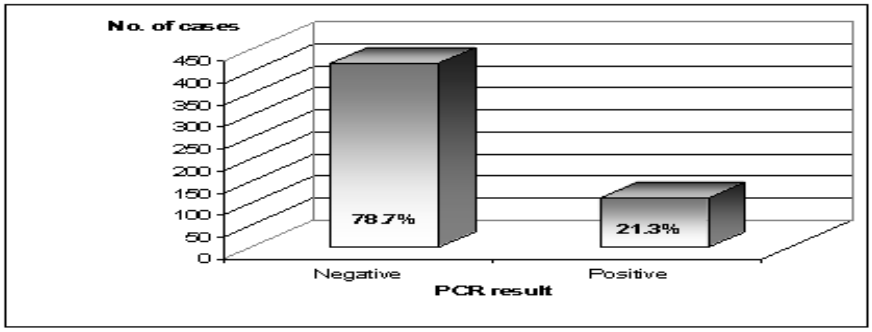

Figure (3) shows the total PCR result of the samples(N=531)

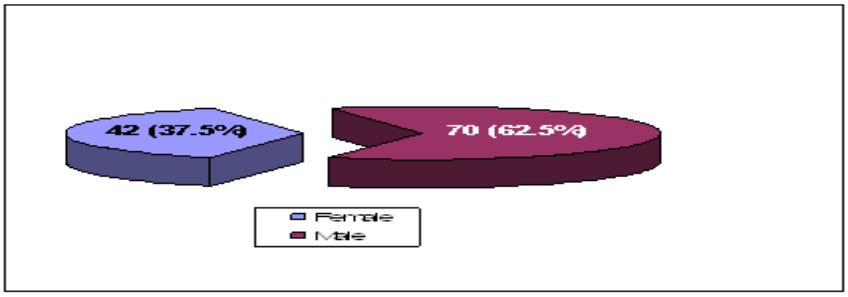

Figure (4) shows Distribution of sex among positive cases (N=112)

EDITORIAL

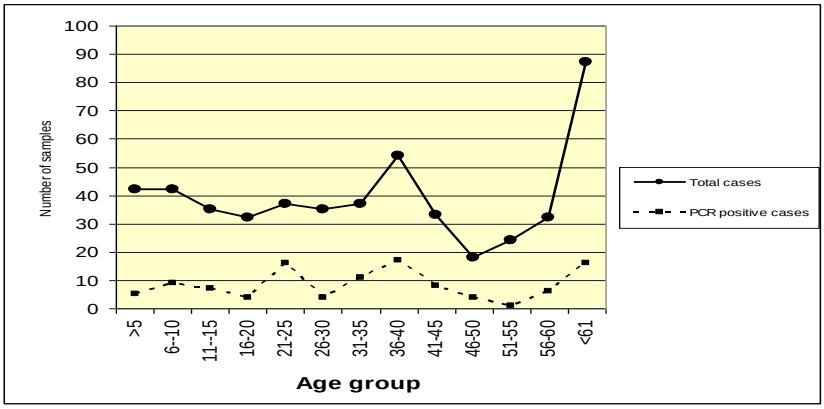

Figure (5) :The Distribution of age group in the all (N=531) and positive cases (N=112)

Table (1): The Distribution of PCR positive result in Different types of samples

Type of sample	Positive (%)	Negative (%)	Total samples (%)
Sputum	64 (22.3)	221(77.7)	285 (53.7)
Acetic fluid	15 (18.1)	69 (81.9)	84 (15.8)
Pleural fluid	12 (23.1)	41 (76.9)	53 (10.0)
Blood	15 (34.1)	26 (65.9)	41 (07.7)
Gastric wash	1 (03.3)	29 (96.7)	30 (05.6)
Others	5 (13.2)	33 (86.8)	38 (07.2)
Total	**112 (21.3)**	**419 (78.7)**	**531(100)**

DISCUSSION

Current global TB control efforts are based on diagnosis of cases followed by adequate treatment. It is important that diagnosis be established early and efficiently in order to prevent misdiagnosis and continued transmission. In environments with high prevalence of TB, better tests and more-efficient diagnostic processes are needed.

Microscopy and culture are still the major backbone for laboratory diagnosis of tuberculosis (TB), but the molecular methods for identifying organisms, strain typing, and determining drug susceptibilities are extremely valuable for pathogens that are impossible to grow on artificial media, slow growing on artificial media, or exceptionally hazardous to grow in a clinical microbiology laboratory (Marks 1993). Conventional methods for the identification of mycobacteria are slow and labour intensive. DNA amplification methods offer rapid sensitive and specific diagnosis (Magana-Arachch *et al*, 2008) test for samples that are difficult to be diagnosed by conventional methods. Our data showed that, the majority of the samples were sputums; however, the blood samples a high positive result. This finding can facilitate the clinical diagnosis of millary TB and bone TB which were found difficult to be diagnosed in other specimens like sputum. In contrast, the samples of gastric wash reported low positive sample. This may be due to the sample handling, collection or dilution.

The routine molecular tests have now been incorporated into laboratory practice allowing the physicians to more rapidly initiate proper drug regimens. In addition, particular emphasis should be applied to quality control and quality assurance programs in the labouratories. Although nucleic acids amplification tests (NAA) have been offered by individual labouratories, approval of commercial kits may result in increased use of NAA in clinical practices in general and for TB control in particular. In our Department, more than ten consultants are referring their samples for molecular diagnosis of TB, this indicates that, there is an

increase of the understanding of the role of the molecular diagnosis techniques. Our results open the way to a simplified diagnostic screening procedure in which the conventional methods can be replaced. However, cost-effectiveness studies and operational studies are required to support an evidence based decision to introduce PCR for TB control in high-burden environments. Also further investigations are needed to assess the PCR kits , different types of samples and negative PCR results the responded to clinical and therapeutic trails

ACKNOWLEDGEMENT

The comments of Professor. Ali Idris Baanaib are gratefully acknowledge

REFERENCES

Cho, S. N. and Brennan, P. J. (2007). "Tuberculosis: diagnostics ".Tuberculosis (Edinb 87) Suppl 1 :S14-7.

Daniel, T. 1990. The rapid diagnosis of tuberculosis: a selective review. J. Lab Clin. Med. 116:277–281.

Glassroth, J. (2005). "Tuberculosis 2004: challenges and opportunities." Trans Am Clin Climatol Assoc 116: 293-310.

Hall, G. S., and Howard. B. J. 1994. Mycobacteria, p. 503–528. In B. J. Howard, J. F. Keiser, T. F. Smith, A. S. Weissfeld, and R. C. Tilton (ed.),Clinical and pathogenic microbiology, 2nd ed. Mosby-Year Book, Inc., St. Louis, Mo.

Kent, P., and G. Kubica. 1985. USDHHS public health mycobacteriology. A guide for the level III laboratory. Centers for Disease Control, Atlanta.

Magana-Arachchi, D., J. Perera, et al. (2008). "Low cost in-house PCR for the routine diagnosis of extra-pulmonary tuberculosis ".Int J Tuberc Lung Dis.80-275 :(3)12

Marks, G. L. (1993). "Genetics of tuberculosis" Med Clin North Am. 77: 1219-34.

Narita, M., Shibata, M., Togashi, T., And Kobayashi, H. 1992. Polymerase chain reaction for detection of Mycobacterium tuberculosis. Acta Paediatr. 81:141–144.

Ongut, G., D. Ogunc, et al. (2006). "Evaluation of the ICT Tuberculosis test for the routine diagnosis of tuberculosis." BMC Infect Dis **6**: 37.

Pai, M., Kalantri, S. and Dheda, K. (2006). "New tools and emerging technologies for the diagnosis of tuberculosis: part I. Latent tuberculosis." Expert Rev Mol Diagn 6 (3): 413-22.

Persing, D. H. Smith, T. F., Tenover, F. C., and White, T. J. (ed.). 1993. Diagnostic molecular microbiology: Principles and applications. American Society for Microbiology, Washington, D.C.

Piper, M., and Unger. M. 1989. Nucleic acid probes: a primer for pathologists, p. 67 69. ASCP Press, Chicago.

Sibata, and T. Togashi. 1992. Nested amplification protocol for the detection of Mycobacterium tuberculosis. Acta Paediatr. 81:997–1001.

Stahl, D., and J. W. Urbance. 1990. The division between fast- and slow growing species corresponds to natural relationships among the mycobacteria. J. Bacteriol. 172:116–124.

Victor, T., R. du Toit, and P. van Helden. 1992. Purification of sputum samples through sucrose improves detection of Mycobacterium tuberculosis by polymerase chain reaction. J. Clin. Microbiol. 30:1514–1517.

Watson, J., M. Gilman, J. Witkowski, and M. Zoller. 1992. Recombinant DNA, p. 79–90. Scientific American Inc., New York.