

Original Research Article

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## Multiplex PCR test for the rapid detection of *Mycobacterium tuberculosis* in Pulmonary tuberculosis patients

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### ABSTRACT

Rapid, accurate and early diagnosis of tuberculosis is a major health concern especially in developing country like Egypt. This study aimed to evaluate Multiplex PCR (MPCR) using both MPB64 and IS6110 gene targets, and to compare it with conventional methods such as microscopy and mycobacteria growth indicator tube (MGIT) culture and also to compare with single target PCR IS6110 or MPB64 for rapid diagnosis of *Mycobacterium tuberculosis* (*M. tuberculosis*) from clinical samples. Sixty samples were processed for Ziehl-Neelsen (ZN smear), MGIT culture and PCR for single target PCR IS 6110 target or MPB 64 gene target and MPCR (IS6110 & MPB 64 gene target). Our study showed that out of 60 samples processed, 32 (53.3%) were positive for AFB, whereas 42(70%) were positive for MGIT culture. PCR targeting IS6110 showed positive results 45 (75%) as compared to PCR assay targeting MPB64 by which 40(66.7%) samples showed positive results while the higher proportion of positive results were detected with MPCR was 49 (81.7% ( samples. when clinical diagnosis was considered as the gold standard. AFB smear examination had a sensitivity of 53.3% and a specificity of 100%. For MGIT culture test, sensitivity was 70% and specificity was 100%. In comparison, single target PCR test was found to have a sensitivity of 75% and 66,7% for PCR 1 (IS 6110) and PCR 2 (MPB64) respectively And specificity was 90% while Multiplex PCR has much higher sensitivity 81.7% and specificity 92.3%. Combined ZN smear & MIGT culture showed 33 (55%) positive smear and culture, 9(15%) smear negative culture positive and 18 (30%) smear negative culture negative samples. Of the 33 samples PCR positivity was found to be 33(100),30(90.9) and 28(84.8) with multiplex PCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) respectively. In the 9 smear negative culture positive samples PCR positivity was observed as 8(88.9) in both PCR 1 (IS 6110) and multiplex PCR while PCR2 (MPB64) positivity 7)77.8%. However the MPCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) positivity was 8(44.4%), 7(38.9%) and 5(27.8%) in smear negative culture negative samples. This study reveals that multiplex TB-PCR, amplifying two targets specific for *M. tuberculosis* has higher specificity and sensitivity and can complement the tests in TB diagnosis and allow an initial therapeutic approach.

### Keywords

Tuberculosis,  
Ziehl-neelsen,  
Multiplex PCR  
and mycobacteria  
growth  
indicator  
tube

### Article Info

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## Introduction

Tuberculosis (TB) remains a public health problem worldwide with a global mortality of 1.2 to 1.5 million in 2010. The situation is further exacerbated with increasing incidence of multi-drug resistant (MDR) and extremely drug resistant (XDR) TB and emergence of HIV (WHO, 2011).

Though early diagnosis is mandatory in control of TB, especially for pulmonary TB as it is transmissible, it has remained enigmatic. Although, the conventional acid fast bacilli (AFB) microscopy has high positive predictive value for detection of causative agent (*M. tuberculosis*), it lacks sensitivity. Lowenstein-Jensen (L-J) culture test though considered as a gold standard is labour intensive and slow. Thus, detection of *M. tuberculosis* in clinical samples containing small numbers of the organism is still a major challenge (Kharibam, *et al.*, 2016).

Polymerase chain reaction (PCR) based assays offer high sensitivity by amplification of small amount of DNA, and have been extensively evaluated for the detection of *M. tuberculosis* from clinical samples (Shinnick and Jonas, 1994). Many of the tests described are based on amplification of IS6110, an insertion element that is believed to be restricted to members of the *M. tuberculosis* complex (Chakravorty, *et al.*, 2005). The presence of multiple copies of this element in the majority of *M. tuberculosis* strains undoubtedly enhances the sensitivity of PCR. However, the discovery of occasional *M. tuberculosis* strains lacking IS6110 (Van Soolingen *et al.*, 1993) implies that relying only on IS6110-based PCR is not advisable. Initially, a PCR test was developed (Kadival, *et al.*, 1993) targeting a house keeping gene of *M. tuberculosis*, for MPB64 gene, involved in

phosphate transport (Harboe and Wiker, 1992).

Single gene target can result in false negative results while more reliable results are obtained by using more than one target gene for amplification. Multiplex PCR (MPCR) in which several target genes are amplified simultaneously is more sensitive and specific in diagnosis of *Mycobacterium tuberculosis* infection (MTB) (Kusum, *et al.*, 2011).

The aim of the present study to evaluate multiplex PCR using both MPB64 and IS6110, and to compare it with conventional methods such as microscopy, and culture and also single target PCR either for IS6110 or MPB64 based PCR in rapid diagnosis of *M. tuberculosis*.

## Materials and Methods

### Studied Subjects

This was a cross-sectional, done in Microbiology and Immunology Departments, Benha University, Egypt from March 2015 to September 2015. Involved clinically suspected 60 cases of pulmonary tuberculosis Sputum samples were collected from subjects aged >18 years presenting to Chest Department and Outpatient Clinic of Benha University Hospital, Benha Chest hospital, Egypt, with fever, weight loss, cough, lymphadenopathy, abnormal physical examination or chest X-ray (CXR) suggestive of TB disease, ESR, positive history of contact/ family history or past history of tuberculosis, Positive Mantoux test, chronic TB (who tested positive after a Category II retreatment regimen), new sputum smear-positive TB (patients who did not convert to negative at 2 months of treatment) or default, relapse or treatment failure (previously treated cases) with a

mean age of 34.7 years ( $\pm 10.4$  standard deviation) and an equal gender distribution. In addition, 36 specimens from non-TB COPD and asthmatic patients were also included in the study as negative controls.

### **Sputum Sample Collection**

Three successive morning sputum samples were collected in sterile containers.

### **Decontamination and Concentration**

sputum was decontaminated with the sodium hydroxide–Nacetyl- L-cysteine (NaOH-NALC) method using BBL MycoPrep™ Specimen Digestion/Decontamination Kits (Becton Dickinson, Sparks, USA). Samples were then centrifuged at  $3000 \times g$  for 15 min.

### **Sputum Smear Microscopy (SSM)**

Two drops of NaOH-NALC decontaminated sample pellet were used for smear microscopy (ZN staining), according to standard protocol. The grading of the AFB results was done in accordance with the WHO/International Union Against Tuberculosis and Lung Disease guidelines and scored as “0” for absence of AFB, scanty 1–9, 1 +, 2 +, or 3 + (World Health Organization, 1998).

### **MGIT Culture (Salman and Sabine, 2005)**

MGIT tube obtained from (Becton Dickinson, Sparks, USA) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains modified Middlebrook 7H9 broth base. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase). Addition of the

MGIT PANTA (Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin)

A lyophilized vial of BBL MGIT PANTA antibiotic mixture was reconstituted with 3 ml of sterile distilled water. The MGIT tube was labeled with specimen number, the cap was unscrewed and aseptically 0.5 ml of MGIT OADC was added, then aseptically 0.1 ml of reconstituted MGIT PANTA antibiotic mixture was added. For best results, the addition of OADC enrichment and PANTA antibiotic mixture was made just prior to specimen inoculation. 0.5 ml of the concentrated specimen suspension was added the tube was tightly recapped and mixed well. Then the tube was incubated at  $37^\circ\text{C}$ . The tube was read daily starting on the second day of incubation using a UV lamp.

Preparation of interpretive negative and positive control tubes: Positive control tube was prepared by Empty broth from an uninoculated MGIT tube. 0.4% sodium sulfite solution was prepared (0.4 g in 100 ml sterile distilled or deionized water) then 5 ml was added to the tube, the cap was replaced, tightened and the tube was allowed to stand for a minimum of 1 h at room temperature before use. Negative control tube: An unopened, uninoculated MGIT tube is used as a control.

Reading the tubes: tubes were removed from the incubator and placed on the UV light next to a positive control tube and negative control. Normal room light is preferred. MGIT tubes that showed bright fluorescence were visually located. Fluorescence was detected as a bright orange color in the bottom of the tube and also an orange reflection on the meniscus. The MGIT tubes were compared to positive control and negative control tubes.

The presence of mycobacteria in the medium was confirmed by the presence of AFB after ZN staining and identification by biochemical tests (niacin accumulation and nitrate reduction).

### **Biochemical Tests**

Niacin accumulation test (Young A *et al.*, 1970)

Niacin TB test strips (Becton Dickinson, Sparks, USA) were used, these strips were dipped downward into the test (3–4 weeks old culture on which sterile distilled water was added) and negative control tubes (containing 0.5 ml sterile DW) and left for 5–10 min. Positive test for niacin was indicated by the appearance of a yellow color in the test culture and no color in the control tube.

### **Nitrate Reduction Test (Virtanen *et al.*, 1960)**

A nitrate test strip (Becton Dickinson, Sparks, USA) was used. This strip was dipped into tube containing 0.5 ml of DW, two spades (at least 50 colonies) from a 4 week old mycobacterial culture, then it was incubated at 37 °C for 2 h. Positive nitrate test was indicated by the appearance of a blue color in the top portion of the strip.

### **DNA Extraction for Molecular Methods**

DNA was extracted from sputum by Thermo Scientific GeneJET Genomic DNA Purification Kit according to manufacturer's instructions and protocols. Eluted DNA was stored at -20°C. To avoid contamination during DNA extraction and amplification, strict precautions were taken, including separate areas for DNA extraction, reagent preparation, amplification and product detection and regular meticulous cleaning of

surface with 10% hypochlorite were also applied to maintain the standard molecular laboratory procedures

### **DNA Amplification of *Mycobacterium tuberculosis***

#### **Amplification of *M. tuberculosis* dna using is6110 primer: (Sigma, bangalore, India)**

First round of PCR was done on all extracted DNA samples. First PCR was carried out using the sequence for IS6110 gene (Tang *et al.*, 2004). Primer 1: 5'-CCT GCG AGC GTA GGC GTC GG-3' Primer 2: 5'-CTC GTC CAG CGC CGC TTC GG-3' Each PCR reaction contained 1x buffer, 200µM dNTPs, 1.5 U Taq Polymerase, 200 ng of each Primers and 5 µl of sample DNA per 50 µl of reaction volume. DNA amplification was performed for 35 cycles following an initial denaturation at 94 °C for 5 min. in a thermal cycler (Biometra, Germany) by using following programme: denaturation at 94 °C for 1.5 min., annealing at 70 °C and extension at 72 °C for 1.5 min with a final extension of 10 min at 72 °C. The amplified product was stored at 4 °C till the detection by electrophoresis.

#### **Amplification of *M. tuberculosis* DNA using MPB 64 primer (Sigma, Bangalore, India)**

Second round of PCR was also done on all extracted samples by using primer for MPB 64 gene (Parekh *et al.*, 2006): Primer 1: 5'-TCC GCT GCC AGT CGT CTT CC-3' Primer 2: 5'-GTC CTC GCG AGT CTA GGC CA-3' Each reaction contained 1X buffer, 250 µM dNTPs, 1.5 U Taq Polymerase 200 ng of each of primer and 5 µl of sample DNA per 50 µl of reaction. DNA amplification was performed for 30 cycle following an initial denaturation step

at 94 °C for 5 min in the thermal cycler (Biometra, Germany) by using the following programme: denaturation at 94 °C for 2 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min with a final extension of 10 min at 72 °C. The amplified product was stored at 4 °C till the detection.

Detection Amplification of bacterial DNA was performed by using multiplex *M.tuberculosis* PCR.

Third round of MPCR was also done on all extracted samples by using primers (Sigma, Bangalore, India) for the IS6110 (IS1: 5'-CCTGCGAGCGTAGGCGT-3' and IS2: 5'-CTCGTCCA GCGCCGCTTCGG-3') and MPB64 (MPB1: 5'- ACC AGGGAGCG GTTCGCCTGG-3' and MPB2: 5'- GATCT GGGGGTCGTCGGAGCT-3') region of the *M. tuberculosis* complex; amplification conditions have been described earlier. (Sharma K. *et al.*, 2006) The amplification protocol in the thermal cycler (Biometra, Germany) involved the following amplifications steps (1 cycle at 94 °C for 15 min; 40 cycles at 94 °C for 30s, 62 °C for 1.30min, 72 °C for 1.30min; 1 cycle at 72 °C for 10 min).

### Detection of PCR Products

Specific bands of 123 bp for IS6110 and 240 bp for MPB64 were visualized using fluorescence of ethidium bromide. The gel was transferred to the UV transilluminator. The (DNA bands) were observed by using ultraviolet light at wave length 312 nm in a dark room as showed in figures 1,2,3. The sample was considered positive for MPCR if a specific band was seen in any or both of the two gene targets chosen. In every MPCR assay, results were compared with one positive (DNA of *M.tuberculosis* H 37 Rv strain) and a negative (PCR-grade water) control. A 100-bp ladder was used as a molecular marker.

### Statistical Analysis

The collected data were tabulated and analyzed using SPSS version 16 (SPSS Inc., Chicago, IL). Qualitative data were presented as frequencies and percentages.

### Results and Discussion

Out of 60 samples processed, 32 (53.3%) were positive for AFB, whereas 42(70%) were positive for MGIT culture. single gene target PCR assay 1 targeting IS6110 showed positive results 45 (75%) as compared to PCR assay targeting MPB64 by which 40(66.7%) samples showed positive results while The higher proportion of positive results were detected with MPCR was 49 (81.7% ( samples. when clinical diagnosis was considered as the gold standard. AFB smear examination had a sensitivity of 53.3% and a specificity of 100%. For MGIT culture test, sensitivity was 70% and specificity was 100%. In comparison, single target PCR test was found to have a sensitivity of 75% and 66,7% for PCR 1 (IS 6110) and PCR 2 (MPB64) respectively And specificity was 90% while MPCR has much higher sensitivity 81.7% and specificity 92.3% (Table 1).

Combined ZN smear & MIGT culture showed 33 (55%) positive smear and culture, 9(15%) smear negative culture positive and 18 (30%) smear negative culture negative samples. Of the 33 samples PCR positivity was found to be 33(100),30(90.9) and 28(84.8) with multiplex PCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) respectively. In the 9 smear negative culture positive samples PCR positivity was observed as 8(88.9) in both PCR 1 (IS 6110) and multiplex PCR while PCR2 (MPB64) positivity 7)77.8%. However the MPCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) positivity was 8(44.4%),

7(38.9%) and 5(27.8%) in smear negative culture negative samples (Table 2).

PCR with its rapidity and higher sensitivity facilitates the early and accurate identification of the causative organism of tuberculosis (i.e. *M. tuberculosis*) and thus very helpful for treatment, prevention and control of this chronic infectious disease. PCR as molecular diagnostic had been used for many years for the detection of Mycobacterial DNA. However, PCR of mycobacterium DNA with one type of primer sometime may cause ambiguity in the detection of MTB-DNA and therefore, should be checked with other primers specific for *Mycobacterium tuberculosis* (Asthana and Madan, 2015).

Out of 60 samples processed, 32 (53.3%) were positive for AFB, whereas 42(70%) were positive for MGIT culture. single gene target PCR assay 1 targeting IS6110 showed positive results 45 (75%) as compared to PCR assay targeting MPB64 by which 40(66.7%) samples showed positive results while The higher proportion of positive results were detected with MPCR was 49 (81.7%) (Gopinath and Singh, 2009) also reported that the multiplex PCR test showed the highest (77.24%) detection rate, while ZN smear examination had the lowest (20%) detection rate, which was bettered by L-J culture (34.4%) and BACTECTM MGIT-960 culture (50.3%) methods (Asthana and Madan, 2015). Also reported that positive results were detected in 70 (70%) of samples PCR assay targeting M IS611, 48% by PCR assay targeting MPB64 and 77% by MPCR. our results also was in agreement with Kidane *et al.*, 2002 who reported PCR positivity results on such samples 83% using IS6110 systems.

In the present study AFB smear examination had a sensitivity of 53.3% and a specificity

of 100%. For MGIT culture test, sensitivity was 70% per cent and specificity was 100%. In comparison, PCR test was found to have a much higher sensitivity of 81.7% and 75% for Multiplex PCR and PCR 1 (IS 6110) respectively. And specificity was 97.3 % for Multiplex PCR and 90% for PCR 1 (IS 6110), while PCR 2 (MPB64) has sensitivity 66.7% and specificity 90%. *M. tuberculosis* detection by PCR was found to be significantly more sensitive and rapid compared to culture and microscopy with sensitivities ranging between 36% and 100% and specificities ranging between 85% and 100% using various PCR targets such as IS6110, MPB64, TRC4, GCRS, hup-B, Pab gene etc.) Bhanu, *et al.*, 2005, Chakravorty, *et al.*, 2005, Sankar S. *et al.*, 2011, Katoch V. *et al.*, 2004 and Jain, 2011). (Deshpande S. *et al.*, 2007) stated that the IS6110 PCR assay was useful in terms of sensitivity (82.4%) and specificity (75.9%) as compared to culture results showing 68.6% sensitivity and 79.3% specificity Therefore, it has been found that IS6110 PCR is a good supportive method for rapid diagnosis of clinically diagnosed TBM. also Most reports of studies using IS6110-based detection have claimed sensitivities of over 75% and specificities approaching 100%. Boondireke *et al.*, 2007 stated the sensitivity (92.1%) and specificity (98.2%) of the multiplex PCR assay developed in their study for detecting *M. tuberculosis* have proved that the assay is reliable enough for diagnostic application.

In our study combined ZN smear & MIGT culture showed 33 (55%) positive smear and culture, 9 (15%) smear negative culture positive and 18 (30%) smear negative culture negative samples. Of the 33 samples PCR positivity was found to be 33(100), 30(90.9) and 28(84.8) with multiplex PCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) respectively. Asthana A and Madan, 2015

also reported that smear and culture positive group showed a sensitivity of 84.3% and 93.7% for single target and multiplex PCR, respectively. Similar results also obtained by (Srivastava R. *et al.*, 2006) who reported the sensitivity of 85 to 100 %. Though patients with positive smears are responsible for the

maximum transmission of TB (Behr *et al.*, 1999) demonstrated that smear-negative TB cases contribute much more to ongoing transmission than was previously believed. Thus early diagnosis of smear negative TB patients may prove to be a more pragmatic approach to control TB.

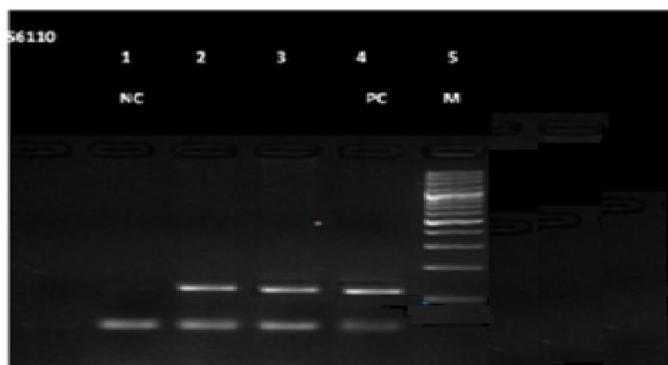
**Table.1** Sensitivity and Specificity Observed with Smear Examination, Migt Culture and Multiplex PCR test.

Method	Sputum samples from PTB patients N=60				
	Positive results	Sensitivity	Specificity	PPV	NPV
<b>AFB smear Microscopy</b>	32(53.3%)	53.3%	100%	100%	56.2%
<b>MIGT Culture</b>	42(70%)	70%	100%	100%	66.7%
<b>Multiplex PCR</b>	49(81.7%)	81.7%	92.3%	94.2%	76.6%
<b>PCR 1 (IS 6110) only</b>	45(75%)	75%	90%	91.8%	70.6%
<b>PCR 2 (MPB 64) only</b>	40(66.7%)	66.7%	90%	88.2%	54.5%

**Table.2** Comparison between Combined Culture & Smear Results and PCR results

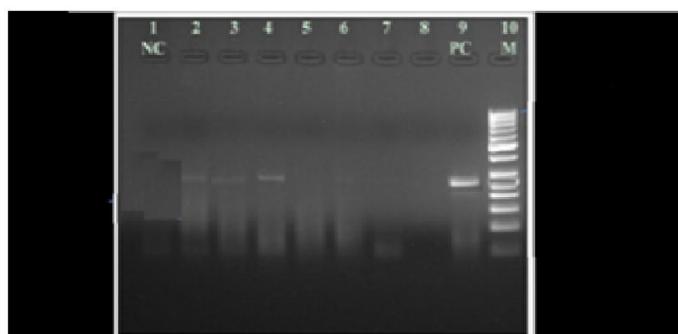
Culture & smear results	PCR 1 (IS 6110) only	PCR 2 (MPB 64) only	Multiplex PCR
<b>Smear positive Culture positive N=33</b>	30(90.9)	28(84.8)	33(100)
<b>Smear negative Culture positive N=9</b>	8(88.9)	7(77.8%)	8(88.9)
<b>Smear negative Culture negative N=18</b>	7(38.9%)	5(27.8%)	8(44.4%)
<b>Control N=36</b>	4(11.1%)	4(11.1%)	3(8.3%)

**Fig.1** IS6110 Gene Targeted In-house PCR for Detection of *Mycobacterium tuberculosis*



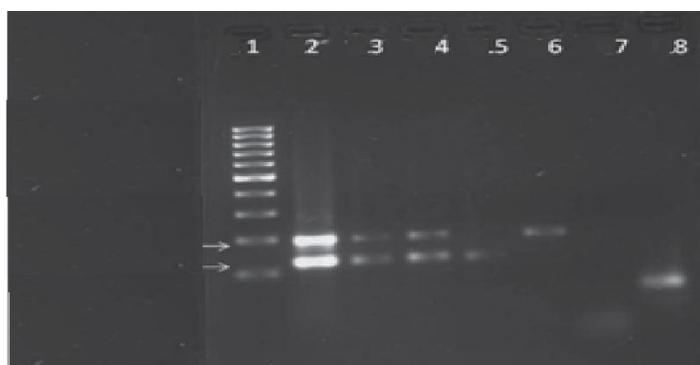
Lanes 1-5. Lanes-1 represents the negative control (nuclease free water), Lane-2 and 3 represent clinical pulmonary samples, Lanes-4 was positive control (*Mycobacterium tuberculosis* culture H37Rv). Lane-5 was 100 bp marker. The presence of a 123 bp amplicon in the lanes 2-4 indicated the presence of the target.

**Fig.2** MPB64 Gene Targeted PCR for Detection of *Mycobacterium tuberculosis*



Lanes 1-10. Lanes-1 represents the negative control (nuclease free water), lane-2 to 8 represents clinical pulmonary samples, Lanes-9 was positive control H37Rv (*Mycobacterium tuberculosis* culture). Lane-10 was 50bp marker. The presence of a 240bp. amplicon in the lanes 2 to 4, 9 indicated the presence of the target.

**Fig.3** A Multiplex PCR Test for the Detection of *Mycobacterium tuberculosis*



LI: 100-bp molecular marker, L2: Positive control, L3,4: Patient sample DNA positive for both the bands, L5: Clinical sample positive for IS6110, L6: Clinical sample positive for MPB64 band, L7: Negative control, L8: Negative patient sample. The presence of a 123 bp a 240bp amplicon in the lanes 2-4 indicated the presence of the target.

In the present study of the 9 smear negative culture positive samples PCR positivity was observed as 8(88.9) in both PCR 1 (IS 6110) and multiplex PCR while PCR2 (MPB64) positivity 7) 77.8%. PCR positivity was observed as 8(88.9) in both PCR 1 (IS 6110) and multiplex PCR while PCR2 (MPB64) positivity 7) 77.8% (in smear negative culture positive. This result was comparable with Negi S *et al.*, 2006 who reported sensitivity of 73.6% with multiplex PCR in smear negative culture positive which is higher than the majority of the studies reporting it at around 60 % or even less by Sarmiento O. *et al.*, 2003. However, the present study shows that the protocol for DNA extraction and PCR used in our laboratory was highly satisfactory and nonspecific inhibition was seen only in 1 out of 9 mycobacteriologically confirmed specimens.

In the present study however the MPCR, PCR 1 (IS 6110) and PCR 2 (MPB 64) positivity was 8(44.4%), 7(38.9%) and 5(27.8%) in the 18 smear negative culture negative sample which probably could be tuberculosis in patients on anti-tuberculosis treatment shedding non-cultivable bacteria. eight of these 18, were positive by multiplex PCR. These 8 smear negative culture negative samples were stained with fluorescein diacetate- ethidium bromide and showed green stained bacilli method indicating presence of live bacilli which were not detected by MGIT culture test (Dezemon Z. *et al.*, 2003.). PCR-positive results without bacteriological confirmation of the specimen are often doubted as false positive, but these apprehensions are not often true. In some cases, this may be the only evidence of infection. Nevertheless, care should be taken while interpreting the PCR results if considered as sole evidence. The criteria of diagnosis of tuberculosis in smear and culture negative pulmonary

tuberculosis suspect's may constitute and the epidemiological, clinical, radiological, tuberculin testing, and response to treatment, along with *M. tuberculosis* PCR. (Katoch *et al.*, 2004)

In conclusion, this study reveals that multiplex TB-PCR, amplifying two targets specific for *M. tuberculosis* has higher specificity and sensitivity and can complement the tests in TB diagnosis and allow an initial therapeutic approach. and also significant multiplex TB-PCR has utility in the early diagnosis (by decreasing time duration) of pulmonary smear negatives tuberculosis which is not diagnosed by conventional test or low detection rate by ZN smear and mycoacterial culture, more work is needed to evaluate the using of multiplex PCR for diagnosing tuberculosis in pulmonary cases in Egypt.

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