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MOLECULAR DETECTION OF INSERTION SEQUENCE 6110 OF *MYCOBACTERIUM TUBERCULOSIS* IN PATIENTS WITH PULMONARY TUBERCULOSIS AND TUBERCULOUS PLEURITIS IN ANBAR GOVERNORATE, WEST OF IRAQ

***DR. MUSHTAK T.S. AL-OUQAILI**

Professor, Vice-Chancellor for Scientific Affairs, Department of Microbiology, College of Medicine, University of Anbar, Iraq.

ABSTRACT

Accurate identification and early diagnosis of tuberculosis especially latent and active infection is the key to prevention of the disease. This study was conducted to detect the accuracy of conventional and real time polymerase chain reaction in the diagnosis of pulmonary tuberculosis caused by *Mycobacterium tuberculosis* in sputum and blood samples using 123 bp gene of repetitive insertion sequence 6110 (IS 6110) of bacterial genome and tuberculous pleuritis using pleural effusion**.** Sixty five patients who have clinical suspicion of pulmonary tuberculosis, fifty patients with suspicion of TB pleurisy and twenty patients with non-tuberculous mycobacterial pulmonary disease as control were studied during the period from April, 2012 to December, 2015. They were admitted to Department of Internal Medicine in Ramadi Teaching Hospital and Clinical Private. EDTA-Peripheral blood and sputum samples had been taken from patient with pulmonary tuberculosis. Also, pleural effusion was obtained from patients with tuberculous pleuritis. They were subjected to DNA extraction, amplification of the target DNA by conventional PCR and qualitative real time PCR. Of the 65 sputum samples from patients with pulmonary tuberculosis, 37 (56.9%) were AFB smear-positive. Of these, 32 (86.5%) and ?? (94.6%) were positive for PCR and RT-PCR respectively. Further, out of 28 negative smear, 7 (25%) and 8 (28.6%) smears were PCR and RT-PCR positive respectively. Further, in peripheral blood based PCR study, out of 37 patients whose sputum were positive by AFB smear, only 25 (67.6%) of them were positive for PCR. Peripheral blood based PCR and RT-PCR essays were negative for all AFB smear negative cases. In suspected tuberculous pleuritis, 8 (16%), 9 (18%) patients reveal positive result for both of conventional and real time PCR respectively. Also, all twenty spontaneous sputum samples (controls)were negative for PCR. The study concluded that PCR provides a sensitive and specific means for laboratory diagnosis of pulmonary tuberculosis and qualitative real time PCR is a more confirmatory test but the molecular diagnosis of the tuberculosis should be based on the combined analysis of baciloscopia, clinical manifestations and therapeutic proves. Further, it was concluded that PCR and RT-PCR based- peripheral blood leukocytes is of little value for specific diagnosis of pulmonary tuberculosis. Regarding tuberculous pleuritis, PCR test revealed low sensitivity and high specificity using pleural fluid. The sensitivity was higher in cases in which the bacillary load was high-in acid fast bacilli-positive samples.

KEYWORDS: ***PCR, RT-PCR, Pulmonary tuberculosis, TB pleurisy***

DR. MUSHTAK T.S. AL-OUQAILI *

Professor, Vice-Chancellor for Scientific Affairs, Department of Microbiology, College of Medicine, University of Anbar, Iraq.

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INTRODUCTION

The investigation on a rapid, accurate, yet inexpensive test for the diagnosis of tuberculosis (TB), begun almost a century ago has become the equivalent to the search for the Holy Grail[1].Tuberculosis remains a major global health problem which causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus. The latest estimates included in World Health organization (WHO) [2]reveal that there were 8.6 million new TB cases in 2012 and 1.3 million TB deaths. The number of TB deaths is unacceptably large given that most are preventable if people can access health care for a diagnosis and the right treatment is provided[2]. Because of the slow growth rate of the causative agent *Mycobacterium tuberculosis*, isolation, identification, and drug susceptibility testing of this organism can take several weeks orlonger[3]. Despite the enormous global burden of TB and the overall low rates of case detection, conventional approaches to diagnosis continue to rely on tests that have major drawbacks. For example, sputum smear microscopy is insensitive; culture is technically complex and slow; determination of drug susceptibility is even more technically complex and slower yet; chest radiography is nonspecific; and tuberculin skin testing is imprecise, and the results are often nonspecific. In view of these limitations, there is a need for less complicated and more accurate tests[4]. Chain reaction of polymerase (PCR) and its variations have been outstanding as promising molecular techniques for the fast diagnosis of tuberculosis[5]. It is able to define the etiologic agent of this disease efficiently[6]. Schluger and colleagues[7] reported that use of PCR techniques with phenol chloroform-purified DNA from a buffy coat layer of cells makes the diagnosis of pulmonary tuberculosis easier. In the examination on blood cells using a primer pair which was specific for repetitive insertion element IS6110, the researchers concluded that all patients with active pulmonary infections were positive for PCR positive and all controls[7]. In contrast, Kolk and colleagues[8] have pointed out that PCR assay of peripheral blood lymphocytes may only be useful for diagnosis of tuberculosis in immune-compromised patients. The diagnosis of patients at an earlier stage while still smear negative, would be advantageous because they are less contagious[9]and have lower morbidity and mortality[1].In the light of this, the application of molecular biology technique is recommended and it allows overcoming the difficulties in the diagnosis of *Mycobacterium tuberculosis*. The molecular diagnosis of the tuberculosis should be based in the combined analysis of several parameters, as the baciloscopia, culture, clinical manifestations, therapeutic proves and previous history of tuberculosis[5].Nucleic acid amplification (NAA) methods allow for detection of mycobacterial DNA directly from the specimens before the culture results are available[3].The objective of this study is to evaluate the efficiency of molecular tool (PCR with primers specific for IS6110 of the *Mycobacterium tuberculosis* complex) and advanced qualitative real time polymerase chain reaction in the detection of this bacterium in patients with pulmonary tuberculosis using sputum and lymphomonocytic blood cells samples and tuberculous pleuritis using pleural fluid.

Patients and methods

Sixty five patients who have clinical suspicion of pulmonary tuberculosis, fifty patients with suspicion of tuberculous pleuritis were admitted to Department of Internal Medicine and Thoracic Surgery in Ramadi Teaching Hospital and Clinical Private and studied during the period from April, 2012 to December, 2015. They were well diagnosed by experienced clinicians. As a negative control for the PCR analysis, 20 other spontaneous sputum samples from patients with viral respiratory infections were incorporated. These samples were collected from patients with viral respiratory infection. EDTA-Peripheral blood and sputum samples were taken from patient with pulmonary tuberculosis and control subjects. Also, pleural effusion was obtained from patients with tuberculous pleuritis.

Ziehl-Neelsen stain

All respiratory specimens (three consecutive early morning sputum samples) were digested and decontaminated with a solution of 2% sodium hydroxide (NaOH) and N-acetyl-L-cysteine (NALC), then centrifuged for 15 min at 3000rpm. The supernatant was removed and the remaining sediment was mixed in a 1:10 dilution with sterile water. The processed sample was stained with Ziehl–Neelsen acid-fast stain and examined under microscope carefully[10, 11, 12].

Molecular part of study

The target gene

The study target DNA is a 123-base pair (bp) segment of repetitive insertion sequence (IS6110), which is repeated in the *M. tuberculosis*

chromosome and is specific for the *M. tuberculosis* complex using specific primers and probes by conventional and real time PCR.

Conventional polymerase chain reaction

DNA extraction

The genomic DNA was extracted according to the following procedure published by manufacturing kit. Briefly, the required quantity of 1.5 ml polypropylene tubes was prepared and included one tube for negative control of extraction.100µl of internal control and 300 µl of lysis solution was added in a certain tube, followed by 10 µl of samples add to the tube[13].The controls were prepared as follow: - 100 µl of negative control were added to the tube labeled C-neg. The tubes were vortexed and incubated for 5 min at 65°C followed by centrifugation for 7-10 seconds at 12000=16000g). The sorbent was vortexed vigorously and 20 µl was added to each tube. After that, Vortexing for 5-7 seconds was done and incubated all tubes for 3 min at room temperature. This step was repeated twicely. All tubes were centrifuged for 30 sec at 5000g and by using a micropipette with a plugged aerosol barrier tip, carefully the supernatant was removed and discard from each tube without disturbing the pellet. Also, 300µl of washing solution was added to each tube. They vortexed vigorously and centrifuged for 30 seconds at 8000g and the supernatant was removed and discarded form each tube. 500µl of washing solution was added to each tube and vortex vigorously and centrifuged for 30 seconds at 8000g followed by removing and discarding supernatant from each tube. All tubes were incubated with open cap for 5 min at 65°C. The pellet was re-suspended in 50 µl of DNA-eluent and incubated for 5 min at 65°C and vortex periodically. The tubes were centrifuged for 1 min at 12000g.The supernatant contains DNA ready for the amplification[13].

Amplification of the target gene

The required quantity of tubes PCR-mix-1 was mixed. For each sample in the new sterile tube 10*(N+1) µl of 2,5 x PCR-buffer-blue and 0,5*(N+1) µl of hot start polymerase was added. Also, 10 µl of reaction mix into each sample tube was added in addition to one drop (15 µl = of mineral oil). Ten µl of DNA sample obtained after sample preparation were eluted to appropriate tube and 10 µl of DNA-buffer was added to the tube for negative control of amplification. Further, 10 µl of DNA positive control was added to the tube for positive control of amplification.PCR-mix-1 tubes were closed and the contents were transferred into the thermocycler only when temperature reached 95°C and the following program was started as follows:- Initial denaturation, 15 min at 95°C. Then the thermocycling profile was also included 20 sec. at 95°C, 20 sec. at 70°C, 20 sec at 72°C for 42 cycles followed by 2 minutes at 72 °C and finally storage at 10 minutes as hold temperature. The results were analysed was based on the presence or absence of specific bands of amplified DNA in agarose gel (2%). The length of specific amplified DNA fragment in *Mycobacterium tuberculosis* was 390 bp and 750 bp in an internal control.

Real time polymerase chain reaction for detection of Mycobacterium tuberculosis complex

Principle

Mycobacterium tuberculosis DNA is extracted from sample amplified using real time amplification and detected using fluorescent reporter dye probes specific for *M. tuberculosis* and *M. tuberculosis* internal control (IC). *M. tuberculosis* IC is DNA fragment of IS 6110 insertion of this bacterium modified and cloned in bacteriophage λ, containing DNA fragment used in the kit as matrix for primers. Internal control (IC) serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. IC is detected in a channel other than the *M. tuberculosis* DNA[13]. MTB Real-TM kit, Sacace Biotechnologies, Italy was used in this study for amplification of the target gene, IS6110. This kit contains UDG-Enzyme which is added to the reaction mix. Since deoxyuridine triphosphate (dUTP) is only present in amplicons while deoxyuridine triphosphate (dTTP) is present in MTB DNA the use of UDG enzyme degrades only amplicons generated from previous runs avoiding possibility of amplicon contamination. UDG is active at room temperature during master mix preparation, while during amplification is inactive, not affecting the correct and wanted experiments amplicon[13].

DNA isolation

DNA/RNA Prep (Sacace, REF K-2-9) kit was used for DNA isolation at which it is fully optimized for real time amplification by Smart cycler instrument used in this study. DNA extraction was carried out according to the manufacturer's instruction (Sacace Biotechnologies, Italy). Briefly, 10 µl of MTB IC (internal control) was added to each tube followed by 300 µl of Lysis solution. 100 µl of samples add to the appropriate tubes using pipette tips with aerosol barriers. Controls was prepared as follows:- 100 µl of negative control was placed on the tube

labeled negative control. After that, the tubes vortexed and incubated for 5 min at 65ºC then centrifuged for 7-10 seconds was done. 400 µl of prec sol added and mixed by vortex. Centrifugation of all tubes at 13.000 r/min for 5 min was done using a micropipette with a plugged aerosol barrier tip. Supernatant was carefully removed and discarded from each tube without disturbing the pellet. 500 µl of wash solution was placed into each tube and vortexed vigorously to ensure pellet washing, and all tubs were centrifuged at 13000 r/min for 60 seconds using a micropipette with a plugged aerosol barrier tip, carefully supernatant was removed and discarded from each tube without disturbing the pellet. 200 µl of wash solution 4 placed into each tube and vortexed vigorously to ensure pellet washing. Then, centrifugation for all tubes at 13000 round/min for 60 seconds was done and incubated all tubes with open caps at 65ºC for 5 min. After that, the pellet in 50 µl of RE-buffer was re-suspended and incubated for 5 min at 65ºC and vortexed periodically. The tubes were centrifuged at 13000g for 60 seconds was achieved. The supernatant that contains RNA/DNA is now ready for amplification[13, 14].

Protocol for amplification program

Briefly, in the new sterile tube for each sample 10*(N+1) µl of PCR-mix-1, 5*(N+1) µl of PCR Buffer flu, 0.5*(N+1) µl of Taq F DNA polymerase and 0.5*(N+1) µl of UDG-Enzyme was prepared. They were vortexed and centrifuged briefly.15 µl of Reaction Mix was added to each tube. Then, 10 µl of extraction DNA was added to appropriate tubes. Controls panel were prepared as follows: 10 µl of DNA-buffer was placed on the tube labeled amplification negative control. Also, 10 µl of C+MTB &IC was added to the tube labeled amplification positive control and the tubes were inserted in the thermocycler.

Amplification program

1-Temperature profile was created on Smart Cycler instrument as represented in the following table

Table 1

The amplification program for the target gene including temperature profile and cycle repeats.

Stage	Temp. (ºC)	Time	Fluorescence detection	Cycle repeats
Hold	95	15 min	-	1
Cycling	95	15 s	-	5
	65	30 s	-	
	72	15 s	-	
Cycling 2	95	15 s	-	40
	65	30 s	FAM(Green) CY3 (Yellow)	
	72	15 s	-	

Fluorescence was detected at the step of cycling 2 stages (60ºC) in FAM/Green and CY3/Yellow fluorescence channels. For data analysis, the fluorescent signal intensity is detected in two channels:- *Mycobacterium tuberculosis* is detected on the FAM (Green) channel, IC DNA on the CY3(Yellow) channel. For analysis of result for control samples, result of the analysis is considered reliable only if the results obtained for positive and negative controls of amplification as well as negative control of extraction are correct as represented in the following tables

Table 2

The suspected results for controls and their interpretation represented in CY3 channels.

Control	Stage for control	Ct channel FAM (Green)	Ct channel Cy3	Interpretation
NCE	DNA extraction	Neg	<36	Valid result
NCE	Amplification	Neg	Neg	Valid result
C+	Amplification	Neg	<34	Valid result

Ct: Threshold of cycle; NEC: Negative control; C+: Positive control

Table 3
The interpretation of result for the test samples represented in FAM channels.

Ct Value FAM (Green)	Ct Value Cy3	Validity	Interpretation
≤38	≤38/<38	Valid	*M. tuberculosis* complex is detected
-	≤38	Valid	*M. tuberculosis* complex is not detected
- / > 38	- / > 38	Invalid	Invalid (repeat material sampling)
< 38	≤38	Invalid	Equivocal (repeat material sampling)

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). Data were analyzed using the Student *t* test for continuous variables and Fisher's exact test and Pearson's $\chi 2$ test for categorical variables. Significance was set at $p < 0.05$ using two-sided comparisons. In addition to Microsoft Excel, the above statistical analysis were used to detect sensitivity, specificity, positive and negative predictive values for both of PCR and RT-PCR in addition to percentages and necessary curves including histograms to reveal distribution of results among study techniques. The values for FAM and CY3 threshold cycles (Ct) for amplified selected samples were detected using software analysis of Smart cycler, Cepheid.

RESULT

Of 65 sputum samples tested from patients in whom pulmonary tuberculosis was diagnosed, 37 (56.9%) were smear-positive for acid-fast bacilli (Ziehl-Neelsen) while 28 (43.1%) were smear negative as represented in the following figure

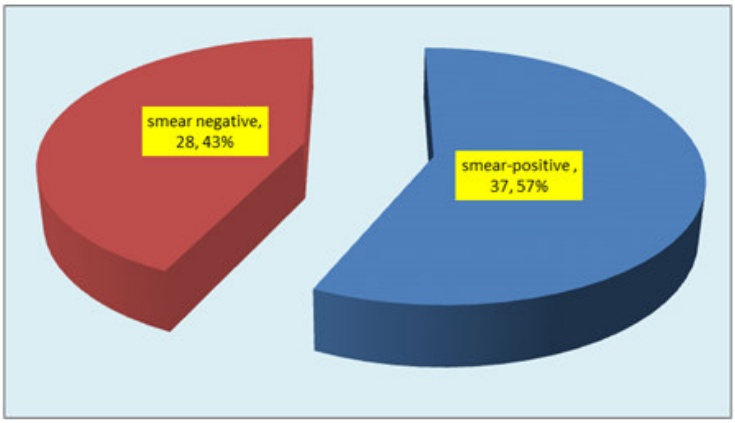


Figure 1
Distribution of smear positive and negative Ziehl-Neelsen stain among study sputum samples.

Out of smear positive cases, 32 (86.5%) and 35 (94.6%) were positive for PCR and RT-PCR respectively while 5 (13.5%) and 2 (5.4%) of them were PCR and RT-PCR negative respectively. While out of 28 smear negative for Ziehl-Neelsen, 7 (25%) and 8 (28.6%) of them were PCR and RT-PCR positive respectively while 21 (75%) and 20 (71.4%) were negative for both techniques respectively as represented in the following tables (4 and 5) in addition to figure 2.

Table 4
The result of PCR and RT-PCR in acid fast bacilli smears positive and negative specimens among 65 patients with suspected pulmonary tuberculosis using sputum sample.

AFB Smear positive 37 (56.9%)		AFB Smear negative 28 (43.1%)		Sensitivity	Specificity
Positive PCR	Negative PCR	Positive PCR	Negative PCR	82%	80%
32 (86.5%)	5 (13.5%)	7 (25%)	21 (75%)		
Positive RT-PCR	Negative RT-PCR	Positive RT-PCR	Negative RT-PCR	81%	90%
35 (94.6%)	2 (5.4%)	8 (28.6)	20 (71.4%)		

Table 5
The sensitivity, specificity and predictive value for both of two study tests (PCR and RT-PCR).

Method	Sensitivity (%)	Specificity (%)	Predictive value	
			Positive (+ve)	Negative (-ve)
PCR	82%	80%	42.6%	22%
RT-PCR	81%	90%	89.5%	20%

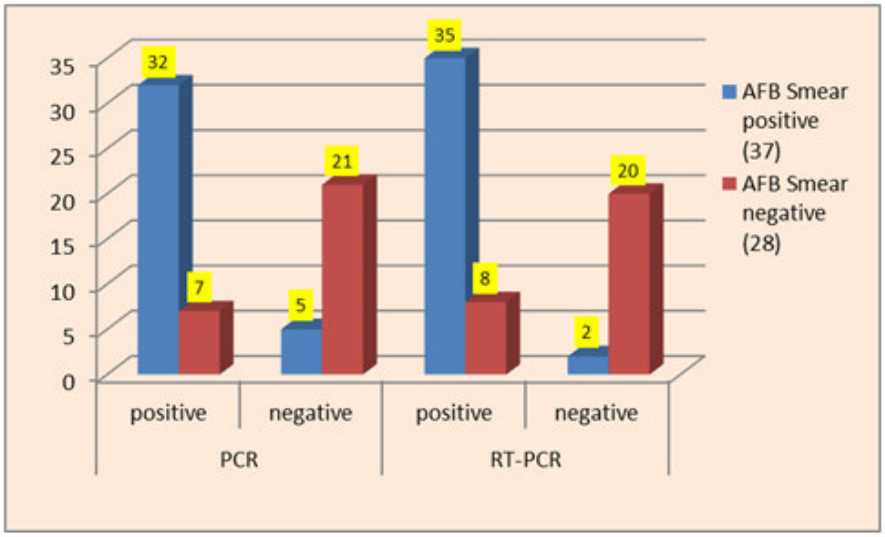


Figure 2
Distribution of results of PCR and RT-PCR in smear positive and negative Ziehl Neelsen stain among study patients with pulmonary tuberculosis using sputum sample.

Further, in peripheral blood based PCR study, Out of 37 patients whose sputum were positive for Ziehl-Neelsen stain and PCR, only 25 (67.6%) of them were positive for PCR while 12 (32.4%) were negative as reflected in table 6. Regarding real time PCR assay, out of 37 patients who sputum were positive for Ziehl-Neelsen stain and PCR, 28 (75.7%) of them were positive for PCR while 9 (24.3%) were negative. On the other hand, peripheral blood based PCR and RT-PCR essays were negative for all AFB smear negative cases as represented in table 6 and figure 3.

Table 6
The result of peripheral blood based PCR and RT-PCR in acid fast bacilli smears positive and negative specimens among patients with pulmonary tuberculosis.

AFB Smear positive 37 (56.9%)		AFB Smear negative 28 (43.1%)		Sensitivity	Specificity
Positive PCR	Negative PCR	Positive PCR	Negative PCR	100%	70%
25 (67.6%)	12 (32.4%)	0.0 (0.0%)	28 (100%)		
Positive RT-PCR	Negative RT-PCR	Positive RT-PCR	Negative RT-PCR	100%	75%
28 (75.7%)	9 (24.3%)	0 (0.0%)	28 (100%)		

Regarding suspected TB pleurisy, 8 (16%) and 9 (18%) patients reveal positive result for tuberculosis in pleural effusion by both of conventional and real time PCR respectively while 42 (84%) and 41(82%) were negative for both techniques respectively.

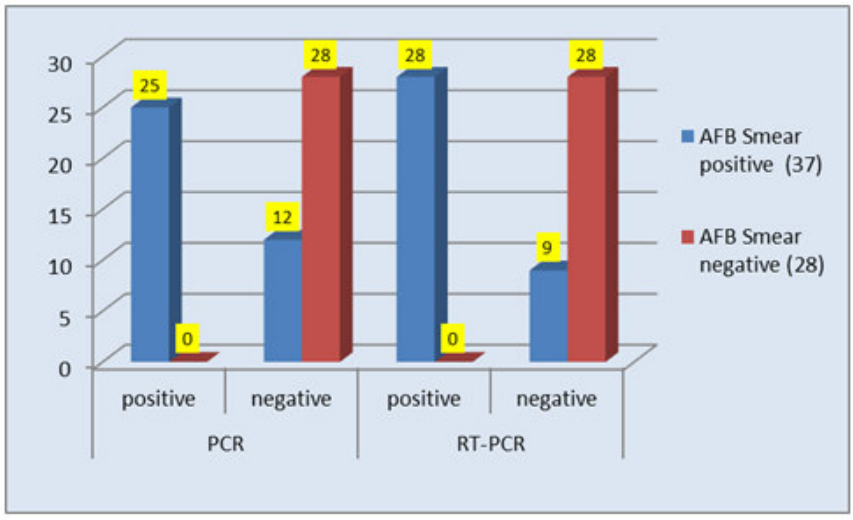
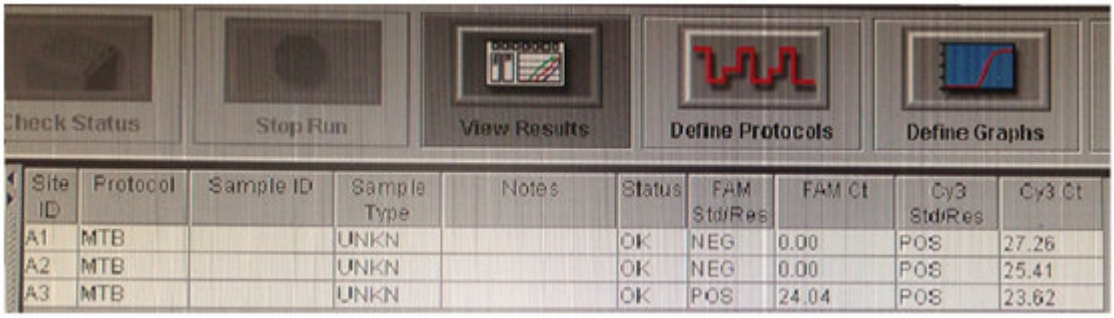


Figure 3

Distribution of results of PCR and RT-PCR in smear positive and negative Ziehl Neelsen stain among study patients with pulmonary tuberculosis using peripheral blood.

On the other hand, regarding the twenty specimens from patients with non-tuberculous mycobacterial pulmonary disease, all were PCR negative.



Site ID	Protocol	Sample ID	Sample Type	Notes	Status	FAM Std/Res	FAM Ct	Cy3 Std/Res	Cy3 Ct
A1	MTB		UNKN		OK	NEG	0.00	POS	27.26
A2	MTB		UNKN		OK	NEG	0.00	POS	25.41
A3	MTB		UNKN		OK	POS	24.04	POS	23.62

Samples A1 and A2 appeared to be negative for Mycobacterium tuberculosis at which the result in the column std/Res FAM was indicated as negative (value of FAM Ct=zero)and in the column Std/Res CY3 was positive (CY3 Ct=27.26 and 25.41 for A1 and A2 respectively). Sample A3 was positive for Mycobacterium tuberculosis at which the result in the column std/Res FAM was indicated as positive (value of FAM Ct=24.04) and in the column Std/Res CY3 was also positive CY3 Ct=23.62).

Figure 4

The values for FAM and CY3 threshold cycles (Ct) for amplified selected three sputum samples using software analysis of Smart cycler, Cepheid.

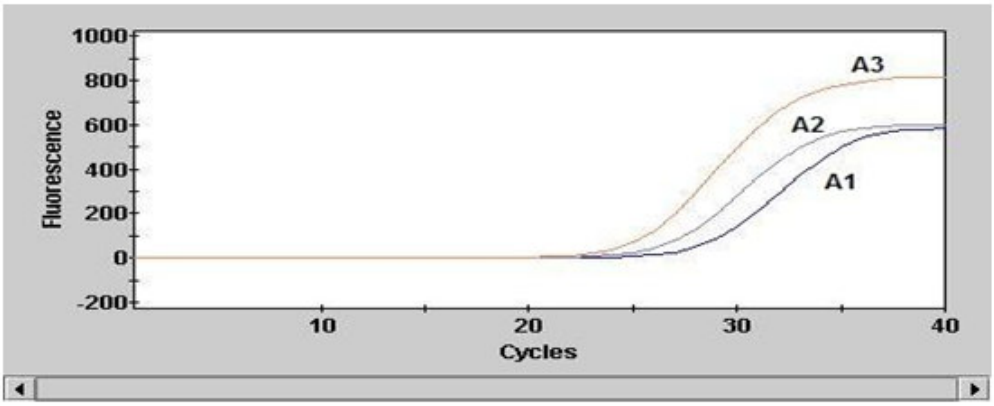


Figure 5

The amplification curve for internal controls detected in CY3 channel for three selected sputum samples described above at which all were positive (CY3 Ct values were 27.26, 25.41 and 23.62 for A1, A2 and A3 respectively).

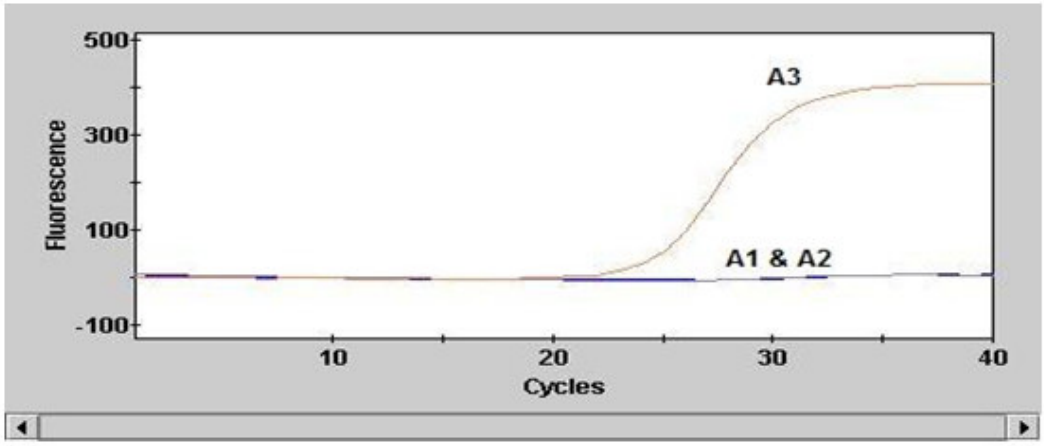


Figure 6

The amplification curve for DNA samples detected in FAM channel obtained from three selected sputum samples. A3 sample was positive for Mycobacterium tuberculosis (FAM Ct= 24.04) while A1 and A2 were negative for this bacterium at which FAM Ct for both of them equal zero.

DISCUSSION

It is well realized that World Health organization reported that the early, rapid and accurate detection of TB and drug resistance relies on a well-managed and equipped laboratory network. The laboratory confirmation of TB and drug resistance is critical to ensure that people with TB signs and symptoms are correctly diagnosed and have access to the correct treatment as soon as possible[3]. .Further, the early diagnosis of tuberculosis makes effective treatment possible and increases the probability of clinical outcome owing to quite effective anti-tuberculosis therapy; however, the diagnosis of tuberculosis has certain difficulties. According to international standards, tuberculosis diagnosis must be confirmed either by bacteriology or by histological studies, but the bacteriological methods do not always allow detecting *Mycobacterium tuberculosis* in people infected with pulmonary tuberculosis and especially with extra pulmonary tuberculosis. Among the available laboratory technique for TB diagnosis, the polymerase chain reaction (PCR) stands out. This technique provides results in a period of time similar to that of sputum smear microscopy, using mycobacteria concentration similar to that used for culture and PCR is capable for defining the causative agent[6]**.** Detection of *M. tuberculosis*-specific DNA sequences might represent a more sensitive and fast diagnostic target[15, 16]; however, the successful use of DNA amplification techniques is strongly dependent on the choice of the target sequence[17]**.** Moreover, since respiratory tract specimens are naturally contaminated by many different species of commensal and pathogenic microorganisms, a high degree of specificity for *M. tuberculosis* recognition is mandatory[16].The target molecular marker used in this study is the repetitive insertion sequence 6110 (IS 6110) of *Mycobacterium tuberculosis*. However, the target most frequently amplified is the IS 986 or IS 6110 repetitive element, which is present in multiple copies (up to 20) in most strains of *M. tuberculosis* complex. DNA from the bacteria present in clinical specimens has been extracted by numerous methods ranging from simple boiling or shaking with glass beads to more complex extraction procedures. Due to the risk of cross contamination, some scientists have performed PCR with d DTP instead of dTTP allowing for decontamination with uracil-N'-glycosylase. Up to one fifth of clinical specimens have been reported to contain inhibitors of Taq polymerase and prudent scientists have devised internal quality control strategies to identify the presence of inhibitors to prevent false negative reporting[18]. Out of smear positive cases, 32 (86.5%) and 35 (94.6%) were positive for PCR and RT-PCR respectively while 5 (13.5%) and 2 (5.4%) of them were PCR and RT-PCR negative respectively. Clarridage and coworkers,[19]; Forbes and Hicks[20] documented that developed nucleic acid amplification techniques have specificity more than 95% and are more than 95% sensitive in smear positive specimens. Other studies revealed that several different PCR systems have produced widely differing results with regard to specificity and sensitivity in the detection of *Mycobacterium tuberculosis* in clinical samples[21, 22, and 23]. Petanik and associates[24] documented that slide microscopy for the detection of AFB is a convenient, rapid and economic test used for determination of M *tuberculosis* infection but it lacks sufficient sensitivity and specificity and some studies have shown that up to half of the cases of

tuberculosis are smear negative .The study observation concentrates on 28 patients with smear negative smear at which 7 (25%) and 8 (28.6%) of them revealed positive result for PCR and RT-PCR respectively. False negative AFB smear in clinically TB suspected and PCR positive patients may be due to low concentration of bacilli in pulmonary secretions of the indigenous patients as those observed by Santos, et al.[6].The focusing point is that successful detection of DNA by amplification methods depends on purity and quality of DNA template in sputum samples[14]. On the other hand the study result was in consistent with those observed by Kocagoz and associates[25] who observed that four out of nine samples which were both smear and culture negative were positive for PCR. This result suggested that PCR assay is probably more sensitive by detecting nonviable and/or fewer viable organisms. For smear negative specimens, PCR is the only current available method that can provide the clinical diagnosis of pulmonary tuberculosis. Forbes and Hicks[20] documented that using an internal control, 52% of respiratory specimens interfere and inhibit PCR assay in the direct detection of *M tuberculosis* in clinical specimens. In addition to that presence of PCR inhibiting substances may also interact with PCR processing at which Buck and associates[26] reported that the origin of PCR inhibiting substances in sputum. This may interpret false negative PCR in 5 (13.5%), 2 (2.4%) out of 37 AFB smear positive by both of PCR and RT-PCR respectively. Also, other factors which play an important role as the major adverse factors in *M tuberculosis* DNA identification in sputum by PCR are the low numbers of mycobacteria and the presence of endogenous PCR inhibitors that may reach up to 20% false negative[27, 28, 29]. On the other hand, the presence of positive PCR in AFB smear negative at which clinical situation and decision prone to be weak suspicion of pulmonary tuberculosis may be due to cross contamination with molecular target sequence because of an error in handling the samples[25]**.** Peripheral blood based PCR assay was incorporated in study design based on the previous studies which concluded that the blood of patients with no trace of extra-pulmonary disease or a military pattern were PCR positive for *M tuberculosis* supports the hypothesis that this bacterium escape from the alveolar spaces to the blood circuit more often than previously thought[30] but the presence of heme compounds in blood specimens have been identified as inhibitors of PCR amplification[20]**.** The presence of false negative peripheral blood based PCR assay in AFB smear positive may be due this reason. The study hypothesized that the investigation of one gene region of *M tuberculosis* genome did not improve the positive detection rate of this bacterium DNA in peripheral blood. On the other hand, positive PCR assay in this population reflecting increasingly high numbers of *M tuberculosis* DNA copies circulating in the blood[30]**.**The other researcher concluded that non quantitative PCR assay of peripheral blood leucocytes seems to be of little value for the diagnosis of pulmonary tuberculosis in immunocompetent patients[30]**.** The term extra-pulmonary tuberculosis (EPTB) has been used to describe isolated occurrence of tuberculosis at body sites other than the lung. However, when an extra-pulmonary focus is evident in a patient with pulmonary tuberculosis, such patients have been categorized under pulmonary tuberculosis as per the guidelines of the World HealthOrganization[31]. Tuberculosis in pleural effusion is categorized as extra-pulmonary despite an intimate anatomic relationship between pleura and the lungs[32, 33]. Tuberculosis (TB) has traditionally been one of the major causes of pleural disease and until the earlier decades of the past century held as a principal paradigm of “pleuritis. Indeed in the presence of a distinctly exudative effusion and a compatible clinical presentation the widely used term “pleuritis exudativa” insinuated a tuberculous etiology and has therefore been understood to be synonymous with pleuritis exudative tuberculosa[34]. Nucleic acid amplification (NAA) assays amplify *M. tuberculosis*-specific nucleic acid sequences with a nucleic acid probe. This allows direct detection of *M. tuberculosis* in clinical specimens like pleural fluid within hours of their receipt[35]. In this study, low percentage for recovery in study cases for tuberculous pleural effusion was observed by both of PCR and RT-PCR. A pooled analysis of the data from 20 studies assessing the use of pleural fluid NAA tests concluded that these tests demonstrated reasonably high specificity(97% for commercial and 91% for in-house tests), but generally poor and variable sensitivity (62% for commercial and 76.5% for in-house tests)[36]. An earlier meta-analysis of 40 studies came to very similar conclusions[37]. The disappointingly low sensitivities of NAA techniques might be due to the presence of inhibitors in the pleural fluid or to intracellular sequestration of the mycobacteria[35].

CONCLUSION

The study concluded that PCR provides a sensitive and specific means for the laboratory diagnosis of

pulmonary tuberculosis and TB pleurisy within 24hrs. Also, qualitative real time PCR was more confirmatory molecular in the diagnosis of pulmonary tuberculosis and TB pleurisy test but the molecular diagnosis of the tuberculosis should be based in the combined analysis of several parameters like baciloscopia, clinical manifestations, therapeutic proves. Further, it is concluded that non-quantitative PCR and RT-PCR of peripheral blood leukocytes is of little value for the specific diagnosis of pulmonary tuberculosis. Regarding extra pulmonary tuberculosis, the PCR test revealed low sensitivity and high specificity for the diagnosis of TB pleurisy using pleural fluid. The sensitivity was higher in cases in which the bacillary load was high-in acid fast bacilli-positive samples.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. AL ZAHRANI K, AL JAHDALI H, POIRIER L, RENÉ P, GENNARO ML, MENZIES D. Accuracy and Utility of Commercially Available Amplification and Serologic Tests for the Diagnosis of Minimal Pulmonary Tuberculosis. Am J Respir Crit Care Med [Internet]. 2000;162(4):1323–9. Available from: http://dx.doi.org/10.1164/ajrccm.162.4.9912115
2. World Health Organization. Diagnostics and laboratory strengthening. In: WHO Global tuberculosis report. Geneva. 2013 p. 59-67.
3. Soini H MJ. Molecular diagnosis of mycobacteria. Clin Chem. 2001;47(5):809–14.
4. Nahid P. Advances in the Diagnosis and Treatment of Tuberculosis. Proc Am Thorac Soc [Internet]. 2006;3(1):103–10. Available from: http://dx.doi.org/10.1513/pats.200511-119jh
5. NC S. Molecular diagnosis of pulmonary tuberculosis. J Bras Pathol Med Lab. 2007;43(1):1–7.
6. Santos RMC, Ogusku MM, Miranda JM, dos-Santos MC, Salem JI. Evaluation of polymerase chain reaction in the diagnosis of pulmonary tuberculosis in indigenous and non-indigenous patients. J Bras Pneumol [Internet]. 2006;32(3):234-40 Available from: http://dx.doi.org/10.1590/s1806-37132006000900010
7. Schluger NW, Condos R, Lewis S, Rom WN. Amplification of DNA of Mycobacterium tuberculosis from peripheral blood of patients with pulmonary tuberculosis. Lancet [Internet]. 1994;344(8917):232–3. Available from: http://dx.doi.org/10.1016/s0140-6736(94)92999-8
8. Kolk AHJ, Kox LFF, Kuijper S, Richter C. Detection of Mycobacterium tuberculosis in peripheral blood. Lancet [Internet]. 1994;344(8923):694. Available from: http://dx.doi.org/10.1016/s0140-6736(94)92135-0
9. Behr MA, Warren SA, Salamon H, Hopewell PC, de Leon AP, Daley CL, et al. Transmission of Mycobacterium tuberculosis from patients smear-negative for acid-fast bacilli. Lancet [Internet]. 1999;353(9151):444–9. Available from: http://dx.doi.org/10.1016/s0140-6736(98)03406-0
10. Baron EJ, Peterson LR, Finegold SM. Baily and Scott's diagnostic microbiology. 19th ed. Toronto: Mosby company; 1994. p. 389-400.
11. Anon . Diagnostic Standards and Classification of Tuberculosis in Adults and Children. Am J Respir Crit Care Med [Internet]. 2000;161(4):1376–95. Available from: http://dx.doi.org/10.1164/ajrccm.161.4.16141
12. Gillespie SH. Mycobacterium tuberculosis [Internet]. Principles and Practice of Clinical Bacteriology. John Wiley & Sons, Ltd; 2nd ed. 2006. p. 159–69. Available from: http://dx.doi.org/10.1002/9780470017968.ch10
13. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 4th ed. Cold Spring harbor laboratory press, Cold Spring harbor, New York; 2012.

14. Amita J, Vandana T, Guleria RS VR. Qualitative evaluation of mycobacterial DNA extraction protocols for polymerase chain reaction. Mol Biol Today. 2002;3(2):43–9.
15. GL W. Molecular techniques in mycobacterial detection. Arch Pathol Lab Med. 2001;125(1):122–6.
16. Broccolo F, Scarpellini P, Locatelli G, Zingale A, Brambilla AM, Cichero P, et al. Rapid Diagnosis of Mycobacterial Infections and Quantitation of Mycobacterium tuberculosis Load by Two Real-Time Calibrated PCR Assays. J Clin Microbiol [Internet]. 2003;41(10):4565–72. Available from: http://dx.doi.org/10.1128/jcm.41.10.4565-4572.2003
17. Kafwabulula M, Ahmed K, Nagatake T, Gotoh J, Mitarai S, Oizumi K ZA. Evaluation of PCR-based methods for the diagnosis of tuberculosis by identification of mycobacterial DNA in urine samples. Int J Tuberc Lung Dis. 2002;6(8):732–7.
18. A R. PCR for diagnosis of tuberculosis: where are we now? Indian J Tuberc. 2000;47(2):79–82.
19. Clarridge JE, Shawar RM, Shinnick TM PB. Large-scale use of polymerase chain reaction for detection of Mycobacterium tuberculosis in a routine mycobacteriology laboratory. J Clin Microbiol. 1993;31(8):2049–56.
20. Forbes BA HK. Direct detection of Mycobacterium tuberculosis in respiratory specimens in a clinical laboratory by polymerase chain reaction. J Clin Microbiol. 1993;31(7):1688–94.
21. Noordhoek G, Kolk J, Bjune G, Catty D, Dale JW, et al. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among even laboratories. J. Clin. Microbiol. 1994;32(2):277-84.
22. Kox L, Rhienthong D, Miranda AM, Udomsantisuk N, Ellis K, et al. A more reliable PCR for detection of *Mycobacterium tuberculosis* in clinical samples. J. Clin. Microbiol. 1994;32(3):672-78.
23. Beige J, Lokies J, Schaberg T, Finckh U, Fischer M, Mauch H, Lode H, Ko¨hler B, Rolfs A. Clinical evaluation of a *Mycobacterium tuberculosis* PCR assay. J Clin. Microbiol. 1995;33(1):90-5.
24. Patnaik M, Liegmann K, Peter JB. Rapid Detection of Smear-Negative Mycobacterium tuberculosis by PCR and Sequencing for Rifampin Resistance with DNA Extracted Directly from Slides. J Clin Microbiol [Internet]. 2001;39(1):51–2. Available from: http://dx.doi.org/10.1128/jcm.39.1.51-52.2001
25. Kocagöz T, Yilmaz E, Ozkara S, Kocagöz S, Hayran M, et al. Detection of Mycobacterium tuberculosis in sputum samples by polymerase chain reaction using a simplified procedure. Journal of clinical microbiology. 1993;31(6):1435-8.
26. Buck GE, O'Hara LC, Summersgill JT. Rapid, simple method for treating clinical specimens containing Mycobacterium tuberculosis to remove DNA for polymerase chain reaction. Journal of clinical microbiology. 1992 May 1;30(5):1331-4.
27. Nolte FS, Metchock B, McGowan JE, Edwards A, Okwumabua O, Thurmond C, Mitchell PS, Plikaytis B, Shinnick T. Direct detection of Mycobacterium tuberculosis in sputum by polymerase chain reaction and DNA hybridization. Journal of clinical microbiology. 1993 Jul 1;31(7):1777-82.
28. Soini H, Skurnik M, Liippo K, Tala E, Viljanen MK. Detection and identification of mycobacteria by amplification of a segment of the gene coding for the 32-kilodalton protein. Journal of clinical microbiology. 1992 Aug 1;30(8):2025-8.
29. Amicosante M, Richeldi L, Trenti G, Paone G, Campa M, Bisetti A, Saltini C. Inactivation of polymerase inhibitors for Mycobacterium tuberculosis DNA amplification in sputum by using capture resin. Journal of clinical microbiology. 1995 Mar 1;33(3):629-30.
30. Rolfs A, Beige J, Finckh U, Köhler B, Schaberg T, Lokies J, Lode H. Amplification of Mycobacterium tuberculosis from peripheral blood. Journal of clinical microbiology. 1995 Dec 1;33(12):3312-4.
31. Maher D, Chaulet P, Spinaci S, Harries A. Treatment of tuberculosis: guidelines for national programs. Geneva: World Health Organization; 1997.
32. VALDES L. Tuberculous pleural effusions. Eur J Intern Med [Internet]. 2003;14(2):77–88. Available from: http://dx.doi.org/10.1016/s0953-6205(03)00018-9
33. Sharma SK, Mohan A. Extra-pulmonary tuberculosis. Indian J Med Res. 2004;120:316-53.
34. Frank W. Tuberculous Pleural Effusion. In:

Tuberculosis- Current Issues in Diagnosis and Management. By: Frank; licensee In Tech. 2013 http://dx.doi.org/10.5772/54955

35. LIGHT RW. Update on tuberculous pleural effusion. Respirology [Internet]. 2010;15(3):451–8. Available from: http://dx.doi.org/10.1111/j.1440-1843.2010.01723.x
36. Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, et al. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol Assess (Rockv) [Internet]. 2007;11(3). Available from: http://dx.doi.org/10.3310/hta11030
37. Pai M, Flores LL, Hubbard A, Riley LW, Colford JM. Nucleic acid amplification tests in the diagnosis of tuberculous pleuritis: a systematic review and meta-analysis. BMC Infect Dis [Internet]. 2004;4(1). Available from: http://dx.doi.org/10.1186/1471-2334-4-6