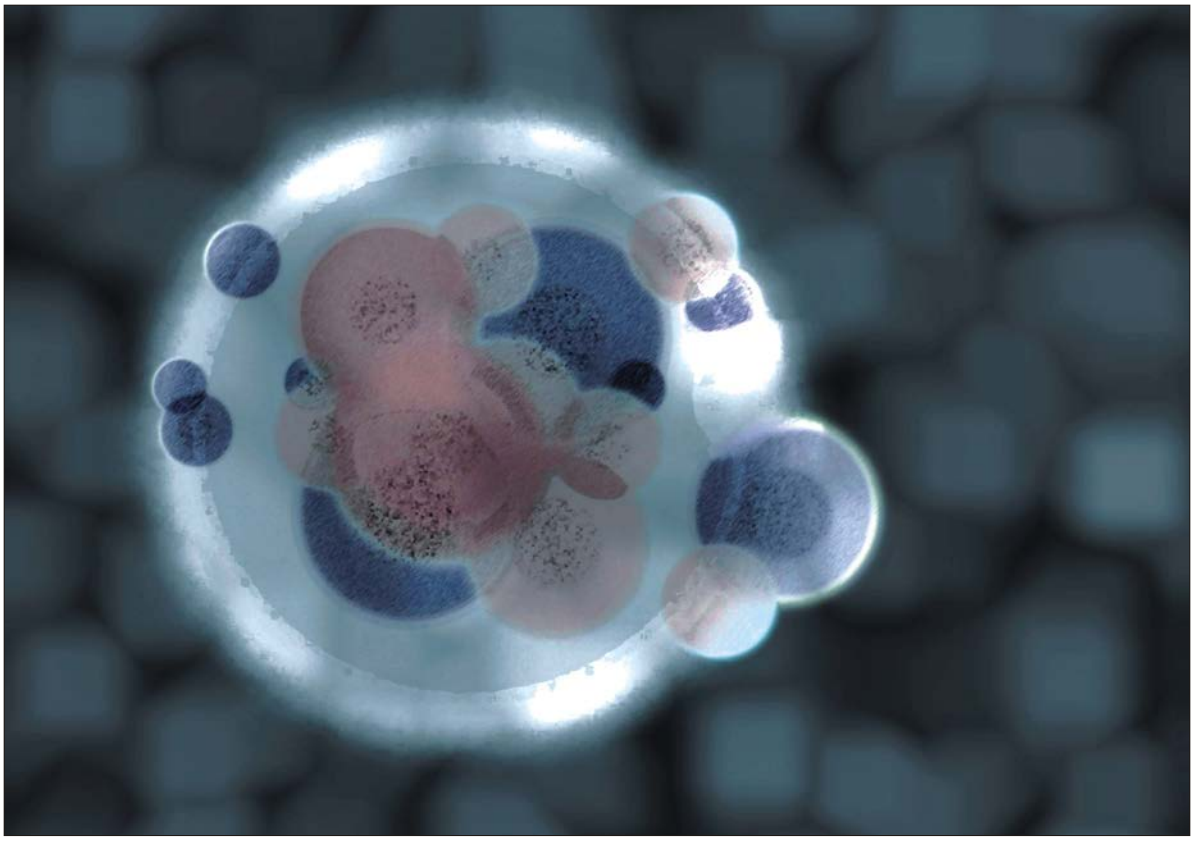

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 6 (1): 121-126, 2006
ISSN 1727-3048

Sensitivity of IS6110, mtp40 and 85B-RNA Based Amplification Assays in the Diagnosis and Treatment Follow up of Pulmonary *Mycobacterium tuberculosis*

[1]Maha A. El Demellawy, [1]Abeer Abdel Wahab, [2]Essam M. Emad, [2]Kamal M. Kandeel,
[3]Ashraf A. Tabll and [3]Mostafa K. El Awady
[1]Department of Medical Biotechnology, Genetic Engineering and Biotechnology Research Institute, Mubarak City for Scientific Research and Technology Applications, New Borg El Arab, Alexandria, Egypt
[2]Department of Biochemistry, Faculty of Science, Alexandria University, Alexandria, Egypt
[3]Department of Biomedical Technology, National Research Center, Cairo, Egypt

Abstract: This study was designed to compare microscopy, culture and 3 nested Polymerase Chain Reaction (PCR) based assays using as templates either of the followings: IS6110, mtp40 or 85B-RNA in the diagnosis and treatment follow up of pulmonary tuberculosis. Sputum specimens from 250 patients clinically diagnosed to have pulmonary tuberculosis were utilized. Samples were categorized into 4 groups. Group I: Samples from 120 patients with suspected TB infection; Group II: Samples from 70 patients relapsed after treatment, Group III: Samples from 30 patients not responding to treatment and Group IV: Samples from 30 patients subjected to follow up every two months during the treatment. The results of this study revealed that PCR is equally sensitive in all groups studied. TB DNA detection by PCR is more sensitive than ZN staining when taking culture plus clinical investigations as a gold standard method. In those patients with negative ZN, negative culture but have clinical and/or radiological evidence for the disease, PCR and RT-PCR methods were able to detect TB DNA and TB RNA at sensitivities of 96 and 100%, respectively. False positives were observed in TB DNA by IS6110 PCR at the end of successful treatment (probably due to detection of DNA from dead bacilli). On the contrary, RT-PCR of 85B-RNA is more specific and sensitive method for detection of viable mycobacteria. Present data altogether indicate that amplification of mtp40 and 85B-RNA are fast, sensitive and specific methods for diagnosis and follow-up of TB infection with slightly more specificity of 85B-RNA than mtp40 DNA.

Key words: *Mycobacterium tuberculosis*, diagnosis, polymerase chain reaction IS6110, mtp40, 85B-RNA

INTRODUCTION

Tuberculosis (TB) has again become one of the most life threatening health problems all over the world. Annually, TB infection led to the death of approximately 3 million people worldwide. At least 8 million new TB infected patients were reported worldwide[1]. Theoretically, one TB patient can transfer infection to at least 10-20 people from his surroundings. Therefore early and quick diagnosis will be of great help to isolate the patients and control the disease. The standard methods for identification of the organism are by acid fast staining (Ziehl-Neelsen stain, ZN) and by culture on Lowenstein-Jensen (LJ) media[2-4]. BACTEC system has been utilized to detect drug susceptibility; the last two procedures need at least 2 to 3 weeks to get information, besides the need to use specific media or exclusive instrument. The use of nucleic acid amplification methods to identify the TB bacilli in clinical samples has become acceptable approach worldwide[5,6]. Recently, Negi *et al.*[7] reported that, PCR is a rapid and sensitive method for the early diagnosis of pulmonary and extrapulmonary tuberculosis. Few private laboratories and laboratories of university hospitals are using the commercially available kits, developed by Roche, Abbott, Gene-probe, INNO-LIPA[8] or Gene-trak for PCR amplification of TB DNA besides the culture and smear examination. However, such tests may be misinterpreted because of either cross-contamination, presence of DNA polymerase inhibitors, or inappropriate treatment of the clinical samples. It is of great importance to standardize protocols for nucleic acid amplification for the detection of TB[9]. Until achieving quality-controlled procedure for TB detection, it has been always advised to use culture and smear examination of clinical samples to

Corresponding Author: Dr. Mostafa K. El Awady, Department of Biomedical Technology, National Research Center, Tahrir Street, Dokki, Cairo 12622, Egypt Tel: (+2)(02)(3362609) Fax: (+2)(02)(3370931

detect the presence of TB bacilli. On the other hand, detection of 85B antigen expression which is an enzyme involved in cell wall biosynthesis and represents a major target of the host immune response[10]; can be of great help in TB diagnosis. Depending on the fact that the life span range of 85B mRNA is only 3 min, any assay targeting this mRNA as a template would provide excellent method to detect only viable mycobacterium[11]. In the present study we compare DNA amplification methods, using either IS6110 or mtp40 genes as templates, with RNA amplification of 85B-RNA as template. Microscopic examination of ZN stained samples and long term culture of mycobacteria on LJ solid medium were used as references for comparison. The diagnostic efficiencies of the studied methods have been evaluated before and after receiving standard anti-TB treatment.

MATERIALS AND METHODS

Patients: A total of 250 patients (male: female ratio was 67: 33%; with age range of 15-75 years old) were utilized for study. All patients were admitted to Maamora Chest hospital in Alexandria, Egypt. Each patient was subjected to detailed history taking and clinical examination, routine laboratory investigations and chest X-ray. Sputum samples were collected at three consecutive days and examined by direct acid fast stain smear test using Ziehl Neelsen (ZN) stain and cultured on LJ medium. The patients were classified into 4 groups according to results of fresh smear test as follows:

Group I: Includes 120 patients with acute cases admitted to the hospital for the first time

Group II: Includes 70 patients who experienced relapse after withdrawal of treatment

Group III: Includes 30 patients chronic TB with no response to treatment

Group IV: Includes 30 patients who were freshly diagnosed and received the first line anti-TB drugs. These patients were subjected to follow up and sputum samples were examined every two months during the 9 month treatment course. Sputum samples from healthy and non TB patients were used as controls.

Decontamination of specimens: Sputum specimens was decontaminated as previously described[12]. Briefly, sputum was mixed with equal volume of 4% NaOH in a closed container, incubated for 15 min at 37°C with shaking and centrifuged at 2000 x g for 20 min. Supernatant was discarded and the pellet was neutralized with 8% HCl using phenol red as a pH indicator. Swabs were taken from the sediment for the examination of ZN and culture on LJ medium.

Nested PCR amplification: DNA extraction was performed according to Sambrook *et al.*[13]. Amplification of IS6110 insertion sequence and mtp40 gene were performed using sequence specific primers in a nested PCR reaction as described[6,12,14,15] (Table 1). The first PCR amplification of IS6110 insertion sequence was carried out in a mixture containing 70-100 ng of DNA, 1X PCR buffer, 200 μM dNTPs (Amersham Biosciences, Freiberg, Germany), 1 μM of each of the external primers TB_1 TB_2, 15 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen Life Technologies, Scotland, UK). The amplification was initiated by one cycle consisting of denaturation at 95°C for 5 min, annealing at 65°C for 1 min, then extension at 72°C for 1.5 min followed by 30 cycles, each consists of denaturation at 95°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1.5 min A final extension at 72°C for 15 min was performed. Size of the amplified fragment after this round was 580 bp. Nested PCR amplification was carried out using 5 μL of the first PCR amplified product in a PCR mixture containing 1X PCR buffer, 200 μM dNTPs, 1 μM of each of the internal primers TB_3 and TB_4, 2.5 mM $MgCl_2$ and 1 U Taq DNA polymerase for 20 cycles, each consists of denaturation at 95°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1.5 min followed by extra extension at 72°C for 15 min this step will give amplified fragment of 181 bp.

Amplification of mtp40 was performed using sequence specific primers in a nested PCR reaction. The first PCR amplification was carried out using 70-100 ng extracted DNA, 1X PCR buffer, 200 μM dNTPs (Amersham Bioscences), 1 μM of each of the external primers PT1 and PT2, 15 mM $MgCl_2$ and 1 U Taq DNA polymerase (Invitrogen Life Technologies, Scotland, UK).

Table 1: Primers used for amplification of TB DNA (IS6110 and mtp40 genes) and RNA (85B gene)

Gene	Primer direction		Primer Sequence *	Product size (bp)
IS6110	First	TB1	5' - GGA CAA CGC CGA ATT	580
		TB2	TGC GAA GGGC 3	
			5'-TAG GCG TCG GTG ACA	
			AAG GCC ACG-3'	
	Nested	TB3	5' - ACG ACC ACA TCA ACC - 3'	181
		TB4	5' - AGT TTG GTC ATC AGC - 3'	
mtp40	First	PT1	5' - CAA CGC GCC GTC GGT GG – 3'	396
		PT2	5' - CCC CCC ACG GCA CCG C – 3'	
	Nested	PT3	5' - CAA CGC GCC GTC GGT GG – 3'	223
		PT4	5' - CCC CCC ACG GCA CCG C – 3'	
85B	First	MRL41		
		MRL42	5' - GAG TAC CCT GCA GGT GCC	593
			GTC GCC GTC–3'	
			5'-CCG GGT GTT GTT TGC GAC CAG	
			CTT G–3'	
	Nested	MRL43	5' - GAC TTA CAA GTG GGA AAC C–3'	216
		MRL44	5' - CCG ATC AGG GTA GGC CCC-3'	

Primer Sequence as described previeosly[6,11,12,14,15,24]

The amplification was initiated by one cycle consisting of denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1.5 min A final extension at 72°C for 10 min was performed. The amplified product was 396 bp in length. The nested PCR amplification was done using 5 μL from the first PCR product in a mixture containing 1X PCR buffer, 200 μM dNTPs, 1 μM of each of the internal primers (PT3 and PT4), 2.5 mM $MgCl_2$ and 1 U Taq DNA polymerase for one cycle by denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and extension at 72°C for 1 min A final extension at 72°C for 10 min was performed. The size of the nested product was 223 bp.

Nested RT-PCR for detection of 85B-RNA: Extraction of RNA was performed according to Chomczynski and Sacchi[16] using single-step method of RNA isolation by acid Guanidinium thiocyanate-phenol-chloroform extraction. Briefly, 1 mL of extraction solution (4 M Guanidinium isothiocyanate, 25 mM Sodium Citrate, 5% Sarcosyl, 0.72% mercaptoethanol) was added to 0.5 mL of sputum sample in a sterile tube. The solution was mixed by inverting the tube several times until complete liquefaction of sputum. The sample was then mixed with equal volume of phenol (pH 4.5), 1/10 volume of 2 M sodium acetate (pH 4.5) and 1/5 volume of chloroform/ isoamyl alcohol (1: 24) and incubated in ice for 15 min. The two layers were separated by centrifugation at 12,000 rpm at 4°C for 15 min, where the aqueous (upper) layer was transferred to a sterile tube. An equal volume of isopropanol was then added and incubated overnight at -20°C. Precipitated RNA was centrifuged at 12,000 rpm for 30 min at 4°C. The pellet was then washed twice with ice cold 70% ethanol, dried in a water bath at 65°C and then dissolved in 20 μL sterile distilled water and stored in -20°C until used.

Reverse transcription and first PCR amplification were carried out in a single step using RT-PCR beads (Amersham Biosciences), briefly, 1 μM of each external primer MRL41 and MRL42[11], 10% formamide and 1 μg extracted RNA were added to RT-PCR beads and incubated for 30 min at 42°C then for 5 min at 94°C to stop the reverse transcriptase reaction. The synthesized first strand cDNA was then amplified for 30 cycles of denaturation at 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min. For nested reaction, 5 μL from the first PCR reaction was mixed with 1X PCR buffer, 200 μM dNTPs, 1 μM of each internal primer, MRL43 and MRL44, 2.5 mM $MgCl_2$ and 1 U Taq DNA polymerase for 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min then extension at 72°C for 1 min. A final extension at 72°C for 10 min was performed. The nested amplified fragment was 216 bp in length. Different concentrations of purified TB DNA (from 10 to 100 ng) were used as templete for PCR reaction using IS6110 and mtp40 primers to determine the concentration that can be used for production of amplified production which can be visualized in agarose gel.

RESULTS

The mtp40 method gave positive results using only 10 ng of chromosomal of mycobacterium while the IS6110 method required at least 70 ng of chromosomal DNA for positive amplification (Data not shown).

In sputum samples negative for ZN as well negative culture methods, and clinically positive, PCR and RT-PCR were able to detect TB DNA and TB RNA demonstrating sensitivity with 96 and 100%, respectively.

All the three nucleic acid techniques had equally highest sensitivities in relapsed patients with slightly more sensitivity of mtp40 than IS6110 based method. Among acute and relapsed patients groups, ZN had the lowest sensitivity compared with nucleic acid based methods. In chronic patients all methods used had 100% sensitivities (Table 2). In follow up patients group (30 cases), It is obvious that IS6110 is the less specific nucleic acid based method compared with the mtp40 or 85B-RNA methods (Table 3 and Fig. 1). Table 3 demonstrate that ZN and culture have the highest specificities as indicated from the lowest rates of false positives during the last 3 months of treatment coarse. The mtp40 based assay reached 100% specificity, similar to 85B-RNA assay, only after 9 months of 9 months of treatment. The IS6110 based assay had less specificity among all the three nucleic acid based assays since it was still detecting TB DNA in 9 out of 30 cases after treatment whereas all the other 4

Table 2: Diagnosis of TB infection using different detection methods in patients with various presentations of the disease

	Patient group					
	Acute (120)		Relapsed (70)		Chronic (30)	
Detection method	TB +	TB -	TB +	TB -	TB +	TB -
ZN*	70	50	55	15	30	0
	58%		78%		100%	
Culture**	90	30	57	13	30	0
	75%		81%		100%	
DNA (IS6110)	111	9	65	5	30	0
	92%		93%		100%	
DNA (mtp40)	115	5	66	4	30	0
	96%		94%		100%	
RNA (85B)	115	5	65	5	30	0
	96%		93%		100%	

*ZN: Ziehl-Neelsen stain(acid fast staining)
** Culture on Lowenstein-Jensen (LJ) media

Table 3: Comparison between TB detection methods throughout nine month duration of therapy

	Months after treatment											
	0		2		4		6		8		9	
Detection method	TB+	TB-	TB+	TB-	TB+	TB-	TB+	TB-	TB+	TB-	TB+	TB-
ZN*	30	0	19	11	3	27	0	30	0	30	0	30
	100 %		63%		10%		0.0 %		0.0 %		0.0 %	
Culture**	30	0	16	14	11	19	0	30	0	30	0	30
	100 %		53 %		37%		0.0 %		0.0 %		0.0%	
DNA (IS6110)	30	0	30	0	30	0	20	10	15	15	9	21
	100 %		100%		100 %		67%		30%		30%	
DNA (mtp40)	30	0	30%	0	21%	9	15	15	11	19	0	30
	100%		100%		70%		30%		37%		0.0%	30
RNA	30	0	30	0	30	0	22	8	7	23	0	30
(85B)	100%		100%		100%		73%		23%		0.0%	

*ZN: Ziehl-Neelsen stain(acid fast staining), ** Culture on Lowenstein-Jensen (LJ) media

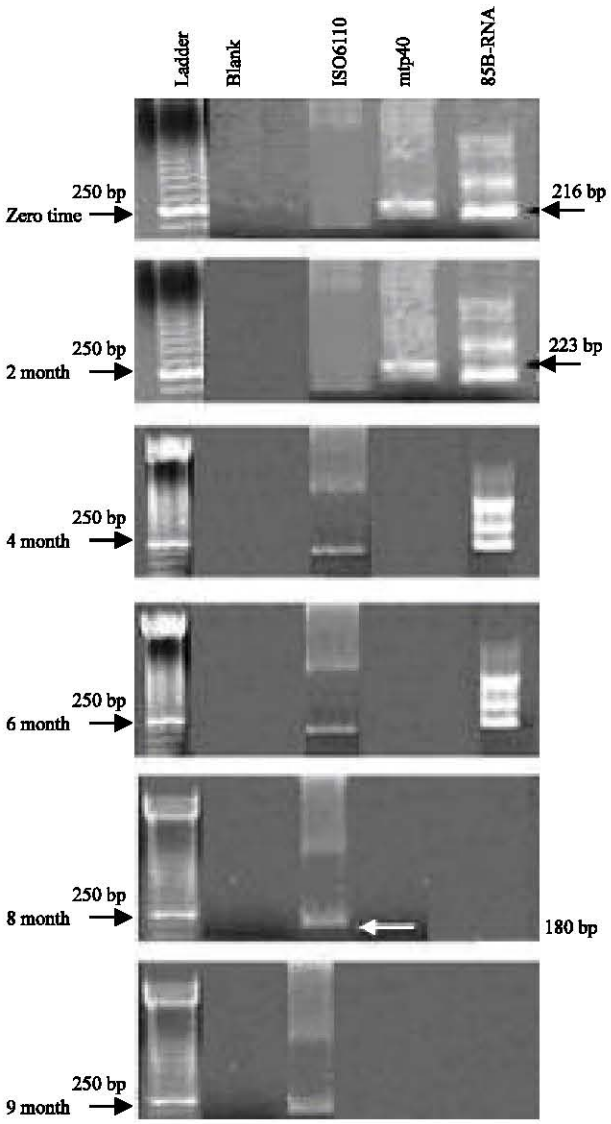

Fig. 1: Comparison of TB nucleic acid amplification methods during 9 months duration of treatment ISO6110 shows bans at 180 bp mtp40 shows bans at 223 bp, 85B-RNA shows bans at 216 bp

methods did not detect TB, presumably due to retaining of dead bacilli at the end of treatment and partly due to mispriming of IS6110 primers.

DISCUSSION

The significance of the present study stands on assessing the sensitivities and specificities of classical microscopic and microbiological methods with three different nucleic acid amplification protocols. The fundamental differences between the two DNA amplification protocols lies on the use of different bacterial genes as templates; the first is IS6110, a gene that exists in strains of mycobacterium, including human and bovine strains[17-19]. The second is mtp40 gene that is characteristic to the human *Mycobacterium tuberculosis*[20]. Since proper database on which strains infect the Egyptian TB patients is necessary for development of disease control strategies, the methodological comparison held in the present study seems to be valid at this stage to unravel the nature of infection in the studied patient sample. In those patients who display initial response to combination therapy, the mycobacterial DNA of the dead organisms have always been detectable in clinical samples particularly sputum. This has drawn the attention of investigators towards searching for a reliable marker for viable bacilli. Jou *et al.*[11] demonstrated that the mRNA specific for the 85B proteins has a life span of only 3 min. Therefore, the development of an 85B mRNA based procedure is of a paramount importance in treatment follow up and it can give a quick identification of patients with drug resistance. Present results demonstrated that ZN and culture on LJ medium had poor sensitivities in patients with acute infection and those who were relapsed after termination of treatment. Whereas, these two methods displayed 100% sensitivities similar to nucleic acid based procedures in diagnosis of chronic TB infection. These findings support provision reported by Garcia-De-Lomas and Navarro[21] and Quintamilla *et al.*[22].

Utilization of mtp40 gene as a PCR template displayed more sensitivity than IS6110 gene in diagnosis of acute infection. Similarly, the use of 85B mRNA as template in RT-PCR based procedure, showed equal sensitivity to mtp40 DNA based assay. Interestingly, DNA based assays namely mtp40 and IS6110 were less specific than 85B mRNA based method, particularly, by the end of treatment, where DNA from dead bacilli was still detectable only by DNA based assays after 6 to 9 months. This phenomenon is confirmed by results described by Jou *et al.*[11] as well as by the present study on treatment follow up where IS6110 assay remains positive in all cases for the first 4 months and the detectability declines gradually till the end of treatment where 30% were still falsely detected indicating less specificity not only when compared with other nucleic acid based assays but also when compared with ZN and culture on LJ medium. On the other hand, when comparing mtp40 with 85B mRNA based assays; the latter assay demonstrated higher sensitivity during the first 6 months. Furthermore, 85B mRNA assay demonstrated more specificity than mtp40 assay after 8 months of treatment. The reason might be that 4 out of 11 mtp40 still positive cases after 8 months of treatment are dead bacilli, which were not detected with 85B mRNA based assay. Although, the difference in specificity between the two assays disappeared at the 9th month of treatment, the major disadvantage of this lack of specificity is the more expenses in a significant portion of patients due to un-necessary extension of treatment duration. On the other hand ZN and culture methods could not detect bacilli in any of the cases after 6 months of treatment, indicating a striking lack of sensitivity in both methods with a subsequent high risk for relapse[23]. In general, the IS6110 based assay displays false positive results longer than mtp40 in patients after treatment. In conclusion, both ZN and LJ culture demonstrate considerable sensitivity and specificity only in cases with chronic TB infection. However, both methods suffer poor sensitivity and specificity in acute infection and in cases with disease relapse. Among the nucleic acid amplification assays, IS6110 had less specificity with more tendency to amplify DNA from non-human strains as well as dead bacilli than did the other two PCR methods. Both mtp40 DNA and 85B-RNA based assays have equally demonstrated the best sensitivity and specificity with slight preference of the latter method in detecting the viable bacilli during the course of combination antibiotic therapy.

ACKNOWLEDGMENTS

We thanks staff members of Maamora Chest hospital in Alexandria, Egypt in particular Drs. Somaia Hussein and Maged Hindi for being helpful in the clinical investigations

REFERENCES

1. Anonymous, 1997. Group at risk: WHO report on the tuberculosis epidemic. Geneva, Switzerland: World Health Organization.
2. Yeager, H., J. Lacy, L. Smith and C. LeMaistre, 1967. Quantitative studies of mycobacterial populations in sputum and saliva. Am. Rev. Resp. Dis., 95: 998-1004.

3. Hobby, G.L., A. Holman, P., M. Iseman and J. Jones, 1973. Enumeration of tubercle bacilli in sputum of patients with pulmonary tuberculosis. Antimicrob Agents Chemother., 4: 94-104.
4. Laidlaw, M., 1989. Mycobacterium: Tubercle Bacilli. In Mackie and Mc Cartney. Practical Medical Microbiology. (Collee, J.G., J.P. Duguid, A.G. Fraser and B.P. Marmion, Eds.). 13th Edn., Churchill Livingstone, New York, 25: 399-416.
5. Thierry, D., N. Brisson, N.L. Freubault, S. Nauyen and B. Gicquel, 1990. Characterization of Mycobacterium tuberculosis insertion sequence IS6110 and its application in diagnosis. J. Clin. Microbiol., 28: 2668-2673.
6. Hellyer, T., L. Desjardin, M. Assaf, J. Bates and K. Eisenach, 1996. Specificity of IS6110 based amplification assay for *Mycobacterium tuberculosis* complex. J. Clin. Microbiol., 34: 2843-2846.
7. Negi, S., S. Khan, S. Gupta, S. Pasha, S. Khare and S. Lal, 2005. Comparison of the conventional diagnostic modalities, bactec culture and polymerase chain reaction test for diagnosis of tuberculosis. Ind. J. Med. Microbiol., 23: 29-33.
8. Johansen, I., B. Lundgren, A. Sosnovskaja and V. Thomsen, 2003. Direct detection of multidrug-resistant *Mycobacterium tuberculosis* in clinical specimens in low- and high-incidence countries by line probe assay. Clin Microbiol., 41: 4454-4456.
9. Noordhoek, G., J. Van Embden and A. Kolk, 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: An international collaborative quality control study among 30 laboratories. J. Clin. Microbiol., 34: 2522-2525.
10. Wilkinson, R., L. DesJardin, N. Islam, B. Gibson and R. Kanost *et al*., 2001. An increase in expression of a *Mycobacterium tuberculosis* mycolyl transferase gene (fbpB) occurs early after infection of human monocytes. Mol. Microbiol., 39: 813-821.
11. Jou, N., T.R. Yoshimori, G. Mason, J. Louie and M. Liebling, 1997. Single-tube, nested reverse transcription PCR for detection of viable *Mycobacterium tuberculosi*. J. Clin. Microbiol., 35: 1161-1165.
12. Kennedy, N., S. Gillespie and A. Saruni, 1994. Polymerase Chain Reaction for assessing treatment response in patients with pulmonary tuberculosis. J. Infect. Dis., 170: 713-716.
13. Sambrook, J., E. Fritsch and T. Mamiatis, 1989. Molecular: A Laboratory Manual, 2nd Edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
14. Del Portillo, P., L. Murillo and M. Patarroyo, 1991. Amplification of a species-specific DNA fragment of *Mycobacterium tuberculosis* and its possible use in diagnosis. J. Clin. Microbiol., 29: 2163-2168.
15. Querol, J., J. Minguez, E. Garcia-Sanchez, M. Farga, C. Gimeno and J. Garcia-de-Lomas, 1995. Rapid diagnosis of pleural tuberculosis by polymerase chain reaction. Am. J. Resp. Crit. Care Med., 152: 1977-1981.
16. Chomczynski, P. and N.N. Sacchi, 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159.
17. Tzoanopoulos, D., K. Mimidis, S. Giaglis, K. Ritis and G. Kartalis, 2003. The usefulness of PCR amplification of the IS6110 insertion element of *M. tuberculosis* complex in ascitic fluid of patients with peritoneal tuberculosis. Eur. J. Intern. Med., 14: 367-371.
18. Ocepek, M., M. Pate, M. Zolnir-Dovc and M.M. Poljak, 2005. Transmission of *Mycobacterium tuberculosis* from human to cattle. J. Clin. Microbiol., 43: 3555-3557.
19. Zumarraga, M., V. Meikle, A. Bernardelli, A. Abdala, H.M.H. Tarabla, A. Romano and A. Cataldi, 2005. Use of touch-down polymerase chain reaction to enhance the sensitivity of *Mycobacterium bovis* detection. J. Vet. Diagn. Invest., 17: 232-238.
20. Koivula, T., S. Svenson and G. Kallenius, 2002. The mtp40 gene is not present in *Mycobacterium bovis*. Tuberculosis (Edinb), 82: 183-185.
21. Garcia-de-Lomas, J. and D. Navarro, 1997. New directions in diagnostics. Pediat. Infect. Dis. J., 16: S43-48.
22. Quintamilla, A., L. Garcia, G. Tudo, M. Navarro, J. Gonzalez and JimenezDe-Anta 2000. Single tube balanced hemi-nested PCR for detecting *Mycobacterium tuberculosis* in smear negative samples. J. Clin. Microbiol., 38: 1166-1169.
23. Sinha, S., M. Chatterjee, S. Bhattacharya, S. Pathak, R. Mitra, K. Karak and M. Mukherjee, 2003. Diagnostic evaluation of extrapulmonary tuberculosis by Fine Needle Aspiraton (FNA) supplemented with AFB smear and culture. J. Ind. Med. Ass., 101: 590-591.