Artigo Original

Analysis of different *primers* used in the PCR method: diagnosis of tuberculosis in the state of Amazonas, Brazil

MAURICIO MORISHI OGUSKU, JULIA IGNEZ SALEM

Background: Various *primers* are being tested for the detection of *Mycobacterium tuberculosis* DNA. The accuracy of the polymerase chain reaction (PCR) depends on the target sequence used and whether the test will be performed in culture or in clinical specimens.

Objectives: To identify DNA sequences, specifically those commonly reported as targets for diagnosis of tuberculosis (TB), in clinical samples of *M. tuberculosis* strains.

Method: Eighty-one clinical samples from suspected TB patients were initially processed and submitted to bacilloscopy (smear) and culture, and PCR was performed with specific *primers* for the following targets: IS*6110*, 65 kDa, 38 kDa and MPB64.

Results: Smear and culture results were negative in 24 samples, as was the PCR. The 19 samples testing smear positive, as well as the isolated strains, were 100% positive on PCR, with the exception of the 89.4% result from PCR with MPB64 primers. In the 38 smear negative and culture positive samples, PCR results were inconsistent. The *primers* specific for amplifying the 123 bp IS*6110* fragment gave the highest positivity (92.1%), diagnostic agreement (0.9143), co-positivity (94.7%) and co-negativity (100%).

Conclusion: The IS*6110*, 38 kDa, MPB64 and 65 kDa sequences were found in the genome of all *M. tuberculosis* strains isolated in patients from the state of Amazonas. The protocol for processing the clinical samples prior to PCR analysis and the specific primers used to amplify the 123bp IS*6110* fragment showed a greater efficiency in diagnosing pulmonary (paucibacillary) tuberculosis in comparison to published data.

Key words: *Primers*/PCR. Diagnosis/Tuberculosis. *Mycobacterium tuberculosis*.

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Correspondence to: Mauricio Morishi Ogusku. Instituto Nacional de Pesquisas da Amazônia – INPA. Av. André Araújo, 2936 – Bairro do Aleixo. CEP 69060-001 - Manaus, AM – Brazil - Phone: 55 92 643 3058. E-mail: mmogusku@inpa.gov.br
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INTRODUCTION

Polymerase chain reaction (PCR) is a molecular technique whose accuracy is determined by the choice of the target DNA and the definition of the primers within the DNA sequence. The PCR method has been used as an alternative that presents high sensitivity and specificity for the rapid diagnosis of infectious diseases. However, the use of PCR in the detection of *Mycobacterium tuberculosis* (Mtb) has produced varying results, especially in relation to the sensitivity of the test[1,2,3]. Various target sequences, such as insertion sequence *6110* (IS*6110*), 65 kDa (GroEL), 38 kDa (PhoS, CIE Ag78 or Pab) and MPB64 (23 kDa), have therefore been used. Among these, IS*6110* is more commonly used because it is a repetitive sequence in the Mtb genome. This characteristic helps increase the sensitivity of PCR over that obtained in the amplification of single DNA sequences[4]. However, the IS*6110* sequence was reported to be absent from an Mtb strain isolated in India[5], and homologous sequences have been detected in potentially pathogenic mycobacterium strains such as *M. intracellulare*, *M. fortuitum*, *M. kansasii*, *M. xenopi*, *M. malmoense* and *M. chelonae*[6,7]. In Brazil, the existence of Mtb strains lacking IS*6110* has never been reported, and some of the species mentioned above are frequently isolated in the state of Amazonas[8,9,10].

These facts led us to ask the following questions regarding the use of PCR for the diagnosis of tuberculosis (TB) in the state of Amazonas: Would the sequences reported in the literature also be found in the Mtb strains isolated in the state of Amazonas? Which primers would be most recommended for PCR of clinical samples collected in the state of Amazonas?

In an attempt to address these questions, we investigated the presence of the DNA target sequences most commonly reported in the literature. A set of Mtb strains, as well as the clinical samples from which the strains had been isolated, were analyzed.

METHOD

Clinical samples were collected from 81 suspected TB patients living in the state of Amazonas and were sent to the Laboratory of Mycobacteriology of the *Instituto Nacional de Pesquisas da Amazônia* (INPA, National Research Institute of Amazônia) for study. The samples were submitted to acid-fast bacilli (AFB) smear microscopy and Mtb culture. Smear microscopy and culture results were negative in 24 samples and positive in 19 samples (multibacillary samples). In 38 samples, the smear microscopy was negative and the culture was positive (paucibacillary samples).

Smear microscopy was performed in accordance with the guidelines of the *Ministério da Saúde* (Ministry of Health)[11]. Cultures were performed as follows: samples were transferred to graduated 15-mL conical centrifuge tubes and an equal volume of 4% NaOH solution was added. The samples were agitated and left to settle for 15 minutes. Sterile distilled water was added up to a combined volume of 12 mL with. Samples were then centrifuged at 3000 x g and the supernatant was set aside. The sediment was suspended in 2 mL of sterile distilled water, neutralized with 4% HCl solution, and 0.2-mL aliquots were transferred for culture in Löwenstein-Jensen culture medium (three tubes per sample). The remainder of the suspension was stored for later PCR analysis. The culture tubes were incubated at 37ºC for up to 60 days. Identification of the isolated mycobacterium strains as Mtb was performed as recommended by David et al. [12]

Suspensions from the clinical samples and from the 57 strains isolated from these samples were analyzed using PCR in order to identify the following targets: IS*6110*, 65 kDa, 38 kDa and MPB64. Two distinct pairs of primers were tested for the detection of the IS*6110* region: one that promotes amplification of a 123-bp fragment[4] and another that promotes that of a 541-bp fragment[13]. Amplification of nucleotide sequences of the targets 65 kDa and 38 kDa was performed through two consecutive PCRs – PCR and nested PCR – using two pairs of specific primers for each test. When the target was 65 kDa[14], PCR of the external primer pair amplified a fragment of 383 bp, whereas nested PCR of the internal primer pair

Abbreviations used in this paper:

AFB	– Acid-fast bacilli
bp	– Base pairs
IS*6110*	– Insertion sequence *6110*
Mtb	– *Mycobacterium tuberculosis*
PCR	– Polymerase chain reaction
TB	– Tuberculosis

amplified a fragment of 155 bp. When the target was 38 kDa[15], amplified products from standard PCR and nested PCR produced fragments of 419 bp and 322 bp, respectively. Primers for the MPB64 region amplified a fragment of 240 bp[16].

DNA extraction from sample sediment was performed in accordance with the Ogusku et al. protocol[17]. The DNA was purified with phenol-chloroform extraction and absolute ethanol precipitation, in accordance with the Davis et al. method[18]. For DNA extraction from the 57 Mtb strains isolated from the culture samples, we used an adaptation of the Perosio et al.[19] protocol, in which approximately 1 mg of each strain suspension was transferred into a screw cap tube containing glass beads. The samples in these tubes were agitated on a vortex mixer for cell separation, and sterile distilled water was added to adjust the turbidity of the suspension to a 1.0 McFarland standard. From each suspension, a 50-µ L aliquot was used for DNA extraction. To each aliquot, 50 µ L lysis buffer solution (200 mM Tris-HCl pH 8,0, and 800 µ g/mL Proteinase K) was added. Aliquots were incubated at 37°C overnight and subsequently at 100°C for 10 minutes, then centrifuged at 14.000 x g for 15 seconds. We used 5 µ L of the supernatant to perform PCR with the various primers.

We verified the intrinsic sensitivity of strains in accordance with the protocol devised by Bollela et al.[20] in order to establish the minimum concentration of bacilli that would be detectable by PCR with the various primers. Therefore, a suspension of Mtb H37Rv was prepared and adjusted to a 1.0 McFarland standard turbidity level (equivalent to 3×10^8 bacilli/mL). This suspension was subjected to successive 1:10 dilutions in sterile distilled water – up to a concentration of 3×10^{-1} bacilli/mL. For DNA extraction, each dilution was heated at 100ºC for 10 minutes and centrifuged at 13,500 rpm for 10 minutes. We then used 5 µ L of the supernatant for PCR. Intrinsic specificity of primers was verified in relation to the DNA of the following microorganisms: *Mycobacterium fortuitum, Mycobacterium avium-intracellulare, Shigella sonnei, Salmonella paratyphi A, Escherichia coli* and *Staphylococcus aureus*. The above-mentioned protocol was used for DNA extraction from these species.

The PCR for each target sequence in the DNA extracted from clinical samples and from the isolated strains was performed using a solution containing 10 mM of Tris-HCl at pH 8.3, 50 mM of KCl, 0.01% gelatin; 2 mM of $MgCl_2$, 200 µ M of dNTP (Sigma, St. Louis, MO, USA), 0.1µ M of each primer, 2U of *Taq* DNA Polymerase (Invitrogen, Melbourne, Australia) and 5 µ L of the extracted DNA (from the isolated strain, clinical sample or PCR product) – in a final volume of 50 µ L. Primer sequences for the respective target DNA and amplification parameters are described in Table 1. Primers were synthesized by Invitrogen, and we used a GeneAmp PCR System 2400 thermal cycler (Applied Biosystems, Foster City, CA, USA). A positive control containing Mtb H37Rv DNA and a negative control (reaction without DNA) were incorporated into each DNA amplification session.

Amplified products were electrophoresed on a 1.5% agarose gel. The agarose gel was subsequently stained with ethidium bromide and PCR products were displayed using an Eagle Eye II ultraviolet transilluminator (Stratagene, La Jolla, CA, USA).

Results were analyzed using the Kappa index to determine concordance between the two diagnostic methods (culture and PCR) and among the various primers. Co-positivity and co-negativity were analyzed using 2 X 2 tables.

RESULTS

Intrinsic sensitivity of the primers tested extended to a dilution of 3×10^0, that is, 3 bacilli/mL, except for MPB64 primers, which showed lower sensitivity since positive results were only obtained at a dilution of 3×10^2 bacilli/mL. Specificity of the various primers was considered high since no amplification occurred in DNA extracted from *M. fortuitum, M. avium-intracellulare, S. sonnei, S. paratyphi A, E. coli* and *S. aureus*.

In the sediment of clinical samples that were negative in smear microscopy and culture, PCR results were also negative with all primers tested. On the other hand, all AFB smear-positive clinical samples, as well as all isolated strains, were 100% positive in PCR, with the exception of the 89.4% result from PCR with MPB64 primers. Clinical samples that were negative for AFB, as well as the strains isolated from them, presented inconsistent PCR results depending on the primers tested.

TABLE 1
Sequences of primers and polymerase chain reaction parameters

Targets	Sequences of primers	Amplified product	Cycles	
IS*6110*	5´- CTC GTC CAG CGC CGC TTC GG - 3´	123 pb	94ºC – 1 min	
	5´- CCT GCG AGC GTA GGC GTC GG - 3´		66ºC – 1 min	35
			72ºC – 1 min	
IS*6110*	5´- GTG CGG ATG GTC GCA GAG AT - 3´	541 pb	94ºC – 1 min	
	5´- CTC GAT GCC CTC ACG GTT CA - 3´		65ºC – 1 min	35
			72ºC – 1 min	
	External:	419 pb	94ºC – 1 min	
38	5´- ACC ACC GAG CGG TTC GCC TGA - 3´		63ºC – 1 min	35
kDa	5´- GAT CTG CGG GTC GTC CCA GGT - 3		72ºC – 1 min	
	Internal:	322 pb	94ºC – 1 min	
	5´- TGA CGT TGG CGG AGA CCG - 3´		63ºC – 1 min	35
	5´- ATG GTG CCC TGG TAC ATG - 3´		72ºC – 1 min	
	External:	383 pb	94ºC – 1 min	
65	5´- GAG ATC GAG CTG GAG GAT CC - 3´		60ºC – 1 min	35
kDa	5´- AGC TGC AGC CCA AAA GGT GTT - 3´		72ºC – 1 min	
	Internal:	155 pb	94ºC – 1 min	
	5´- CCA TCG ATC CGA GAC CCT GCT CAA GGG C - 3´		60ºC – 1 min	35
	5´- TGC TCT AGA CTC CTC GAC GGT GAT GAC G - 3´		72ºC – 1 min	
MPB64	5´- TCC GCT GCC AGT CGT CTT CC - 3´	240 pb	94ºC – 1 min	
	5´- GTC CTC GCG AGT CTA GGC CA - 3´		50ºC – 1 min	35
			72ºC – 1 min	

Percentages of positivity for these samples are shown in Figure 1.

Table 2 shows the results of the concordance analysis between TB diagnosis through culture and that made by PCR in relation to the various primers studied, as well as the results of co-positivity and co-negativity testing, both using the isolation of Mtb as a diagnostic standard.

Concordance of TB diagnoses using PCR with the various primers studied, as well as diagnoses made through co-positivity and co-negativity testing, both using IS*6110* primer as a diagnostic standard due to its having the highest percentage of positivity, are shown in Table 3.

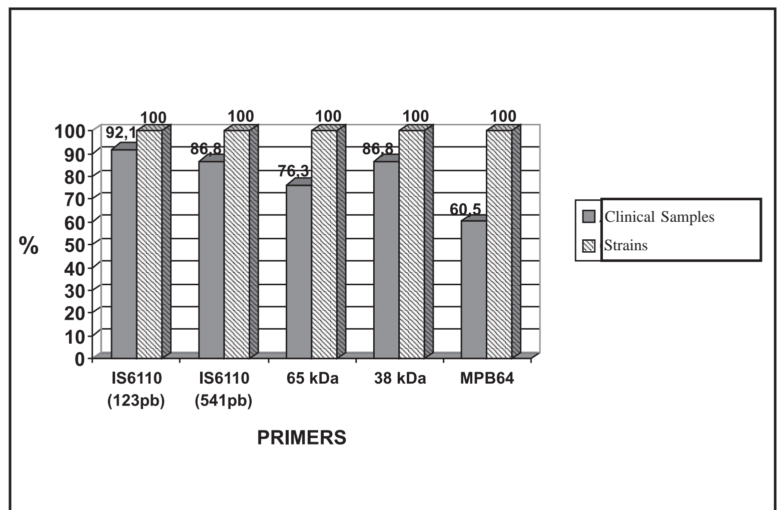

Figure 1. Percentages of PCR positivity in relation to the primers used in paucibacillary clinical samples and in *Mycobacterium tuberculosis* strains

DISCUSSION

Our results show that the target sequences IS*6110*, 65 kDa, 38 kDa and MPB64 were preserved in all strains studied and that the respective primers studied can be used in the diagnosis of TB using

PCR in clinical samples. In multibacillary samples, only primers for the MPB64 sequence (240 bp) were less than 100% efficacious, amplifying only 89.4% of such samples. In paucibacillary samples, percentages of positivity varied depending on the primers studied, as shown in Figure 1.

The percentage of positivity for the amplification of the 123-bp fragment of target IS*6110* in the present study (92.1%) was higher than that reported by Nolte et al.[21], Shawar et al.[22] or Montenegro et al.[2], who obtained 57.0%, 53.0%, and 76.7%, respectively. When we used primers that amplified a 541-bp fragment of target IS*6110*, we obtained 86.8% positivity. The proportions we noted were higher than those reported by Abe *et al.*[13], who obtained only 50% with these primers. Using 65-kDa primers, positivity after nested-PCR (65 kDa – 155 bp) was 76.3%, higher than that reported by Pierre et al.[14], who reported only 40%. As for 38-kDa primers, nested-PCR positivity was 86.8%, higher than the 38.4% reported by Miyazaki et al.[15]. The PCR for MPB64 presented the lowest positivity (60.5%) in paucibacillary samples. Our result was lower than the 70% reported by Martins et al.[23], after the inclusion of the nested-PCR step. However, in the present study, this step was not performed for MPB64.

The differences in positivity percentages between the present study and those in the literature may be related to slight changes in the adopted protocols, such as sample decontamination

TABLE2
Concordance and accuracy of polymerase chain reaction with various primers in relation to culture in Löwenstein-Jensen medium

		PCR									
		IS*6110* (123 bp)		IS*6110* (541 bp)		65 kDa		38 kDa		MPB64	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Mtb	Pos	54	03	52	05	48	09	52	05	32	25
culture	Neg	0	24	0	24	0	24	0	24	0	24
Total		81		81		81		81		81	
Observed concordance		96.3%		93.8%		88.9%		93.8%		69.1%	
Expected concordance		56.8%		55.8%		53.7%		55.8%		45.7%	
Kappa index (K)		0.9143		0.8604		0.7596		0.8604		0.4313	
Interpretation (K)		Excellent		Excellent		Good		Excellent		Normal	
Co-positivity		94.7%		91.2%		84.2%		91.2%		56.1%	
Co-negativity		100%		100%		100%		100%		100%	

PCR: polymerase chain reaction; Pos: Positive; Neg: Negative; IS*6110*: insertion sequence *6110*; Mtb: *Mycobacterium tuberculosis*

TABLE 3
Concordance and accuracy of DNA amplifications with various primers in relation to insertion sequence *6110* primers (123 bp)

Primers		IS*6110* (541 bp)		65 kDa		38 kDa		MPB64	
		Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
IS*6110*(123 bp)	Pos	51	03	47	7	51	3	32	22
	Neg	01	26	01	26	01	26	0	27
Total		81		81		81		81	
Observed concordance		95.0%		90.1%		95.0%		72.8	
Expected concordance		54.7%		53.1%		54.7%		46.5%	
Kappa index (K)		0.8909		0.7895		0.8909		0.4923	
Interpretation (K)		Excellent		Good		Excellent		Normal	
Co-positivity		94.4%		82.4%		94.4%		59.2%	
Co-negativity		96.3%		96.3%		96.3%		100%	

Pos: Positive; Neg: Negative; IS*6110*: insertion sequence *6110*

processes and variations in the composition of lysis buffer solutions. These results corroborate the statement made by Butcher et al. [24] to the effect that PCR positivity varies widely in samples that test negative in smear microscopy and positive in culture for Mtb, to which we add that variations in PCR protocols may also affect positivity.

Concordance of diagnosis, co-positivity and co-negativity among primers and cultures, using Mtb isolation as a standard (Table 2), shows that the primers amplifying the 123-bp IS*6110* fragment are highly indicated for the diagnosis of TB from clinical samples, independently of the smear microscopy results. Although diagnosis of TB was not confirmed through the use of these primers in 3 samples proven to contain Mtb (94.7% co-positivity), no false-positive results were found (100% co-negativity). It is important to highlight the fact that no false-positive results were found with any of the primers tested.

In the PCR analysis of the samples studied, only one of the primers specific for the amplification of the 123-bp IS*6110* fragment was unable to detect Mtb DNA, which was only detected by the primers amplifying the 541-bp IS*6110*, the 65 kDa and the 38 kDa target fragments, as shown in Table 3 (analysis of concordance between primer amplifications of the DNA). This occurred in a paucibacillary sample with the lowest number of isolated colonies per sample (four colonies in the three Löwenstein-Jensen culture tubes).

Multiple copies of the IS*6110* sequence are frequently present in the Mtb genome. This results in higher PCR sensitivity when compared to amplifications of single copy sequences (38 kDa, 65 kDa, and MPB64), even when these are submitted to two consecutive PCR reactions, such as in the PCR-nested PCR technique. The high percentage of positivity in the samples isolated in the state of Amazonas may indicate that the Mtb strains present a higher frequency of copies of this sequence in their genome, which can be verified through the restriction fragment length polymorphism. This premise, as well as the differences in PCR protocols, may explain the fact that proportions of positivity found in the present study were higher than those reported in the literature.

Our results show that the primer pairs studied are useful for the identification of Mtb genome sequences in colonies isolated from cultures, a strategy recommended by Kontos et al.[25]. The identification protocol using PCR within 24 to 48 hours after culture becomes more essential when there is an insufficient number of colonies since, in such a case, identification tests can only be performed after subculture, delaying the identification of the mycobacterium by at least 21 days.

Despite the small number of samples studied, we can conclude that, in the state of Amazonas, IS*6110* (123-bp) primers are the most highly recommended for PCR in clinical samples and in Mtb strains obtained from health care services that perform laboratory diagnosis of TB. In order to confirm these results, a greater number of samples and strains should be analyzed using the primers studied and that other laboratories in the state should also begin to employ this technique.

REFERENCES

1. García-Quintanilla A, Garcia L, Tudó G, Navarro M, González J, Anta MTJ. Single-tube balanced heminested PCR for detecting *Mycobacterium tuberculosis* in smear-negative samples. J Clin Microbiol 2000; 38:1166-9.
2. Montenegro SH, Gilman RH, Sheen P, Cama R, Caviedes L, Hopper T, *et al.* Improved detection of *Mycobacterium tuberculosis* in Peruvian children by use of a heminested IS*6110* polymerase chain reaction assay. Clin Infect Dis 2003; 36:16-23.
3. Shingadia D, Novelli V. Diagnosis and treatment of tuberculosis in children. Lancet Infect Dis 2003; 3:624-32.
4. Eisenach KD, Cave MD, Bates JH, Crawford JT. Polymerase chain reaction amplification of a repetitive DNA sequence specific for *Mycobacterium tuberculosis*. J Infect Dis 1990; 161:977-81.
5. van Soolingen D, de Haas PW, Hermans PW, Groenen PMA, van Embden JDA. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J Clin Microbiol 1993; 31:1987-95.
6. Kent L, McHugh TD, Billington O, Dale JW, Gillespie SH. Demonstration of homology between IS*6110* of *Mycobacterium tuberculosis* and DNAs of other *Mycobacterium* spp. J Clin Microbiol 1995; 33:2290-3.
7. McHugh TD, Newport LE, Gillespie SH. IS*6110* homologs are present in multiple copies in Mycobacteria Other Than Tuberculosis-causing mycobacteria. J Clin Microbiol 1997; 35:1769-71.
8. Salem JI, Marója MF, Carvalho FF, Lima MO, Feuillet A. Mycobacteria other than tubercle bacilli in sputum specimens from patients in Manaus Amazonas, Brasil. Acta Amazonica 1989; 19:349-54.

9. Salem JI, Gontijo Filho PP, Frebault VL, David HL. Isolation and characterization of mycobacteria colonizing the healthy skin. Acta Leprologia 1989; 7:18-20.
10. Fandinho FCO, Salem JI, Gontijo Filho PP, Marója MF, David HL. Mycobacterial flora of the skin in leprosy. Int J Leprosy 1991; 59:569-5.
11. Campelo CL, Vieira FD, Salem JI, Telles MAS, Maia R, Jardim SBV, *et al.* Tuberculose – diagnóstico laboratorial – Baciloscopia. Série Telelab. Brasília: Ministério da Saúde; 2001.
12. David LH, Lévi-frébault V, Thorel MF. Mèthode de Laboratoire pour Mycobacteriologie Clinique. Paris: Institut Pasteur, 1989; 39-58.
13. Abe C, Hirano K, Wada M, Kazumi Y, Takahashi M, Fukasawa Y, *et al*. Detection of *Mycobacterium tuberculosis* in clinical specimens by polymerase chain reaction and Gen-Probe amplified *Mycobacterium tuberculosis* direct test. J Clin Microbiol 1993; 31:3270-4.
14. Pierre C, Lecossier D, Boussougant Y, Bocart D, Joly V, Yeni P, *et al.* Use of a reamplification protocol improves sensitivity of detection of Mycobacterium tuberculosis in clinical simples by amplification of DNA. J Clin Microbiol 1991; 29:712-7.
15. Miyazaki Y, Koga H, Kohno S, Kaku M. Nested polymerase chain reaction for detection of *Mycobacterium tuberculosis* in clinical samples. J Clin Microbiol 1993; 31:2228-32.
16. Shankar P, Manjunath N, Mohan KK, Prasad K, Behari M, Ahuja, GK. Rapid diagnosis of tuberculous meningitis by polymerase chain reaction. The Lancet 1991; 337:5-7.
17. Ogusku MM, Sadahiro A, Hirata MH, Hirata RDC, Zaitz C, Salem JI. PCR in the diagnosis of cutaneous tuberculosis. Braz J Microbiol 2003; 34:165-70.
18. Davis LG, Kuehl WM, Battey JF. Basic methods in biology molecular. Connecticut: Appleton & Lange, 1994; 16-21.
19. Perosio PM, Frank TS. Detection and species identification of mycobacteria in paraffin sections of lung biopsy specimens by the polymerase chain reaction. Am J Clin Pathol 1993; 100:643-7.
20. Bollela VR, Sato DN, Fonseca BAL. McFarland nephelometer as a simple method to estimate the sensitivity of the polymerase chain reaction using *Mycobacterium tuberculosis* as a research tool. Braz J Med Biol Res 1999; 32:1073-6.
21. Nolte FS, Metchok B, McGowen JE, Jr. Edwards A, Okwumabna O, Thurmond C, *et al.* Direct detection of *Mycobacterium tuberculosis* in sputum by polymerase chain reaction and DNA hybridization. J Clin Microbiol 1993; 31:1777-82.
22. Shawar RM, El-Zaatari FAK, Nataraj A, Clarridge JE. Detection of *Mycobacterium tuberculosis* in clinical samples by two step polymerase chain reaction and nonisotopic hybridization methods. J Clin Microbiol 1993; 31:61-5.
23. Martins LC, Paschoal IA, Von Nowakonski A, Silva SAB, Costa FF, Ward LS. *Nested-PCR* using MPB64 fragment improves the diagnosis of pleural and meningeal tuberculosis. Rev Soc Bras Med Trop 2000; 33:253-7.
24. Butcher PD, Hutchinson NA, Doran TJ, Dale JW. The application of molecular techniques to the diagnosis and epidemiology of mycobacterial diseases. in: Mycobacterial disease - old problems, new solutions. (Suplement). J Appl Bacteriol 1996; 81:53S-71S.
25. Kontos F, Petinaki E, Nicolaou S, Gitti Z, Anagnostou S, Maniati M, *et al.* Multicenter evaluation of the fully automated Bactec MGIT 960 system and three molecular methods for the isolation and the identification of mycobacteria from clinical specimens. Diag Microbiol Infect Dis 2003; 46:299-301.