Standardization of in-house Polymerase Chain Reaction for the Identification of *Mycobacterium tuberculosis* at the Reference Tropical Disease Hospital in the State of Goiás, Brazil

Márcia Alves Vasconcelos Rodrigues/+, Álvaro Bisol Serafini, Marieta de Souza Pereira, Thathiane Dias da Silva*, Marcelo Fouad Rabahi, Suely Lemes de Alves***, André Kipnis**

Departamento de Microbiologia, Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás, 74605-050 Goiânia, GO, Brasil *Secretaria Estadual da Agricultura **Hospital de Doenças Tropicais, Secretaria Estadual de Saúde ***Laboratório Central, Goiânia, GO, Brasil

This study compares smear, growth in Lowenstein-Jensen medium, and in-house polymerase chain reaction (PCR) techniques for the detection of Mycobacterium tuberculosis. *A total of 72 specimens from 72 patients with clinical symptoms of tuberculosis, including 70 sputum and two bronchial aspirate samples, were tested in parallel by smear, culture, and in-house PCR techniques. From these, 48 (66.6%) were negative by the 3 methods, 2 (2.8%) were smear positive and negative by culture and in-house PCR, 11 (15.3%) were both smear and culture negative, and in-house PCR positive, 7 (9.7%) were positive by the 3 methods, 2 (2.8%) were positive by smear and culture, and negative by PCR, 2 (2.8%) were positive by culture and PCR, but smear negative. After the resolution of discrepancies in PCR results, the sensitivity and specificity for in-house PCR technique to* M. tuberculosis *relative to the culture, were 81.8% and 81.9%, respectively. These results confirm that this method, in-house PCR, may be a sensitive and specific technique for* M. tuberculosis *detection, occurring in both positive and negative smear and negative cultures.*

Key words: *Mycobacterium tuberculosis* - polymerase chain reaction - tuberculosis - culture - smear

The increased tuberculosis (TB) incidence and treatment drop-out rate, added to the appearance of multi-drug resistance prompted the World Health Organization (WHO) to consider TB as a worldwide urgency in 1993, and its control has again become a public health issue (Raviglione et al. 1995). According to this report at least 30 million people died from TB in the1990's (Dolin et al. 1994). It is estimated that one third of the world population, almost 2 billion people, is infected by *Mycobacterium tuberculosis*, and currently, approximately 8.8 million new cases and 1.6 million deaths are attributed to tuberculosis (WHO 2004).

In Brazil, TB prevalence in all clinical forms hit, in the year 2001, 71,080 cases, that is, 418.5/100.000 inhabitants. In the Center-West region of the country, there were 2341 cases, or 20.1/100.000 inhabitants, and the state of Goiás reported 1197 cases, or 23.92/100.000 inhabitants (Brasil 2002).

The TB diagnostic techniques currently used are slow and have sensitivities and specificities that need to be improved (CDC 1989). Although the presumptive diagnosis of TB may be obtained through clinical history and radiological findings, the final diagnosis still depends on smear and culture. The smear is an inexpensive and fast method, but presents low sensitivity and specificity since a minimum of 5000 bacilli are needed for a positive test. Although the culture is the reference test for diagnostic confirmation (Kox et al. 1994, Katila et al. 2000, Morán Moguel et al. 2000), it takes 4 to 8 weeks to reach a final result (Koneman et al. 1997).

The most promising technique for a fast diagnosis is based on the polymerase chain reaction (PCR), which is theoretically capable of detecting one copy of DNA of any cell (Bates 1979, Barnes & Barrows 1993). Besides the high sensitivity and specificity, this technique can produce results in few hours, and is being used in the diagnosis of a number of infectious diseases (Kocagoz et al 1993).

The aim of this work was to evaluate a in-house PCR procedure by comparing it to smear and culture in patients with suspicious TB. In addition, to improve TB diagnosis in order to assist a rapid and reliable diagnosis of patients from public hospitals from the Center-West region in Brazil.

MATERIALS AND METHODS

Patients and clinical specimens - The analyzed samples were sputum and bronchial aspirates collected from TB suspected patients at the state reference center, Hospital of Tropical Diseases (HDT), Goiás, Brazil, and obtained according to the medical ethics conduct code, and approved by the ethical research committees of this hospital and the Federal University of Goiás. The clinical assessment included the patient's medical history, signs,

Financial support: Funape (Brazilian Agency), proc. 66365
+Corresponding author. Fax: +55-62-202.12.36. E-mail: malves@iptsp.ufg.br
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symptoms, chest X-ray, pathology, and microbiology results, in addition to follow-up observations. All patients were from both sexes and none were human immunodeficiency virus type 1 infected or suffered from acquired immunodeficiency syndrome (AIDS). Patients with immunosuppression due to medications or radiotherapy are also excluded. Seventy-two samples were analyzed between March, 1999 and August, 2000. All samples were identified with patient's name and collection date, and sent to the Instituto de Patologia Tropical e Saúde Pública, of the Federal University of Goiás, to proceed with culture and molecular procedures.

Acid fast bacilli smear, culture and identification - Samples were decontaminated by the sodium lauryl sulfate method, and concentrated by centrifugation (13,000 x g for 5 min), examined by Ziehl-Neelsen staining, and inoculated at the same time in 2 tubes with Lowenstein-Jensen medium slants (L-J) and another 2 with L-J plus thiophene-2-carboxilic acid hydrazide (T2H) and p-nitrobenzoic acid (PNB). After weekly examination, the cultures that showed growth at 37ºC for 2 months were submitted to colony counting, and to biochemical identification based on growth time, presence of inhibitors, pigment production with PNB and T2H, and niacin production (Brasil 1994).

Standardization of PCR technique - Dilutions (on the number 1 MacFarland scale) were made from a pure culture of *M. tuberculosis*, producing serial dilutions 1:2; 1:4; 1:8; and 1:16. After, these dilutions were centrifugated and re-suspended with 100 µl of lysis buffer (50 mM of Tris.HCl at pH 8.0; 50 mM of KCl; 2.5 mM of $MgCl_2$; 0.45% of Tween 20; 0.45% of Nonidet P40, and 100 µg/ml of proteinase K). Lysis was carried out by incubation at 56ºC for 3 h, with enzyme inactivation by incubation at 95ºC for 15 min. From each dilution 2 µl were used for the PCR reaction to determine DNA detection sensibility of *M. tuberculosis*. The initial conditions for PCR were: 10 ng of each primer, Taq polymerase reaction buffer with 1.5 mM of $MgCl_2$, and one unit (U) of TaqDNA polymerase. The thermal cycler (Gene-Ataq-PharmaciaÒ) was programmed to promote a first denaturation at 94ºC for 1 min, and then 35 cycles of denaturation temperature of 92ºC for 1 min, annealing temperature of 60ºC for 1 min, and extension at 72ºC for 1 min.

The primers (Gibco BRL, US) used in the DNA amplification were specific to the *hsp*65 gene sequence of the members of *M. tuberculosis* complex. The pair of primer A and B (5'ACCAACGATGGTGTGTCCAT3' and 3' CTTGTCGAACCGCATACCCT5', respectively) amplify a 439 bp fragment of *hsp*65 gene. Next, a nested PCR was performed using primers pairs C and D (5'GAGATCGA GCTGGAGGATCC3' and 3'AGCTGCACCCCAAAG GTGTT5', respectively) to originate a 383 bp fragment inside the 439 bp product (Totsch et al. 1996).

PCR products were analysed in 2% agarose, containing ethidium bromide (1 mg/ml), and visualized on a UV transilluminator to verify the production of 2 fragments of 439 and 383 bp, to the first and second PCR, respectively.

After the definition of maximum dilution of detectable DNA, the optimization of $MgCl_2$ concentration was conducted by preparing different PCR tests from the minimum amplifiable dilution, with testing performed with 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; and 4.0 mM of $MgCl_2$, for the first and second PCR procedures. Similarly, the most cost-effective concentration of TaqDNA polymerase for 0.5; 1.0; 1.5; and 2.0 U was determined, using the best dilution of DNA and $MgCl_2$ as described above.

All batches of PCR reactions performed contained the DNA obtained from the pure culture in the first stage as a positive control, a negative sample consisted of DNA obtained from the clinical material of one individual without TB clinical suspicion, as well as another negative control consisted of ultra pure water instead of DNA (Kox et al. 1994).

DNA extraction method from clinical specimen - A second aliquot from the decontaminated clinical sample was stored at –20ºC for ulterior DNA extraction. The samples were concentrated by centrifugation, re-suspended in 100 µl of the same lysis buffer containing 100 µg of proteinase K/ml, and incubated for 3 h at 56ºC. After proteinase K inactivation by incubation at 95ºC for 10 min, lysed aliquots of 2.5 µl at 1/10 were used in PCR tests (Folgueira et al. 1993) using the same conditions as determinated by the standardization of culture sample.

Added to all experiments were 100 ng of DNA extracted from a *M. tuberculosis* strain cultured in L-J medium used as positive control, ultra pure water (MilliQ®, Millipore Corp.) as a negative control, and a sample of DNA of Lambda bacteriophage as molecular mass marker (Amersham-Pharmacia Biotech®) was also applied (Sambrook et al. 1989).

Determination of sensitivity and specificity - The sensitivity and specificity patterns of PCR compared to the *M. tuberculosis* culture in L-J medium were analysed and results evaluated in a 2 X 2 standard table (EPINFO v. 6.04).

RESULTS

The $MgCl_2$ range used varied from 0 mM to 4 mM, with 0.5 mM increments with optimum amplification resulting from 2 mM. Subsequent reactions were made from a Taq polymerase buffer containing Mg 2.5 mM in the final concentration (Fig. 1).

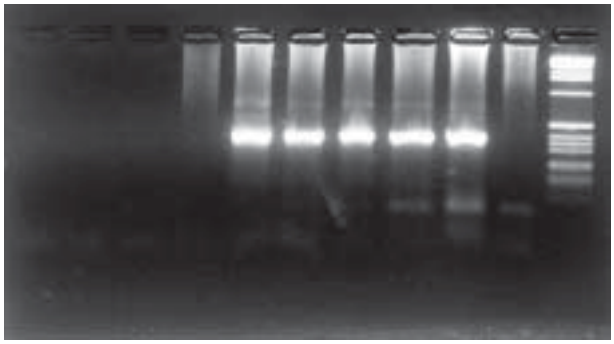

Fig. 1: concentration curve of magnesium for the determination of the optimum concentration. Band 1 to 9: 0; 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0 mM MgCl respectively; 10: negative control (water), 11: 1 kb ladder marker (Amersham-Pharmacia Biotech®)

To determine the technique's sensitivity, control *M. tuberculosis* DNA with serial dilutions was used (Fig. 2), and the highest dilution where a visible amplification was obtained was 10^{-8}, corresponding to a concentration of 1 pg/µl, and as 2 µl per DNA reaction were used, the sensitivity limit was of 2 fg of DNA. Although this sensitivity is very high, DNA extracted from the culture was used, which may present a different reality from the one found in the clinical samples due to the presence of interfering agents. From these results our positive control was used at 10^{-6} dilution (0.1 pg/µl).

The positive results for nested PCR of the samples from the patients with suspected TB (Fig. 3) show the amplification derived from the sputum samples with the size of the amplified fragment after the nested PCR from the *hsp*65 gene. In all three figures the arrow indicates the expected size of the amplified fragment after the nested PCR of this gene.

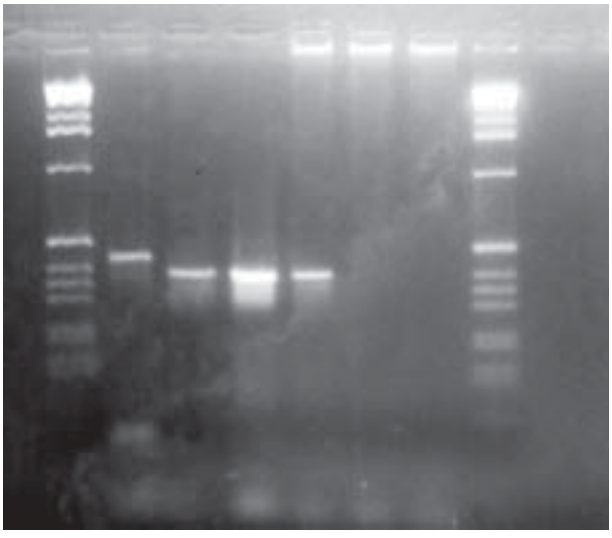

Fig. 2: determination of the detection sensitivity of *Mycobacterium tuberculosis* complex DNA. Band 1 and 7: 1 kb ladder marker (Amersham-Phamacia Biotech®); 2 to 6 dilutions: 10^{-4}; 10^{-6}; 10^{-8}; 10^{-10}; 10^{-12}, respectively; 8: negative control (water)

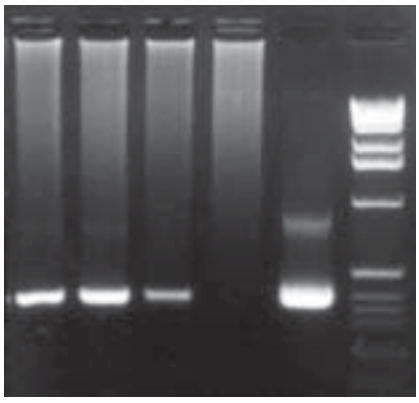

Fig. 3: result of the nested polymerase chain reaction of the *hsp*65 gene of some clinical samples from acid fast bacilli positive patients (bands 1-3) and AFB negative by smear or culture, (band 4); band 5: positive control; band 6: 1 kb ladder marker (Amersham-Phamacia Biotech®)

Our preliminary study of 72 respiratory specimens that included sputum and bronchial aspirates (Table I) from 72 patients showed that none of them were with antibiotic treatment. From these, 48 (66.6%) were negative by smear, culture and PCR techniques, 2 (2.8%) were smear positive and negative to both culture and PCR, 11 (15.3%) were smear and culture negative and positive by PCR, 2 (2.8%) were positive to smear and culture, but PCR negative, 2 (2.8%) were smear negative and positive to culture and PCR, and 7 (9.7%) were positive by the three methods.

The results provided a sensitivity of 81.8% (IC 95%: 47.7 - 96.8) and specificity of 81.9% (IC 95%: 69.6 - 90.2) in relation to the culture (Table II).

TABLE I

Number of positive smear, culture, and polymerase chain reaction (PCR) samples of clinic specimens

Clinical specimens	Smear - Culture - PCR -	Smear + Culture - PCR -	Smear - Culture - PCR +	Smear + Culture + PCR +	Smear + Culture + PCR -	Smear - Culture + PCR +	Total
Sputum	46	2	11	7	2	2	70
Bronchial aspirate	2	-	-	-	-	-	2
Total	48	2	11	7	2	2	72

-: negative; +: positive

TABLE II

Comparison of polymerase chain reaction (PCR) results and those of the culture for detecting the *Mycobacterium tuberculosis* complex

	Cultures with *M. tuberculosis*			
PCR reaction	Positive	Negative	Sensitivity (%)	Specificity (%)
Total n = 72			81.8	81.9
Positive	9	11		
Negative	2	50		

DISCUSSION

This study was conducted to validate a diagnostic protocol that we could use on AFB smear- and culture-negative specimens.

The observation of AFB in sputum samples or other biological material should be considered as TB suspected, but smear does not specifically identify *M. tuberculosis*. Moreover, this procedure is considered the least sensitive methodology for TB diagnosis because the estimated number of bacteria for positive smear is 10^3-10^5/ml (Kent & Kubica 1985, Forbes 1987, Brasil 1994). This justifies the negative smear result in two samples, in contrast with positive results in culture and PCR, which are more sensitive techniques (Morán Moguel et al. 2000).

Smear and culture showed negative results in 11 cases, while positive by PCR, probably due to the fact that the amount of mycobacteria present was not enough to be detected by these techniques, while the number for detection by PCR is in the order of 20 cells (Eisenach et al. 1990, Altamirano et a1. 1992) and in our own studies we could amplify from as low as one single bacterium in culture. Furthermore, the contamination of samples before, during, or after amplification could result in PCR positivity, while the conventional tests were negative (Kox et al. 1994, Morán Moguel et al. 2000), but we have ruled out this possibility with the inclusion of negative controls.

The 2 positive samples for smear and culture, with negative PCR, might have been developed by the fact that the protocol was specific to MTB complex, as well as the fact that the stain and the culture were not specific to the genus and species (Morán Moguel et al. 2000). On the other hand, the false-negative PCR result, in a negative smear sample and positive to *M. tuberculosis* in culture, could be explained by the presence of inhibitors of the amplifying system sample, that may be observed in 2.9% to 20% of the cases (Clarridge et al. 1993, Nolte et al. 1993, Burkardt 2000). Furthermore, these inhibitors were common in sputa samples, the same specimen where the PCR negative result was obtained in this study (Clarridge et al. 1993, Pfyffer et al. 1996). We have tried to ruled out PCR inhibitors by further diluting the samples that resulted negative in the PCR assay without any difference in results.

The PCR positive results may not necessarily reflect an active infection by *M. tuberculosis* because it is not possible to distinguish a previous infection from a minimum quantity of bacteria that does not have clinical significance and does not need treatment (Morán Moguel et al. 2000). Therefore, an approach to PCR positive results in asymptomatic patients, prior to considering them false-positive, must first discard the possibility of sample contamination, look into the patient's clinical history or whether the patient is in treatment (Cousins et al. 1992, Kox et al. 1994).

Sensitivity of the culture is considered low when biological samples are analyzed with a small number of mycobacteria that must necessarily be viable to yield positive results (Eisenach et al. 1993). On the other hand, in this study, the only criterion for sample selection was TB suspicion in any of its forms, because it is probable that, if more specific clinical criteria are established for a sample for PCR, there will be a significant increase in positive results, mainly when negative smears occur (Kritski et al. 1997).

Therefore, the PCR protocol showed a better sensitivity and specificity to TB diagnosis than the culture. In addition it can be executed in less than 48 h, making it possible to get faster results and minimizing the number of false-positive and -negative results (Cartuyvels et al. 1996, Morán Moguel et al. 2000). Finally, when negative PCR results display a discrepancy in relation to other clinical or laboratory criteria, the false-negative results should be discarded due to the presence of inhibitors, to the lack of any basic reaction component, to the use of inappropriate reagent concentrations, or to wrong temperatures (Eisenach et al. 1991, Clarridge et al. 1993).

The sensitivity (81.8%) and specificity (81.9%) of the PCR protocol used in this study is within the limits described by other authors for similar procedures (60% to 100%) (Beige et al. 1995, Moore & Curry 1995, Su et al. 2000), although Lim et al. (2000) found a low sensitivity (44%) and a similar specificity (99%). Padilla et al. (2001), when using the PCR with reverse hybridization line-probe assay to *Mycobacterium* sp., obtained a sensitivity and specificity of 100%.

Similarly, Mehrotra et al. (2002) developed an in-house PCR procedure, a less expensive method, for detection of *M. tuberculosis* in cytological specimens, and obtained the same results when compared to the Roche Amplicor kit. Kontos et al. (2003) identified all recovered cultured mycobacteria by a in-house molecular method.

In conclusion, our study shows that the PCR technique have advantages over AFB smear and culture for predicting diagnosis results in specimens containing a low number of organisms, and this situation could lead to earlier initiation of appropriate therapy and epidemiological intervention. The technique should be used together with the traditional methods of tuberculosis diagnosis until some of the technical limitations of this technique are solved.

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