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Article

Clinical Evaluation of an In-House IS*6110* Polymerase Chain Reaction for Diagnosis of Tuberculosis

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Abstract The aim of this study was to clinically validate a heminested polymerase chain reaction (PCR) method, based on the IS*6110* insertion segment of *Mycobacterium tuberculosis* complex, for the diagnosis of tuberculosis. Samples of pulmonary, extrapulmonary and blood origin were collected prospectively from 331 patients. All samples were processed to detect acid-fast bacilli by direct stain, culture and PCR. The gold standard comparison was a clinically based final case definition of tuberculosis corresponding to group 3 of the American Thoracic Society's classification system. The sensitivities of stain, culture and PCR were 41%, 65% and 59%, respectively. Overall specificity exceeded 97% for all techniques. The combination of PCR and direct stain achieved a sensitivity similar to that of culture alone. The PCR method detected 74 of 95 (78%) culture-positive results. In a hospital setting, PCR could be a useful, reliable tool for diagnosis of tuberculosis and may be introduced as a complementary routine diagnostic laboratory method.

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Introduction

Current policy for control of tuberculosis (TB) consists of early identification and cure of infectious cases. Most infectious cases are detected by direct-smear microscopy examination of sputum, and treatment is usually started before obtaining laboratory confirmation. Microscopy for acid-fast bacilli (AFB) is a rapid, simple diagnostic method. Although its sensitivity ranges from 22% [1] to 78% [2], its specificity remains high (99% [1] to 100% [3]). The sensitivity of direct staining may vary as well, depending on the clinical radiological findings, ranging from 52% in patients with tuberculous caverns to 32% in patients with pulmonary infiltrates only [4]. Culture is more sensitive than direct staining for detection of AFB, but it takes longer. Culture requires an average of 21.7 days to obtain results; even the Bactec radiometric technique (Becton Dickinson, USA) requires 13.4 days to produce results [5]. The sensitivity of culture ranges from 50% [6] to 81% [7], estimates that can also vary depending on the clinical presentation. Thus, a more rapid, sensitive and specific diagnostic assay, such as PCR, could contribute to the control of *Mycobacterium tuberculosis* [8–11]. PCR may overcome some difficulties associated with conventional diagnostic techniques, such as the large number of bacilli required for positive stained smears and the slow growth of mycobacteria in selective media [12].

Several studies validating PCR as a tool for diagnosis of TB have recently been published. Clarridge et al. [13] reported a very high sensitivity using an IS*6110*-targeted PCR system. Overall, the sensitivity and specificity of PCR ranges from 50 to 100% [14–19]. It has been suggested that this wide range may be due to the variability of PCR results in different laboratories [20, 21].

In this study we evaluated the clinical validity and applicability of a PCR method based on the IS*6110* insertion segment for the detection of *Mycobacterium tuberculosis* in HIV-negative patients. The PCR was compared with direct staining and culture. The results of all diagnostic methods were compared with a clinically based case definition of TB, which is our gold standard, and sensitivity and specificity values were calculated.

Materials and Methods

Study Population. The study was conducted at the Hospital Clinic of Barcelona from 1 March 1994 to 29 February 1996. In order to identify all TB patients promptly, a list of those patients admitted to the hospital who were started on isoniazid treatment was obtained from the Pharmacy Department every day. In addition, a list of the patients being followed-up in the TB outpatient clinic was obtained twice a week. On the basis of this information, the patients were classified into the following categories: new HIV-positive TB cases, new HIV-negative TB cases, TB cases with more than 1 week of treatment, patients undergoing prophylactic treatment, or non-TB cases. Following receipt of informed consent, all HIV-negative patients who started anti-TB treatment were included. TB treatment was a short-course, rifampicin-based 6-month chemotherapy regimen (rifampicin, isoniazid and pyrazinamide for the first 2 months, followed by 4 months of rifampicin and isoniazid). Pulmonary or extrapulmonary clinical samples for diagnostic purposes were collected, according to the clinical presentation in each case, at the time of diagnosis and before treatment was initiated. Blood samples were also obtained from all patients at the same time.

Another two control groups of volunteers, who were supposed to be non-TB individuals, were also recruited. Group 1 comprised third-year students attending the School of Medicine, University of Barcelona, and group 2 comprised patients with chronic obstructive pulmonary disease (COPD) without clinical signs of TB who were admitted to the Pneumology Department. HIV antibodies were determined and tuberculin skin tests performed anonymously; signed, informed consent was obtained in all cases.

Gold Standard and Case Definition. The following case definitions [22] were established before the study was started. The final definition (at the end of treatment) was determined by an investigators' consensus, taking the following criteria into consideration: clinical picture, chest radiographic findings, tuberculin skin test, microbiology results, other laboratory findings, computed axial tomography scan (when available) and clinical response to anti-TB drug therapy. According to this data, the cases were classified into group 3 of the American Thoracic Society (ATS) classification described below [23]. Doubtful cases were defined by a lack of consensus of two or more authors. Initial definition represents the situations that clinicians face the first day of treatment. According to this data, the patients were classified as belonging to group 3 of the ATS classification. Classification was determined by two independent TB expert reviewers, based on the initial clinical history recorded, without seeing the patient, and before confirmation by any microbiological laboratory result. Undefined cases were established by lack of agreement between the two reviewers.

The American Thoracic Society [23] classifies people exposed to *Mycobacterium tuberculosis* into six groups. Group 0 comprises persons not exposed, not infected and tuberculin skin test (PPD)-negative. Group 1 comprises those people who have been exposed to tuberculous patients but who are PPD-negative. Group 2 comprises infected (positive PPD test) persons who have no clinical, radiological or microbiological signs of TB. Group 3 comprises patients with active TB who have clinical or radiological evidence of TB. Ideally, the culture should be positive. If the culture is negative, the PPD test must be positive. Group 4 comprises persons with nonactive TB, defined as cases that are PPD-positive, without clinical signs of active TB but with positive microbiological data or radiological signs that suggest TB in the past. Group 5 comprises patients suspected to have TB but for whom confirmation is unavailable and diagnostic studies are incomplete.

Microbiological Processing of Clinical Samples. All clinical samples were processed at the microbiology laboratory of the Hospital Clinic. Laboratory technicians were blinded to the group of patients from which each clinical sample came.

Nonsterile samples were digested and decontaminated by the NaOH-NALC (N-acetyl-L-cysteine) method [24] before culture. Sterile samples were divided into two equal aliquots, one for culture and smear and the other for amplification procedures. The latter aliquot, was submitted to decontamination as a nonsterile specimen in order to obtain an appropriate sample for the amplification technique. Liquid samples such as cerebrospinal fluid, joint fluid, abdominal fluid, pleural fluid and urine were concentrated by centrifugation if more than a 1 ml volume was available [24]. The tissue biopsies were ground and processed as the remaining samples. Smears of all samples (except blood) were prepared and stained with auramine-rhodamine fluorochrome stain. Determination of semiquantitative grade was made in a 1 to 4 scale [24].

For all samples, aliquots of 200 μl were inoculated onto Löwenstein-Jensen slants and incubated at 37 °C for up to 8 weeks. In addition, 500 μl of the sediment was inoculated into radiometric Bactec 12B medium and incubated at 37 °C for 6 weeks. Blood samples were cultured directly by adding 5 ml to a Bactec 13A bottle and incubated following the same scheme. Solid medium slants were read weekly and Bactec cultures were read eight times over 6 weeks. A growth index of 200 on Bactec medium was considered positive and was further confirmed by Ziehl-Neelsen stain. Solid medium slants were considered positive when colonies grew; growth on positive slants was further confirmed as mycobacteria by Ziehl-Neelsen stain.

Strains were identified using the morphological aspect on Bactec (Becton Dickinson, USA) [25] smears and the AccuProbe (Gen Probe, USA) method. When identification was inconclusive, gas chromatography [26] and routine biochemical methods were used [24].

A 0.5 ml aliquot of the pellet remaining after decontamination was obtained for each sample except blood. Blood samples were processed as follows: leukocytes were extracted from 3 ml of total blood by Ficoll-Hypaque (1119) gradient, washed and resuspended in Tris-EDTA buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.0) [27]. Two 0.5 ml aliquots were obtained. Aliquots were stored at –30 °C until amplification procedures were performed.

A single-tube heminested PCR with unlabeled primers was used for amplification [28, 29]. This method is a modification of the tube-nested technique described by Wilson et al. [30].

Aliquots were heated at 95 °C for 30 min and concentrated by centrifugation at 12,500 × *g* for 15 min. The pellet was resuspended in tenfold TE buffer with 50 mg/ml of lysozyme. This suspension was incubated for 90 min at 37 °C, after which it was centrifuged for 10 min. Proteinase K and sodium dodecyl sulfate (SDS) were added to the final concentrations of 250 μg/ml and 1% (w/v) and incubated for 30 min (or overnight) at 43 °C. The suspension was extracted with phenol chloroform (25:24:1) twice and chloroform-isoamyl alcohol (24:1) twice and was precipitated with two volumes of ethanol 100% and 0.2 M NaCl and stored at –20 °C overnight. It was then washed with 70% ethanol, dried and resuspended in 100 μl of distilled water. Ten microliters was used for PCR amplification.

The DNA target used was the repetitive sequence IS*6110*, which is found in the members of *Mycobacterium tuberculosis* complex at varying copy numbers [31, 32]. The outer primers used were Tb294 (5′-GGACAACGCCGAATTGCGAAGGGC-3′) and Tb850 (5′-TAGGCGTCGGTGACAAAGGCCACG-3′), which generate a product of 580 base pairs (bp), and the inner primer used was Tb505 (5′-ACGACCACATCAACC-3′) [30], which generates a final fragment of 369 bp.

The outer primers Tb294 and Tb850 were both used at a concentration of 10 nM, and the inner primer Tb505 was used at a concentration of 500 nM. The amplification was performed in 0.5 ml PCR tubes with a final volume of 50 μl using a 480 thermal cycler (Perkin-Elmer, USA). Each reaction tube contained 2.5 U of *Taq* DNA polymerase (Gibco BRL, USA), 2.5 U of uracil-N-glycosylase (UNG) (Boehringer Mannheim, Germany) in order to avoid contamination with the products of previous reactions [33], 200 μM each of dATP, dCTP, dGTP and 600 μM dUTP (Boehringer Mannheim), final buffer 1×, 2 mM of $MgCl_2$ and 20 μl of sample. After incubating for 15 min at 25 °C to allow UNG to act, the temperature was raised to 94 °C for 5 min to inactivate the enzyme. The thermal cycler is programmed to perform the PCR in two stages that are distinguished by different annealing temperatures. The first stage of amplification involves 30 cycles of denaturation at 94 °C for 45 s, with primer annealing and extension carried out in one step at 72 °C for 1 min 30 s. The second stage included 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 1 min and extension at 72 °C for 30 s, after which the reaction mixture was held at 72 °C.

To detect sample inhibitors, a duplicate tube of 50 μl of PCR mix for each sample was spiked with 5 μl of an aqueous solution containing 43 pg of purified DNA target. In order to maintain reagent proportions, 5 μl of the original sample was added to these duplicate tubes instead of 10 μl. To avoid cross-contamination, DNA purification, amplification and detection was carried out in a separate room from that in which the buffers were prepared. Samples were processed in lots of 20, including one extraction-negative control and one amplification-negative control. A positive control was also included in each lot of samples.

Twenty microliters of amplified product was analyzed by ethidium bromide (0.5 μg/ml; Sigma) electrophoresis in 2% (w/v) agarose gel. The gels were visualized and photographed on an ultraviolet transilluminator. The presence of a band in the same position as that shown by a positive control and spiked duplicates, namely 369 bp, was considered positive.

Specificity Testing of the Amplification Method Using Bacteria and Mycobacterial Strains. The following 13 strains of six mycobacterial species and 18 strains of eight bacterial species were assayed: *Mycobacterium tuberculosis* ($n=5$), *Mycobacterium xenopi* ($n=3$), *Mycobacterium kansasii* ($n=2$), *Mycobacterium avium* ($n=1$), *Mycobacterium scrofulaceum* ($n=1$), *Mycobacterium gordonae* ($n=1$), viridans group streptococci ($n=2$), coagulase-negative staphylococci ($n=3$), *Proteus mirabilis* (n = 1), *Klebsiella pneumoniae* ($n=2$), *Enterococcus* spp. ($n=2$), *Staphylococcus aureus* ($n=3$), *Escherichia coli* ($n=2$) and *Pseudomonas aeruginosa* ($n=3$). All strains were isolated previously in clinical samples. For each strain a suspension of 5 ml of distilled water was prepared for 1 McFarland bacterial density. Dilutions were prepared, and 0.5 ml aliquots for several concentrations were obtained. For *Mycobacterium tuberculosis* the concentrations studied were 10^7 cfu/ml, 10^5 cfu/ml, 10^3 cfu/ml and 10^2 cfu/ml. For the remaining species the concentrations used were 10^7 cfu/ml, 10^5 cfu/ml and 10^3 cfu/ml. The 0.5 ml aliquots were processed for lysis, DNA extraction, amplifications and detection following the same protocol as that used for the clinical samples.

Statistical Methods. Results were expressed as proportions, and 95% confidence intervals were calculated. Sensitivity and specificity were calculated using standard methods [34], comparing the results of each diagnostic method with the case definitions. Statistical differences between sensitivity and specificity of different diagnostic methods were tested by the McNemar exact binomial test. Degree of agreement between the case definitions was measured through Kappa statistic index. A Kappa value of 1 indicates perfect agreement and a value of zero means no agreement. Cases not well defined due to lack of case definition agreement were not considered in the analysis. A significance level of 5% was chosen for all statistical tests, using Bonferroni's correction in multiple tests.

Results

Following the recruitment system described, a total of 553 patients started receiving isoniazid treatment during the study period or were attended in the TB outpatient clinic. One hundred ninety-eight (36%) were found to be HIV-positive, 39 had previously started anti-TB treatment, 53 were receiving TB prophylaxis and 25 were lost to follow-up. Therefore, a total of 238 HIV-negative TB patients were included in the study group. The mean age of patients in the study was 48 years (95% CI, 46–51), with a range of 17 to 91 years. One hundred sixty-six (69%) were male. The other TB-free cohorts comprised 46 students and 47 COPD patients. The students' mean age was 20 years, 67% being female. The mean age of the COPD patients was 68 years, ranging from 42 to 82 years, with only 13% being female.

Clinical samples were collected from 331 study and control individuals (Table 1). In total, 316 samples were available for stain, 319 for culture and 314 for PCR. Additionally, blood samples were collected from 261 individuals.

Clinical samples were of pulmonary origin (sputum, bronchial aspirate, bronchoalveolar lavage and gastric fluid) in 270 individuals, including all controls, and of extrapulmonary origin (lymph nodes, feces, joint fluid, abdominal fluid, cerebrospinal fluid, pleural effusion, bone marrow aspiration, urine, vertebral bone biopsy and pus) in 50 individuals; 11 samples were insufficient

Table 1 Results of stain, culture and PCR, according to sample origin

Type of sample	PCR result	Negative culture			Positive culture			No culture, no stain available	Total
		Negative stain	Positive stain	NA	Negative stain	Positive stain	NA		
Respiratory	negative	173			10	9			192
	positive	2	3		19	40	1		65
	NA	3			1			1	5
Gastric fluid	negative	4			1				5
	positive					3			3
	NA								
Lymph node	negative	3							3
	positive				1	2			3
	NA								
CSF	negative	4		1					5
	positive				1				1
	NA			1					1
Pleural effusion	negative	16			1				17
	positive	3			5				8
	NA								
Urine	negative	2	1						3
	positive								
	NA								
Other[a]	negative	7							7
	positive				1	1			2
	NA								
Insufficient	negative								
	positive								
	NA							11	11
Total		217	4	2	40	55	1	12	331

[a] Includes 2 vertebral bone samples, 1 bone marrow sample, 1 fecal sample, 1 peritoneal fluid sample, 1 joint fluid sample and 3 samples of nonspecific origin

NA, not available; CSF, cerebrospinal fluid

to perform any test. Laboratory results of stain, culture and PCR were negative for all the students and COPD cohort members, although one COPD patient had an isolated positive blood sample result.

One case of cross-contamination during extraction was detected. New aliquots of the samples affected in the same lot were prepared and tested again, and previous aliquots were rejected.

Thirty-four of 314 (10.8%) clinical samples (excluding blood samples) from patients and control individuals showed evidence of inhibition; among blood samples, this percentage was 32.1%.

The presence of inhibitors was demonstrated in five (22.7%) microbiologically confirmed cases for which PCR results were negative. Likewise, 29 (13.8%) of those with negative results by PCR and no microbiological confirmation showed the presence of inhibitors.

The specificity testing of the amplification method used with bacteria other than mycobacteria gave the following results: none of the 18 bacterial strains belonging to eight different species assayed at three different concentrations showed amplification product in the gel electrophoresis analysis. Moreover, no amplification was obtained from the eight strains of five mycobacterial species included in the test. All five *Mycobacterium tuberculosis* isolates amplified the specific band in gel electrophoresis when tested at the 1×10^7 cfu/ml, 1×10^5 cfu/ml and 1×10^3 cfu/ml concentrations. However, only one of the five strains amplified the 1×10^2 cfu/ml concentration. According to these results, the sensitivity limit of this PCR method is around 1,500 to 3,000 cfu/ml.

On the basis of the final case definition, there were 141 TB cases, 85 non-TB cases and 12 poorly defined cases (Table 2). Overall, PCR was more sensitive than stain, reaching statistically significant differences for extrapulmonary ($P=0.035$) and total samples ($P<0.0001$) but not for pulmonary samples. Compared with culture, PCR had a lower non-statistically significant sensitivity. All methods showed a high and similar specificity. Culture appeared to be more sensitive than stain for both pulmonary and total samples ($P<0.0001$), but the difference was not significant for extrapulmonary samples.

Table 3 shows the results of PCR and the PCR-stain combination compared with the results of culture. A total of 96 of 331 culture results were positive for AFB. Overall sensitivity values were below 87%. PCR was more sensitive than stain, reaching statistically significant differences for extrapulmonary ($P=0.024$) and

Table 2 Sensitivity and specificity of each test according to sample origin, compared with the final case definition of tuberculosis

Test	Total samples				Pulmonary samples				Extrapulmonary samples			
	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Stain	57/138 (41.3)	33.0–50.0	75/77 (97.4)	90.9–99.7	53/109 (48.6)	38.9–58.4	57/59 (96.6)	88.3–99.6	4/29 (13.8)	3.9–31.7	18/18 100	81.5–100[a]
Culture	92/140 (65.7)	57.2–73.5	74/78[a] (94.9)	87.4–98.6	80/110 (72.7)	63.4–80.8	55/59[a] (93.2)	83.5–98.1	12/30 (40)	22.7–59.4	19/19 (100)	82.4–100[b]
PCR (main samples)[c]	82/139 (59.0)	50.3–67.3	76/76 (100)	95.3–100[b] (62.4)	68/109	52.6–71.5 (100)	58/58	93.8–100[b] (46.7)	14/30	28.3–65.7 (100)	18/18	81.5–100[b]
Stain & PCR combined	89/137 (65.0)	56.4–72.9	74/76 (97.4)	90.8–99.7	74/108 (68.5)	58.9–77.1	56/58 (96.6)	88.1–99.6	32.5–70.6 (51.7)	18/18	81.5–100[b] (100)	
PCR (blood samples)	13/104 (12.5)	6.8–20.4	52/52 (100)	93.2–100[b]								

[a] Two cases of *Mycobacterium xenopi* and 2 cases of *Mycobacterium kansasii* infection
[b] One side 97.5% confidence interval
[c] Samples other than blood are included

Table 3 Sensitivity and specificity of each test by sample origin, compared with the results of culture

Test	Total samples				Pulmonary samples				Extrapulmonary samples			
	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Stain	55/95 (57.9)	47.3–68	126/130 (96.9)	92.3–99.2	52/83 (62.7)	51.3–73	91/94 (96.8)	91–99.3	3/12 (25)	5.5–57.2	35/36 (97.2)	85.5–99.9
PCR	74/95 (77.9)	68.2–85.8	121/129 (93.8)	88.1–97.3	63/83 (75.9)	65.3–84.6	87/92 (94.6)	87.8–98.2	11/12 (91.7)	61.5–99.8	34/37 (91.9)	78.1–98.3
Stain & PCR combined	82/94 (87.2)	78.8–93.2	119/128 (93)	87.1–96.7	71/82 (86.6)	77.3–93.1	87/92 (94.6)	87.2–98.2	11/12 (91.7)	61.5–99.8	32/36 (88.9)	73.9–96.6

total samples ($P<0.047$) but not for pulmonary samples. Both methods showed a high and similar specificity.

Based on the initial case definition (data not shown), 120 cases were classified as TB cases, 108 as non-TB cases and 10 as undefined cases. Sensitivity, for total samples, was as follows: stain, 35% (95% CI, 26.5–44.4); culture 59% (95% CI, 49.4–67.8), PCR, 53% (95% CI 44.0–62.6); and combined stain-PCR, 58% (95% CI 49.1–67.7). The difference between PCR and stain reached statistical significance for total samples ($P<0.0001$) but not for extrapulmonary and pulmonary samples. PCR and culture had similar sensitivity. No differences in specificity were shown for any method.

In the samples submitted to the three assayed tests, the agreement between culture and both (initial and final) case definitions was as follows: (i) when comparing culture and the final case definition, there was 83.2% agreement and a Kappa index of 0.65 ($P<0.001$); (ii) when comparing culture and the initial case definition, there was 76.7% agreement and a Kappa index of 0.49 ($P<0.001$); (iii) when comparing the initial and final case definitions, there was 87.4% agreement and a Kappa index of 0.74 ($P<0.001$).

Discussion

This study shows that PCR is not more sensitive than culture, but that it is more sensitive than stain for extrapulmonary but not for pulmonary samples. Overall, culture is the most sensitive technique, However, the combination of PCR and stain, both of which are faster than culture, has a sensitivity similar to that of culture. When the non-TB groups were considered, the same pattern was seen. By adding "healthy" individuals, the estimates for specificity were more precise and slightly higher, but the sensitivity parameters remained the same as those presented in Tables 2 and 3. These results are comparable to those published recently and based on in-house methods or commercial kits; sensitivity rates reported ranged from 58 to 64% [14, 35–43]. Reports comparing commercial and in-house methods [44, 45] showed in-house techniques to be more sensitive. The lack of standardization and a poor reproducibility have been argued as limitations for the routine use of in-house techniques [14], but the same phenomenon has been demonstrated with commercial kits [46].

We also studied the value of heminested PCR in blood samples for diagnosis of TB in nonimmunosuppressed individuals (as far as testing for HIV is concerned). Thirteen of 104 blood samples collected from patients with confirmed TB (final definition) were positive by PCR, whereas only one of 23 classified control samples was positive.

The presence of inhibiting substances found in this study is similar to that reported by others, ranging from 5 to 16% [47, 48]. Blood samples showed a higher percentage of inhibition (23%). Inhibition caused a problem of misclassification, which was reflected in the calculation of the sensitivity rate for PCR; the sensitivity of PCR might be higher if inhibiting substances are avoided. Blood is considered to contain a high level of inhibiting substances such as hemoglobin, although recently it has been demonstrated that pretreatment with SDS may remove the hemoglobin, at least partially [49].

We conducted this prospective study within a hospital setting to test the possibility of introducing PCR as part of the routine diagnosis. It is impossible to generalize the results for other settings. Nevertheless, these results are similar to those found elsewhere and also correspond with those found in an epidemiological study of TB in Barcelona [50]. In the design stage, we intended to include some negative controls to validate the PCR test. However, we excluded them from the primary analysis on the basis of the likelihood of introducing bias. Indeed, the total number of controls was arbitrary and we found that the bigger the number, the higher the specificity (data not shown). Nevertheless, given the fact that specificity is, in any case, high, this approach seems to be valid for evaluating sensitivity, the estimates of which did not change at all. The gold standard remains the critical issue. Our data shows that culture and the final definition seem to have similar value as a gold standard for validating a new diagnostic technique. Although this study had a rather long follow-up, its prospective design allowed us to compile all the essential data required to determine the case definition, on which the analysis was based.

We used a diagnostic method developed by our group [28, 29], a one-tube heminested PCR based on a modification of a nested PCR described by Wilson et al. [21]. This technique targeted the most commonly used fragment, the insertion sequence IS*6110* [16–19, 31]. In order to test the specificity of the amplification technique described, several bacterial and mycobacterial strains were tested at different concentrations. None of these strains amplified the 369 bp band. This was confirmed when samples from non-TB patients were tested, showing the high specificity of the amplification technique used. In addition, five *Mycobacterium tuberculosis* strains at four different concentrations of mycobacteria were tested in order to approximate the threshold of this technique. The results of this test showed a sensitivity of around 1,000 to 3,000 cfu/ml, which may be lower than that reported by several authors in the initial studies applying this methodology to the diagnosis of TB [16–18]. However, the clinical evaluation of the method described in the present study showed a sensitivity comparable to that of other methods that were evaluated using clinical cases,

including a representative proportion of smear-positive and smear-negative cases, despite the different protocols applied in extraction, purification and amplification [14, 35–45]. This could mean that, with the current methodology, the sensitivity of PCR techniques is similar to that described here. This conclusion corresponds with the results obtained in a multicentric study using artificial samples with a known number of mycobacteria [21]. On the other hand, it could be argued that our PCR sensitivity estimates could be lower because *Mycobacterium tuberculosis* strains lack the insertion segment IS*6110*, as has recently been found in Asian patients. Nevertheless, the spread of this strain appears to be limited [51–53]: most *Mycobacterium tuberculosis* strains have 2–20 copies, and only 1 to 5 copies are present in *Mycobacterium bovis* strains [16, 31]. In order to overcome the well-known PCR-related sensitivity and specificity problems, the heminested technique includes a second step that enhances the sensitivity by amplifying a second set of primers [54] and that enhances specificity by reducing the need for manipulation and, therefore, decreasing the risk of contamination. This technique has the advantage of a much lower cost when compared with a conventional two-stage nested PCR, which requires more reagents. In the present study, the sensitivity and specificity of the amplification technique used were the main focus, and therefore, the extraction technique used was considered a reference, despite being laborious. However, from a theoretical point of view, other, faster extraction methods could be applied to obtain results the same day.

In conclusion, we consider that the PCR cannot, at present, replace culture for the final confirmation of clinical cases because of its lower sensitivity and the additional need to have growth of *Mycobacterium tuberculosis* colonies for susceptibility testing and other special studies. However, the use of stain and PCR combined greatly increases the number of cases detected before culture results are available; therefore, it is recommended that PCR be introduced into the hospital laboratory as part of the standard procedures for the diagnosis of tuberculosis.

The PCR technique is a fast, reliable method for the diagnosis of TB, but its sensitivity, total automation and cost should be improved in the future. In the future, PCR results will probably need to be considered for the classification and treatment of TB, just as the results of stains are now considered in developed countries.

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