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Rapid Diagnosis of Extrapulmonary Tuberculosis by PCR: Impact of Sample Preparation and DNA Extraction

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In cases of suspected extrapulmonary tuberculosis, rapid and accurate laboratory diagnosis is of prime importance, since traditional techniques of detecting acid-fast bacilli have limitations. The major difficulty with mycobacteria is achieving optimal cell lysis. Buffers used in commercial kits do not allow this complete lysis in a number of clinical specimens. A comparison of two sample preparation methods, pretreatment with proteinase K (PK-Roche) and complete DNA purification (cetyltrimethylammonium bromide [CTAB]-Roche), was conducted on 144 extrapulmonary specimens collected from 120 patients to evaluate the impact on the Cobas-Amplicor method. Thirty patients were diagnosed with tuberculosis, with 15 patients culture positive for *Mycobacterium tuberculosis*. Amplification and detection of the amplicons were impaired by a high number of inhibitory specimens (39 to 52%). CTAB-Roche allowed the detection of more culture-positive specimens by PCR than PK-Roche. Comparison with the final diagnoses of tuberculosis confirmed that CTAB-Roche produced the best sensitivity (53.8%) compared to culture (43.3%), PK-Roche (16%), and smear (13%). However, the specificity of the PCR assay with CTAB-Roche-extracted material was always lower (78.8%) than those with culture (100%) and PK-Roche (96.5%). False-positive specimens were lung biopsy material, lymph node biopsy material and aspirate, or bone marrow aspirate, mainly from immunocompromised patients. Despite the efficiency of complete DNA extraction for the rapid diagnosis by PCR of extrapulmonary tuberculosis, the false-positive results challenge our understanding of PCR results.

In microbiology, DNA amplification using PCR has allowed great progress to be made in the rapid and accurate diagnosis of infections due to organisms that are not cultivable by in vitro means, that require complex media or cell cultures and prolonged incubation times, or for which culture is too insensitive. Amplification techniques for the diagnosis of tuberculosis have attracted considerable interest, particularly with the hope of shortening the time required to detect and identify *Mycobacterium tuberculosis* in respiratory and nonrespiratory specimens (27, 46). However, despite numerous reports in the literature (5, 9, 13, 16, 18, 19, 21, 30, 39, 49), amplification techniques do not yet have an established role in the laboratory for tuberculosis diagnosis, nor have they replaced traditional techniques, in contrast to diagnostic modalities for other pathogens, like *Chlamydia* or *Mycoplasma* (27). In clinical situations in which improvement in techniques is most needed, such as smear-negative tuberculosis, results from current PCR techniques have fallen short of expectations for the diagnosis of tuberculosis (14, 37, 45, 53).

This is specifically the case in suspected extrapulmonary tuberculosis, for which a rapid and accurate laboratory diagnosis is of prime importance, since the traditional techniques for detecting acid-fast bacilli have limitations and delayed chemotherapeutic intervention is associated with poor prognosis (9, 13, 14, 18, 35, 38, 53). In contrast to pulmonary specimens, the lack of sensitivity of PCR performed on extrapulmonary samples might result from the use of very small sample volumes and an irregular dispersion of the organisms in those paucibacillary specimens (27). The second major inconvenience of PCR or extrapulmonary specimens is the presence of inhibitors, which interfere with amplification-based techniques. A multistep process is often required to eliminate inhibitors and to obtain highly purified DNA. To achieve this goal, numerous techniques for sample preparation have been proposed, including boiling, freeze-boiling, shaking with glass beads (29), sonication (7), chloroform (55), proteinase K or Chelex (32), resin treatment (2), the complex nucleic acid extraction method (6a, 41), and, as recently described, a sequence capture procedure for pleural-fluid specimens (34).

The major difficulty with mycobacteria is achieving optimal cell lysis. Commercial kits with protocols have been developed to allow the majority of clinical laboratories access to amplification-based techniques (10, 22, 53, 54), but they have perhaps oversimplified such techniques, and more precisely, the sample preparation and DNA extraction steps. The buffers used in commercial kits do not allow complete mycobacterial cell lysis, a result easily obtained with other pathogens, in a number of clinical specimens (blood, pleural fluid, tissue biopsy specimens, and bone marrow aspirates), even with the proposed pretreatment with proteinase K. Consequently, several studies using exclusively extrapulmonary specimens, with the possible exceptions of cerebrospinal fluid (CSF) (28) and gastric aspirate (14, 38, 45, 53), have shown tuberculosis PCR sensitivity to be extremely low. In addition, Querol et al. have shown that two different extraction methods could lead to variation in sensitivities for pleural-fluid specimens (41). Therefore, there is clearly a need for improved sample preparation techniques.

Our hospital is located in a high-prevalence area for tuberculosis (3), and it deals with a high frequency of patients with

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TABLE 1. Description of study population

Patient group	No. of patients[a]	Median age (range) (yr)	No. from:			No. with HIV status[b]:		
			Africa	France	Other	P	N	ND
Tuberculosis group								
Positive culture	18 (10/8)	42 (23–89)	5	6	7	2	11	5
Positive histology, negative culture	7 (6/1)	40 (26–54)	2	3	2	1	4	0
Treated; negative histology and culture	5 (5/0)	39 (30–56)	5	0	0	2	3	1
Total	30 (21/9)	41 (23–89)	12[c]	9	9[d]	5	18	6
Nontuberculosis group	90 (60/30)	47 (7–91)	29	43	18	19	33	39
Total	120 (81/39)	47	41	52	27	24	51	45

[a] Number in parentheses, male/female.
[b] P, positive; N, negative; ND, not done.
[c] Algeria, Mali, Madagascar, Congo, Morocco, Chad, Liberia, Ivory Coast, and Zaire.
[d] India, Sri Lanka, China, Cambodia, Turkey, and Pakistan.

extrapulmonary tuberculosis (33.3% in 1998 and >50% among human immunodeficiency virus [HIV]-coinfected patients) associated with immunocompromised status (HIV, leukemia, and bone marrow and organ transplantation). Due to a genuine need for a rapid test for the diagnosis of extrapulmonary tuberculosis in such patients, a comparison of two sample preparation methods was conducted with extrapulmonary specimens, such as tissue and skin biopsy materials, pleural and ascitic fluids, bone marrow aspirate, abscesses, and exudates, to evaluate the sensitivity of the Cobas Amplicor MTB assay method (6) and its efficiency relative to sample preparation and DNA purification.

MATERIALS AND METHODS

Patients and specimens. One hundred and fifty-one extrapulmonary specimens sent to the mycobacteriology laboratory for investigation of suspected active tuberculosis were collected during a 2-month period (1 July to 30 August 1998). They originated from 125 patients hospitalized in Saint Louis Hospital (Paris, France). Clinical information about the patients during their hospitalization (fever, weight loss, night sweats, PPD reaction, chest X ray, magnetic resonance imaging or computed tomographic scan, histology, and background, such as previous history of tuberculosis, place of birth, and lifestyle) and follow-up for 1 year were obtained and analyzed using software developed in our hospital (3). All of the specimens were collected prior to the commencement of antituberculosis chemotherapy. The criteria for a positive diagnosis of tuberculosis were as follows: documented tuberculosis with positive culture and/or positive histology or probable tuberculosis with compatible clinical and radiographic evidence along with clinical improvement on a trial of antituberculosis therapy. Seventy-two biopsy specimens (26 lymph node, 24 skin, and 22 other [pleural, liver, lung, peritoneal, gastric, duodenal, and pericardial]) and 79 fine-needle aspirates (13 lymph node, 27 pleural, 19 bone marrow, and 20 other fluids [ascitic, peritoneal, pericardial, and bile]) were evaluated. Five patients (seven specimens) were positive for nontuberculous mycobacteria (*Mycobacterium avium* [two patients], *Mycobacterium intracellulare*, *Mycobacterium kansasii*, and *Mycobacterium marinum*), and they were excluded from the complete analysis, leaving 144 specimens from 120 patients.

Specimen processing. Upon receipt, the specimens were stored at +4°C prior to being processed. Fluid samples were first centrifuged at 3,000 × *g* for 20 min. Bone marrow aspirates were received either in Isolators or in Vacutainers (Becton Dickinson, Le Pont de Claix, France). All specimens were divided after being processed in the laboratory, with approximately two-thirds used for culture and one-third used for DNA extraction or preparation (see below). Tissue specimens were cut and homogenized in a mortar under sterile conditions before being processed. One hundred microliters of these homogenates was applied to chocolate agar (bioMérieux, Marcy l'Etoile, France) and incubated overnight at 37°C. Decontamination was performed only when growth of bacteria or fungi was observed on the medium. For all specimens, half of the sediment or the tissue biopsy specimen was stored at −20°C for the target amplification procedure, and the other half was inoculated onto culture medium and used for acid-fast staining. Acid-fast smears (AFS) were stained with auramine-fluorochrome and examined under a fluorescence microscope (magnification, ×400). All specimens, whether decontaminated or not, were grown on liquid medium (MGIT; Becton Dickinson) and inoculated onto Lowenstein-Jensen slants (Sanofi Diagnostic Pasteur, Marnes la Coquette, France). Liquid cultures were monitored by the automated MGIT 960 (Becton Dickinson) for up to 56 days, blood cultures were monitored for up to 42 days on Bactec 9120 (Myco Lytic; Becton Dickinson), and Lowenstein-Jensen slopes were kept for up to 3 months.

DNA extraction. DNA extraction was performed in an identical manner for all patients samples, with sterile water as the negative control sample. Series of 12 samples were extracted simultaneously: numbers 1, 5, and 12 were always the negative controls; the other numbers corresponded to the patients' samples. One-third of tissue or fluid specimens were resuspended in a final volume of 1 ml of Tris-EDTA, pH 7.6 (Sigma, Saint Quentin en Yvelines, France), containing 10 mg of lysozyme/ml. Samples were then incubated at 37°C for 1 h; 30 μl of proteinase K (14 mg/ml) (Roche Molecular, Paris, France) was added, followed by incubation for 2 to 3 h at 56°C or overnight at 37°C as recommended by the supplier. At this stage, the samples were divided into two aliquots of 500 μl each. The first 500-μl aliquot was processed as recommended by Roche Molecular (referred to hereafter as the PK-Roche method). Briefly, the sample was centrifuged at 13,000 × *g* for 5 min, and the supernatant was discarded, leaving a final volume of 100 μl. Proteinase K was inactivated by 15 min of incubation at 95°C. Further processing was done according to the Amplicor protocol for pulmonary samples (Roche Molecular).

The other 500-μl aliquot was treated as previously described (26) (referred to hereafter as the cetyltrimethylammonium bromide [CTAB]-Roche method). Briefly, 35 μl of 10% sodium dodecyl sulfate was added, and samples were vortexed for a few seconds and incubated for 10 min at 65°C. The samples were incubated in a solution of CTAB-NaCl (50 μl of 5 M NaCl and 40 μl of 10% CTAB) for 10 min at 65°C and then mixed with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]; 700 μl) and centrifuged for 15 min at 14,000 rpm in an Eppendorf centrifuge. The aqueous phase (650 μl) was then separated and mixed with an equal volume of isopropanol. The samples were left at −20°C for 30 min and then centrifuged for 15 min at 13,000 × *g*. The DNA pellet was washed once with 70% ethanol, air dried, and resuspended in a final volume of 100 μl, including equal volumes of lysis buffer and neutralizing reagent (Amplicor; Roche Molecular).

Each step of the extraction protocol was performed in a mycobacterial-DNA-free room, under a safety cabinet, using protected tips and dedicated pipettes.

PCR amplification and hybridization. Fifty microliters of each sample was mixed with an equal volume of amplification buffer and amplified on a Cobas Amplicor (Roche Molecular) as recommended by the manufacturer (6, 12, 42, 47, 48). An internal control template was included in each amplification reaction to allow the detection of inhibitory substances (43). Serial twofold dilutions (1/2, 1/4, and 1/8) were systematically performed on inhibitory specimens to remove inhibitors. Specimens giving an absorbance value of >0.300 were considered positive, as recommended by the supplier.

Sensitivity, specificity, and predictive values were calculated for each extraction method and compared with culture and clinical confirmation values (33).

TABLE 2. Efficiencies of PK-Roche and CTAB-Roche extraction methods for amplification of *M. tuberculosis* DNA compared to culture[a]

Method	Status	No. of specimens culture: (n = 144)	
		P	N
PK-PCR	P	2	3
	N	4	60
	I	9	66
CTAB-PCR	P	4	14
	N	1	69
	I	10	46

[a] P, positive; N, negative; I, inhibitory. Cutoff absorbance value, >0.300.

RESULTS

One hundred and forty-four specimens collected from 120 patients were analyzed. The median age of the study subjects was 47 years, with a majority being men. More than 50% of the patients originated from foreign countries, and 32.9% (24 of 75) of the patients tested for HIV were HIV positive (Table 1), with a range of 4 to 678 CD4 cells/mm^3 and a range of 0 to 4,212,000 HIV RNA copies/ml. No significant differences were observed when results were compared by age, sex, birth origin, or nationality, due mainly to the low number of individuals in each category.

Among the 120 patients, 75% were considered not to have tuberculosis and 25% were considered tuberculosis patients and were treated as such (Table 1). Among these 30 patients, 25 had documented tuberculosis, i.e., 18 patients had positive specimens (15 extrapulmonary and 3 pulmonary) in culture, and 7 patients were documented by a positive histology showing typical tuberculosis granuloma with caseum and necrosis. Five patients were diagnosed on clinical grounds and treated empirically for tuberculosis, with a positive outcome during the course of treatment for four patients, and one patient was lost during follow-up. This gave an overall incidence for tuberculosis in the studied population of 25%. Only 2 samples out of 15 culture-positive extrapulmonary specimens for *M. tuberculosis* were AFS positive.

Amplification and detection of the amplicons were performed on an automated Cobas Amplicor PCR. Materials extracted by the two methods were tested in parallel. As shown in Table 2, this strategy was impaired by a high number of inhibitory specimens: 52.1% (75 of 144) and 38.9% (56 of 144), respectively, for the PK-Roche and CTAB-Roche methods. The highest rate of inhibition was observed in tissue biopsy specimens (54.8% with PK-PCR; 52.1% with CTAB-PCR), particularly cutaneous biopsy specimens (70.8% with PK-PCR; 50% with CTAB-PCR), compared to fluid specimens (43.6% with PK-PCR; 28.2% with CTAB-PCR), particularly bone marrow aspirates (68.4% with PK-PCR; 36.8% with CTAB-PCR) and abscesses (66.7% with PK-PCR; 50% with CTAB-PCR). After twofold serial dilution (up to 1/8), a great reduction in inhibitory specimens was obtained, reaching 7 and 6% for PK-Roche and CTAB-Roche, respectively (Table 3). Seven more culture-positive specimens were detected as positive by PCR at 1/2 dilution, after extraction by the CTAB-Roche method, giving a sensitivity of 84.6% compared to culture (Table 3). Conversely, only one more positive sample was observed after dilution of inhibitory specimens extracted by the PK-Roche method, giving a sensitivity of 27.2% (Table 3).

TABLE 3. Comparative efficiencies of PK-Roche and CTAB-Roche extraction methods for the amplification of *M. tuberculosis* DNA from 144 specimens after removal of inhibitory activity by serial dilution compared to culture[a]

Method	Status	No. of specimens culture:		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		P	N				
PK-PCR	P	3	3				
	N	8	120	27.2	97.6	50	93.8
	I	4	6				
CTAB-PCR	P	11	24				
	N	2	98	84.6	75.5	31.4	98
	I	2	7				

[a] P, positive; N, negative; I, inhibitory; PPV, positive predictive value; NPV, negative predictive value. Cutoff absorbance value, >0.300.

A comparison of the amplification and culture results was then performed, with results from the diagnosis of tuberculosis as a "gold standard" (see Materials and Methods) (Table 4). A final rate of inhibition of 8% for the PK-Roche and CTAB-Roche methods was obtained, and these patients were excluded from the calculation for the diagnostic value of the PCRs. In case more than one specimen was tested for the same patient, only one positive result was considered per patient, and one negative result per patient was considered in cases where all specimens were negative. As mentioned above, extraction of total DNA by the CTAB-Roche method was associated with a higher sensitivity (53.8%) than culture (43.3%), the PK-Roche method (16%), and AFS (13%). However, the specificity of the PCR assay with CTAB-Roche-extracted material was always lower (78.8%) than the specificity of culture (100%) and the PK-Roche method (96.5%). Detailed clinical analysis of false-positive PCR by the CTAB-Roche method showed that 8 out of 18 patients came from areas where tuberculosis is endemic (Algeria, Tunisia, Ivory Coast, etc.); 5 out of the 12 tested patients were HIV positive; 11 patients had leukemia, lymphoma, or cancer; 2 were transplant recipients; 1

TABLE 4. Efficiency of PCR diagnosis versus clinical diagnosis of tuberculosis[a]

Method	Status	No. of patients (n = 120)		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		P	N				
PCR							
PK	P	4	3				
	N	21	83	16	96.5	57.2	79.8
	I	5	4				
CTAB	P	14	18				
	N	12	67	53.8	78.8	43.8	84.8
	I	4	5				
Culture	P	13	0	43.3	100	100	84.1
	N	17	90				

[a] P, positive; N, negative; I, inhibitory; PPV, positive predictive value; NPV, negative predictive value. Cutoff absorbance value, >0.300.

TABLE 5. Description of 18 patients PCR positive (CTAB-Roche) but culture negative with no clinical diagnosis of tuberculosis

Patient	Sample[a]	HIV status[b]	Absorbance value	Origin	Final diagnosis; outcome[c]
1	LNP	N	1.40	France	Hodgkin lymphoma; no active tuberculosis
2	CB[d]	N	1.30	France	Hemorragic rectocolitis, corticotherapy, "pyoderma gangrenosum"; no active tuberculosis
3	LNP	ND	0.95	Algeria	NP cancer, PPD positive; no active tuberculosis
4	PF	ND	0.53	France	Previous tuberculosis in 1956, lung adenocarcinosis; deceased
5	BM	P	1.70	France	*Pneumocystis carinii* infection, pulmonary and cutaneous Kaposi syndrome; lost for follow-up
6	BM	N	0.52	France	Chronic myeloid leukemia; bone marrow allograft (4/98), tuberculosis suspicion (10/98), deceased (11/98)
7	CM	N	0.31	Algeria	Lung adenocarcinosis; no active tuberculosis
8	TA	N	1.2	Algeria	Renal transplantation, anterior thoracic mass, adenocarcinosis; no active tuberculosis
9	PF	N	0.42	France	Chronic myeloid leukemia, nodular pneumopathy; no active tuberculosis
10	NB	ND	0.41	Tunisia	Lung epithelioid carcinoma; deceased (3/99)
11	HB	P	1.10	France	Previous tuberculosis, pulmonary adenocarcinoma, fever, abdominal pain, weight loss (08/98), treated with isoniazid, rifampin, azythromycin, myambutol; deceased (09/98)
12	PF	ND	1.30	France	Sarcoma, acute respiratory distress syndrome (11/98); deceased (11/98)
13	BM	P	0.36	Ivory Coast	Burkitt lymphoma, PPD negative, axillar adenopathy; no active tuberculosis
14	LNP	P	0.46	Ivory Coast	Cervical adenopathy; no active tuberculosis
15	LNP	ND	1.80	Italy	Cervical adenopathy; no active tuberculosis
16	LNB	P	0.44	France	Weight loss, VZV infection, hepatitis B, inginal, axillar and under-clavicular adenopathy; no active tuberculosis
17	SC	ND	1.30	Algeria	Hemoptysis, immunoglobulin synthesis deficit, previous pulmonary and extrapulmonary tuberculosis (1973); no active tuberculosis
18	LNP	N	1.10	North Africa	Cervical adenopathy; lost to follow-up

[a] LNP, lymph node puncture; LNB, lymph node biopsy; BM, bone marrow; PF, pleural fluid; SC, synovial cyst; HB, hepatic biopsy; NB, nodular biopsy; TA,
[b] N, negative; P, positive; ND, not done.
[c] NP, nasopharyngeal; PPD, purified protein derivative (tuberculin test); VZV, varicella-zoster virus; dates are month/year.
[d] Two cutaneous biopsy specimens were tested for this patient.

patient had undergone long-term corticotherapy; 3 had previous histories of tuberculosis; 1 was skin test positive; and 3 presented clinical symptoms like fever, weight loss, or hemoptysis (Table 5). Seven patients out of 18 had pure specimens with an absorbance value after hybridization between 0.3 and 0.6, and one specimen was positive at 0.38 at a 1/2 dilution (Table 5), which can be considered a "grey zone" with a lot of uncertainties. If these are considered negative samples, the specificity and positive predictive value would increase to 88.2 and 58.3%, respectively, without affecting the sensitivity and negative predictive value (86.2 versus 84.8%).

DISCUSSION

A number of unanswered questions regarding the development of molecular techniques in the clinical laboratory for the diagnosis of tuberculosis have yet to be addressed: (i) which method should be used for extraction of mycobacterial DNA; (ii) which specimens should be tested; (iii) whether pulmonary specimens should be tested after decontamination; (iv) which criteria for sensitivity, specificity, speed, simplicity, and clinical relevance are required; and finally, (v) should a positive internal control always be used with molecular methods of this sort, and specifically, should this control be added to the sample preparation at the very start of the process to determine the efficacy of the sample preparation and amplification procedure (27, 50)? The aim of our study was to try to answer several of these questions. We specifically chose to examine extrapulmonary tuberculosis, for which amplification techniques are of considerable interest. The main difficulty with extrapulmonary specimens is that they yield very few tubercle bacilli, as demonstrated by the low sensitivity of AFS and culture. What is more, in paucibacillary tuberculosis, a much longer incubation time is always required for positive growth (52), leaving clinicians with a long delay without a positive answer. With the development of novel techniques in molecular biology, these delays might be shortened.

To assess the influence of sample preparation, we decided to use a modified method of mycobacterial DNA extraction classically performed in research laboratories on *M. tuberculosis* culture (51). One previous report demonstrated improved quality of CTAB-extracted DNA, with 100% amplification of the β-globin gene, compared to PK treatment, which gave 85% amplification (41). The results obtained in our study confirm the efficiency of the CTAB extraction method, with a sensitivity of 85% compared to culture and 54% compared to a diagnosis of tuberculosis on clinical grounds. The latter value was superior to the sensitivity of the smear test (13%) and slightly higher than that for culture (43.3%). The values obtained with the CTAB-Roche method were always higher than those with amplification of PK-extracted specimens.

Optimal sample preparation is required to eliminate inhibitors that interfere with detection. Monitoring or correct evaluation of PCR inhibitors is possible only if an internal control is included during DNA amplification (30, 43). Extraction procedures employing multiple steps to purify DNA, as in our CTAB-Roche method, appear to have lower rates of inhibition (16, 31) than those with fewer steps (19, 29). A comparison of the inhibition rates of the two preparation methods used in this study allowed similar conclusions to be drawn (Table 2). High rates of inhibition were obtained. The highest rate of inhibition was observed in tissue biopsy specimens, particularly cutane-

ous biopsy specimens, compared to fluid specimens, particularly bone marrow aspirates and abscesses. Comparison with published studies is difficult, because the majority of reported studies involved mixed specimens and inhibitory activity was often checked only for discrepant results between culture and PCR, with numerous studies excluding an internal control for PCR-based assays. The phenomenon of interference in amplification-based techniques is not uncommon for pus samples and tissue biopsy specimens, with nearly 20% inhibitory only on discrepant results (9, 29). An underestimate of inhibited PCR assays was further demonstrated by the study of Forbes and Hicks, which showed an increase in inhibition of amplification from 13 to 52% when an internal control was added to the reaction (20). It is difficult to identify the substance(s) responsible for this inhibition, which might be blood, detergents, or heparin. However, none of these products was present after DNA extraction and ethanol precipitation. In purified DNA preparations, eukaryotic DNA in high amounts (>1 to 2 μg) is the only remaining substance, and this has been shown to have inhibitory properties (34). Simple dilution allowed the recovery of seven positive specimens extracted by the CTAB-Roche method after a 1/2 dilution compared to one for the PK-Roche method.

It is frequently observed that attempts to improve the sensitivity of assays are often at the expense of a reduction in specificity, which was a strategy used in a previous study assessing new mycobacterial probes (17). It is not clear in this context whether specimens which are *M. tuberculosis* culture negative but PCR positive are truly false positive or are actually detecting *M. tuberculosis* DNA. Because of the protocol chosen to set up controls (see Materials and Methods), we feel confident that negative controls make contamination less likely. Compared to conventional culture, there were five PCR-positive, culture-negative specimens, which originated from patients with probable tuberculosis. We also observed that 7 "false-positive PCR" patients out of 18 had pure specimens with absorbance values after hybridization between 0.3 and 0.6, and 1 specimen was positive at 0.38 at a 1/2 dilution, which we considered a grey zone with a lot of uncertainties. In addition, these "false-positive" specimens were lung biopsy specimens or lymph node biopsy specimens or aspirates from patients suffering from leukemia or cancer or immunocompromised patients (HIV coinfected, transplant recipients, or on corticosteroid therapy). These specific organs (lung, lymph node, and bone marrow) might be sites of *M. tuberculosis* persistence, where reactivation and replication may take place as soon as host immunity wanes (22, 25, 26). Hernandez-Pando et al. recently demonstrated, with molecular evidence, that normal-appearing human lung tissue rather than granulomatous lesions harbors *M. tuberculosis* DNA, a finding that strongly suggested that latent bacilli reside in apparently normal tissue (25). None of the (Mexican) individuals had shown signs or symptoms of tuberculosis before death, and none of them had shown lesions suggestive of tuberculosis in the lungs at necropsy (25). Similarly, there were no histopathological signs of tuberculosis in the tissue samples, despite the fact that 70% of the PCR-positive patients had died as a consequence of diseases which can cause secondary immunodeficiency (cancer, starvation, or autoimmunity) (25) and thus had increased risk of acquiring tuberculosis, as observed in our study and previously (26). Nevertheless, none of the PCR-positive patients in our population had clinical or microbiological evidence of active tuberculosis. Whether *M. tuberculosis* DNA is a good target, as soon as we improve the sensitivity of the amplification assay, is a critical point. By comparison, detection of RNA, as demonstrated in several elegant studies (11, 23, 24), might allow differentiation between latent bacilli and replicating bacilli in tissue samples. We could not perform such an assay, since the tissue specimens in this study were stored at −20°C (instead of −70°C), but this project is now in progress in the laboratory.

Studies of clinical specimens conducted so far have differed in the type of PCR method used and also in the number and type of samples. A meta-analysis of papers on *M. tuberculosis* PCR has shown a sensitivity of <50% when PCR is performed on smear-negative pulmonary specimens (1), correlating with a smaller number of tubercle bacilli. In specific studies of pulmonary and/or extrapulmonary specimens, sensitivities ranged between 20 and 94% (4, 8, 14, 15, 36, 37, 38, 40, 42, 44, 45, 53, 54) for PCR assays of extrapulmonary specimens. Surprisingly, these values are equivalent to or higher than results observed for smear-negative pulmonary specimens. In addition, the discrepancies observed in these studies could be explained by the prevalence of tuberculosis in the tested population, the number of smear-positive samples, the methods used for extracting mycobacterial DNA, and, more importantly, the type of specimens included in the study. It is worth mentioning that many difficulties are encountered in making a comparison of results from the numerous studies which have mixed respiratory and nonrespiratory specimens. CSF or gastric aspirates are often more positive than tissue or fluid specimens. In our hands, the sensitivity of the Cobas Amplicor assay of CSF is ~89% for optimally processed specimens (unpublished results). The sensitivity values obtained in this study are in the range of published values. However, 65% of the tested specimens were composed of tissue biopsy specimens and bone marrow aspirates (both known to be more inhibitory and paucibacillary), and the remainder were composed of pleural fluid, lymph node aspirates, and other body fluids, confirming the performance of the CTAB extraction method.

In summary, the aim of this study was to demonstrate the importance of optimal preparation of DNA for the efficiency of Roche Cobas Amplicor PCR for the rapid diagnosis of extrapulmonary tuberculosis. The sensitivity obtained in this study for amplification performed on CTAB-Roche-extracted specimens confirms its superiority over conventional AFS. A more rapid answer is possible than with conventional culture, which may avoid the need for other costly investigations. The reagents used in this study are easily available and may even be commercially produced to allow better reproducibility and quality control than reagents used for in-house methods. In addition, the main point is whether it is important to take one more hour to prepare a good DNA sample in order to make a better PCR diagnosis. This assay is not a screening assay, which means that it will be performed only on optimally prescribed extrapulmonary specimens, for which a sensitive and rapid answer is needed by the clinician. However, false-positive DNA amplification results challenge our understanding of PCR results for *M. tuberculosis* and might bring into question the use of the *M. tuberculosis* DNA amplification method com-

pared to RNA amplification for tissue specimens. Finally, it is important that any PCR-based assay include a specific positive internal control to allow proper evaluation of DNA preparation and amplification.

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