

## A More Reliable PCR for Detection of *Mycobacterium tuberculosis* in Clinical Samples

L. F. F. KOX,<sup>1,2\*</sup> D. RHIENTHONG,<sup>3</sup> A. MEDO MIRANDA,<sup>4</sup> N. UDOMSANTISUK,<sup>5</sup> K. ELLIS,<sup>1</sup>  
J. VAN LEEUWEN,<sup>1</sup> S. VAN HEUSDEN,<sup>1</sup> S. KUIJPER,<sup>1</sup> AND A. H. J. KOLK<sup>1</sup>

N. H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute,<sup>1</sup> and Division of Pulmonary Diseases, Academic Medical Centre,<sup>2</sup> Amsterdam, The Netherlands; Department of Microbiology, Faculty of Medicine, Chulalongkorn University,<sup>3</sup> and Tuberculosis Division<sup>3</sup> Bangkok, Thailand; and Instituto Nacional de Saude, Dr. Ricardo Jorge, Porto, Portugal<sup>4</sup>

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**Diagnostic techniques based on PCR have two major problems: false-positive reactions due to contamination with DNA fragments from previous PCRs (amplicons) and false-negative reactions caused by inhibitors that interfere with the PCR. We have improved our previously reported PCR based on the amplification of a fragment of the *Mycobacterium tuberculosis* complex-specific insertion element IS6110 with respect to both problems. False-positive reactions caused by amplicon contamination were prevented by the use of uracil-*N*-glycosylase and dUTP instead of dTTP. We selected a new set of primers outside the region spanned by the formerly used primers to avoid false-positive reactions caused by dTTP-containing amplicons still present in the laboratory. With this new primer set, 16 copies of the IS6110 insertion element, the equivalent of two bacteria, could be amplified 10<sup>10</sup> times in 40 cycles, resulting in a mean efficiency of 77% per cycle. To detect the presence of inhibitors of the *Taq* polymerase, which may cause false-negative reactions, part of each sample was spiked with *M. tuberculosis* DNA. The DNA purification method using guanidinium thiocyanate and diatoms effectively removed most or all inhibitors of the PCR. However, this was not suitable for blood samples, for which we developed a proteinase K treatment followed by phenol-chloroform extraction. This method permitted detection of 20 *M. tuberculosis* bacteria per ml of whole blood. Various laboratory procedures were introduced to reduce failure or inhibition of PCR and avoid DNA cross contamination. We have tested 218 different clinical specimens obtained from patients suspected of having tuberculosis. The samples included sputum (*n* = 145), tissue biopsy samples (*n* = 25), cerebrospinal fluid (*n* = 15), blood (*n* = 14), pleural fluid (*n* = 9), feces (*n* = 7), fluid from fistulae (*n* = 2), and pus from a wound (*n* = 1). The results obtained by PCR were consistent with those obtained with culture, which is the "gold standard." We demonstrate that PCR is a useful technique for the rapid diagnosis of tuberculosis at various sites.**

Timely identification of infection with *Mycobacterium tuberculosis* is important because of the need to make decisions regarding management such as beginning antituberculosis drug therapy, isolation precautions, and prophylaxis. The outcome for the patient could be improved if fast, simple, and reliable tests could be developed. The diagnosis of extrapulmonary tuberculosis and tuberculoid meningitis is difficult, and the definitive diagnosis depends on culture of the mycobacteria, which is time-consuming and not always available.

We and others have recently reported on the use of PCR for the detection and identification of *M. tuberculosis* complex in clinical samples (2-5, 7, 8, 13, 19, 22, 23, 25). This is a rapid and sensitive technique. The exquisite sensitivity of the PCR may also be a drawback: contamination of the laboratory with amplified DNA from previous PCRs (amplicons) may cause false-positive results. In this paper, we describe the use of uracil-*N*-glycosylase (UNG) (10) and dUTP instead of dTTP to prevent false-positive results due to amplicon contamination. We have extended our work on the quality control of the performance of PCR and have developed a DNA purification method for blood and feces samples. We have used this technically improved PCR in clinical samples from patients

suspected of having tuberculosis by comparing the PCR results with the results of Ziehl-Neelsen (ZN) staining and culture.

### MATERIALS AND METHODS

**Bacterial strains.** The same mycobacterial and nonmycobacterial strains as described previously (8) were used for the specificity testing.

**Clinical samples.** Sputum samples from 92 patients suspected of having tuberculosis were obtained from the Tuberculosis Division, Bangkok, Thailand. Samples were transported by air at -20°C. The other clinical samples were sent from various university hospitals in The Netherlands and included sputum (*n* = 53), tissue biopsy samples (*n* = 25), cerebrospinal fluid (*n* = 15), EDTA-blood (*n* = 14), pleural fluid (*n* = 9), feces (*n* = 7), fluid from fistulae (*n* = 2), and pus from a wound (*n* = 1). All samples were kept at -20°C.

**Decontamination of sputum for culture.** The sputum samples from Bangkok were decontaminated with oxalic acid. A swab was inserted into the sputum sample and then placed in 5% oxalic acid for 20 min at 25°C. The swab was then incubated in 5% Na citrate for 10 min at 25°C.

The sputum samples from The Netherlands were decontaminated, depending on the preference of the laboratory, by the NaOH Petroff method (6, 15), by the sodium laurylsulfate method (6, 16), or with NaOH-sodium citrate-*N*-acetyl-L-cysteine (9, 24). In all these cases, samples were concentrated by centrifugation after decontamination.

\* Corresponding author. Mailing address: Royal Tropical Institute, N. H. Swellengrebel Laboratory of Tropical Hygiene, Meibergdreef 39, 1105 AZ Amsterdam, The Netherlands. Phone: (0) 20-5665441. Fax: (0) 20-6971841.

**ZN staining.** At the Tuberculosis Division in Bangkok, sputum smears were microscopically examined after ZN staining (20). In the laboratories in The Netherlands all clinical samples were microscopically examined after ZN staining after being concentrated.

**Routine culture.** In Bangkok, sputum samples were cultured on two Löwenstein-Jensen slants. In the laboratories in The Netherlands, sputum samples were cultured on two different solid media, Löwenstein-Jensen and Colletso's or Ogawa. The samples from patients suspected of having extrapulmonary tuberculosis were cultured in addition on liquid medium, Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) and/or MB-Check (Roche) M, a combined liquid and three solid media (Hoffman-La Roche, Basel, Switzerland). The specimens were incubated at 37°C for 2 months and examined weekly for growth.

**Pretreatment of clinical samples containing blood.** Clinical samples which contained visible traces of blood were treated with a lysis buffer consisting of 1% Triton X-100 in 20 mM Tris-HCl (pH 8.3) as previously described (8).

**DNA purification from clinical samples.** Clinical samples (other than sputum, tissue biopsy samples, blood, and feces) and decontaminated (9) sputum pellets (100 µl) were mixed with 900 µl of a lysis buffer containing approximately 5 M guanidinium thiocyanate, 1% Triton X-100, 50 mM Tris-HCl (pH 6.4), and 20 mM EDTA (L6 lysis buffer [1]). DNA was purified according to the guanidinium thiocyanate method using 20 µl of diatoms (1). DNA was eluted from the diatoms in two steps with 60 and 40 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.3], 1 mM EDTA).

**Treatment of biopsy samples.** Biopsy samples (1 to 15 mg) were incubated for 1 h at 60°C in a mixture of 0.1 mg of proteinase K per ml, 0.5% Triton X-100, and 20 mM Tris-HCl (pH 8.3). The biopsy material was further disrupted by adding 900 µl of L6 lysis buffer and 500 µl of zirconium beads (0.1-mm diameter) (Biospec Products, Bartlesville, Okla.). The mixture was shaken in a mechanical disruptor (MiniBeadBeater model 3110; Biospec Products) for 3 min. After the zirconium beads had settled, the DNA was purified from the supernatant by the guanidinium thiocyanate method (1).

**Treatment of blood and feces.** Blood and feces were treated essentially as described by Meredith and coworkers (12). EDTA-blood (600 µl) or feces (600 µl) was mixed with 250 µl of TE buffer and incubated with 100 µl of a 10-times-concentrated stock proteinase K digestion buffer (1 mg of proteinase K per ml, 5% Triton X-100, and 200 mM Tris-HCl [pH 8.3]) for 1 h at 60°C. DNA was isolated from the digest by phenol-chloroform extraction. A volume of 950 µl of phenol (saturated with 100 mM Tris-HCl [pH 8.3]) was added. After 10 min of shaking on a gyratory shaker at 100 rpm, the mixture was centrifuged for 10 min at 12,000 × g, the aqueous phase was collected, and to this 1 volume of phenol-chloroform (1:1) was added. After shaking and centrifugation as before, the aqueous phase was collected and an equal volume of chloroform was added. The aqueous phase was collected again after a further shaking and centrifugation step. DNA was precipitated by the addition of 0.05 volume of 5 M NaCl and 2 volumes of cold 100% ethanol (-20°C) and then centrifuged at 12,000 × g for 20 min. The pellet was dried and dissolved in 100 µl of TE buffer.

**Solutions for spiking of clinical samples.** For spiking and sensitivity determinations, bacteria and DNA from an *M. tuberculosis* strain that has eight copies of the insertion element IS986 (8) were used. Insertion sequence IS986 is virtually identical to the IS6110 insertion element (11, 22).

**Precautions to prevent contamination.** In our laboratory,

prevention of contamination is accomplished by physical separation of different steps in the PCR procedure, using different pipettes, and wearing separate coats and gloves in each laboratory. The PCR mix is prepared in one laboratory, where work is conducted in a safety cabinet to prevent contamination of the PCR mix by shedding of material from exposed facial skin or hair. Chemicals for use in the PCR and for the treatment of clinical samples are weighed in this laboratory on a balance which is used only for this purpose. Ultrapure water (Baker, Deventer, The Netherlands) is used to make the solutions, which are sterilized in this laboratory. The solutions are stored as aliquots to minimize the number of repeated samplings. Mycobacterial strains or template DNA is not allowed in this laboratory. In a second laboratory clinical samples are treated in a safety cabinet and added to the PCR mix. If possible, we do not open more than one vial at a time in this laboratory. Only concentrations of *M. tuberculosis* DNA lower than 100 pg/ml are allowed in this room. Chemical decontamination of surfaces and equipment is done weekly in both laboratories with 0.5% sodium hypochlorite (15). The PCR and the detection of PCR products, by agarose gel electrophoresis and dot blot hybridization, are done in a third laboratory in a safety cabinet used only for this purpose.

**Synthetic oligonucleotides.** Lyophilized oligonucleotides were purchased from Isogen Bioscience (Amsterdam, The Netherlands). Prior to lyophilization the  $A_{260}$  was determined and the oligonucleotide solution was aliquoted in 10 vials. Lyophilized oligonucleotides were kept at -20°C until use.

The amount of ultrapure water needed to give a 50 µM solution in a vial of lyophilized oligonucleotide was calculated by using the  $A_{260}$  and the millimolar extinction coefficient of the oligonucleotide (17). The resulting solution was aliquoted in volumes of 50 µl and kept at -20°C. One vial was kept at 4°C for immediate use.

**PCR for clinical samples.** Two oligonucleotide primers within the IS6110 insertion sequence, designated primers Pt8 (5'-GTGCGGATGGTTCGAGAGAT-3') and Pt9 (5'-CTCGATGCCCTCACGGTTCA-3'), were used for the PCR, resulting in a 541-bp PCR fragment (10). The composition of the PCR mix was 10 mM Tris-HCl (pH 8.3), 50 mM NaCl, 2.0 mM MgCl<sub>2</sub>, 0.01% (wt/vol) gelatin, 0.2 mM (each) deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dUTP), 0.2 µM (each) primers Pt8 and Pt9, 1 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 0.01 U of UNG, (GIBCO BRL, Gaithersburg, Md.) per 50-µl reaction volume. The PCR mix was aliquoted in a final volume of 40 µl per reaction vial and stored at -20°C. When a test was performed, the reaction vials were removed from the freezer and 50 µl of mineral oil (Sigma, St. Louis, Mo.) was added to each vial before the 10-µl sample was placed under the oil layer. For preparing the PCR mixes and pipetting the mineral oil, positive displacement pipette tips (Microman, Gilson, France) were used. For pipetting clinical samples and DNA solutions, we used DNA-PCR filter tips (Greiner, Nürtingen, Germany). PCR was performed in a water bath thermocycler (Bio-med 60 processor; Bio-med, Theres, Germany) or a heating block thermocycler (Gene ATAQ Controller; Pharmacia LKB, Uppsala, Sweden). The temperature indicators of the thermocyclers were checked monthly with an electrical thermometer (51 K/ Thermometer; Fluke, Everett, Wash.), which in turn was checked twice a year against an officially calibrated mercury thermometer. The PCR incubations were as follows: 5 min at 50°C to allow breakdown of amplicons by UNG, followed by 40 cycles of 1.5 min of denaturation at 94°C, 2 min of annealing at 65°C, and 3 min of primer extension at 72°C. If the PCR products were not

analyzed directly after the final cycle, the vials were incubated for a minimum of 1 h at 72°C.

The quality of the PCR mix was tested by amplification of a series of 1,000, 100, 10 and 1 fg of *M. tuberculosis* DNA per vial. Ten microliters of TE buffer was tested in duplicate to provide a negative control for contamination with *M. tuberculosis* DNA during the preparation of the mix. To control for the activity of the UNG, a vial with 10 µl of a 15-pg/µl 541-bp amplicon solution was amplified in the same assay. This amplicon solution was prepared by using 1,000 fg of *M. tuberculosis* DNA as a template in PCR and subsequently diluting this PCR product 1,000-fold. The PCR mix was considered to be of sufficient quality if 10 fg of *M. tuberculosis* DNA, corresponding to two bacteria, could be detected and if the vials containing TE buffer and the vial containing 150 pg of 541-bp amplicon were negative in PCR.

In all PCR series involving clinical samples, positive controls consisting of a series of vials containing 1,000, 100, 10, and 1 fg of *M. tuberculosis* DNA and two vials with TE buffer were included. Each treated sample from a patient was tested in triplicate. One part (10 µl) was spiked with 10 fg of *M. tuberculosis* DNA, and two parts were not spiked. If all three samples were positive, then the sample was considered positive. The sample was scored as negative if the nonspiked reactions were negative and the spiked reaction was positive. If the results for the nonspiked reactions were ambiguous, the PCR was repeated. If all three reactions were negative, then there were inhibitors in the sample. In such a case, the PCR was repeated with a smaller sample volume; if inhibition recurred, another DNA extraction method was used; if there was still inhibition, then a further sample from the patient was requested.

**Detection of amplified DNA.** The PCR products were analyzed by agarose gel electrophoresis on 2% agarose gels (8). The results were recorded by Bio-print photodocumentation and when necessary stored in the image storage system (Vilber-Lourmat, Marne-la-Vallée, France).

For comparison of PCR results, the intensity of the PCR products was compared visually with the intensity of the PCR products of the positive controls. An intensity equal to or greater than that of the product of 100 fg of *M. tuberculosis* DNA was scored 2+, an intensity between those of the products of 100 and 10 fg of *M. tuberculosis* DNA was scored +, and an intensity less than that of 10 fg of *M. tuberculosis* DNA was scored -. For efficiency calculations, the amount of PCR product was more accurately determined by spot quantification, by interpolation with a standard curve using the Bio-profile software (Vilber-Lourmat). The standard curve was constructed employing the amounts of DNA from the fragments of the *Hae*III-digested  $\phi$ X174 replicative form molecular size marker (New England Biolabs, Beverly, Md.).

The PCR results with clinical samples were confirmed by dot blot hybridization with a biotin-labeled oligonucleotide probe.

**Oligonucleotide labeling with biotin.** The oligonucleotide Pt10 (5'-AGCACGATTCCGAGTGGGCA-3'), which is situated within the 541-bp fragment and corresponds to bp 134 to 153 of the insertion sequence IS6110 of *M. tuberculosis*, was tail labeled at the 3' end of the DNA with biotin-16-dUTP by using the enzyme terminal transferase (18). Biotin-labeled Pt10 was also commercially obtained (Isogen Bioscience). The biotin group was covalently attached to the 5' end of the DNA via a six-carbon spacer arm.

**Dot blot hybridization and colorimetric detection.** Reinforced nitrocellulose membranes (Duralose; Stratagene, La Jolla, Calif.) were cut into pieces (8 by 12 cm) and presoaked in water and then in 10× SSC (1× SSC is 0.15 M NaCl plus

TABLE 1. Efficiency of PCR

Template DNA		PCR product		Efficiency
Amt (fg)	No. of copies of IS6110	Amt (ng)	No. of molecules of 541-bp amplicon (10 <sup>11</sup> )	
10,000	16,000	758 ± 93	13	0.58
1,000	1,600	426 ± 68	7.2	0.65
100	160	197 ± 50	3.3	0.71
10	16	81 ± 65	1.4	0.77

0.015 M Na-citrate [pH 7.0]). Samples of 5 µl of PCR-amplified DNA were made up to a final volume of 50 µl in water and denatured by boiling for 5 min and subsequent cooling on ice. After the addition of 50 µl of an ice-cold solution of 20× SSC, the mixtures were spotted onto a presoaked Duralose (Stratagene) membrane by using a dot blot apparatus (Schleicher-Schüll, Dassel, Germany), and a vacuum of 60 cm Hg (ca. 800 kPa) was applied. The spots were washed with 200 µl of 10× SSC. The DNA was fixed to the membranes by UV (312 nm) cross-linking at 1/cm<sup>2</sup> (Fluo-link TFL20M; Vilber-Lourmat) or by heating at 120°C for 15 min. The membranes were sealed in a plastic bag with 5 ml of 5× SSC-1% (wt/vol) blocking agent (Boehringer, Mannheim, Germany)-0.1% (wt/vol) *N*-laurylsarcosine-0.02% (wt/vol) sodium dodecyl sulfate (SDS) and prehybridized at 50°C for 15 min. After the addition of 1 pmol of biotin-labeled Pt10 from either source to the prehybridization mix, the membranes were incubated at 50°C for another 2 h. The membranes were washed twice at 50°C for 5 min in 50 ml of 2× SSC-0.1% (wt/vol) SDS. The membranes were used directly for the detection of the hybridized probe by incubation with streptavidin-alkaline phosphatase conjugate according to the instructions of the manufacturer (Boehringer) and scored visually (8).

## RESULTS

**Specificity, sensitivity, and efficiency of the PCR.** PCR using the primers Pt8 and Pt9 was specific for the *M. tuberculosis* complex. No PCR products were found with bacteria or mycobacteria other than those belonging to this complex. To determine the sensitivity of the PCR, eight series of 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, 10, and 1 fg of *M. tuberculosis* DNA were amplified. In all eight tests 10 fg of DNA, the DNA equivalent of two bacteria or 16 copies of the IS6110 insertion element, could be detected by agarose gel electrophoresis. PCRs with 1 fg of DNA were positive in two of the eight tests.

The amount of PCR product resulting from the different amounts of template DNA was determined by using the Bio-profile software (Table 1). The efficiency of the PCR amplification was calculated by using the following formula:  $(1 + x)^n = y$ , where  $x$  is the mean efficiency of a cycle,  $n$  is the number of cycles, and  $y$  is the amplification factor after  $n$  cycles. The amount of template DNA and amplicon is expressed as the number of copies of the IS6110 insertion element. Amplification of 10<sup>4</sup>, 10<sup>3</sup>, 10<sup>2</sup>, and 10 fg of *M. tuberculosis* DNA in 40 PCR cycles resulted in mean efficiencies of, respectively, 58, 65, 71, and 77% per cycle. Large quantities of DNA are amplified less efficiently than small amounts of DNA because the plateau effect occurs earlier for larger amounts of template DNA.

**Detection of amplified DNA.** The results obtained by agarose gel electrophoresis were confirmed by dot blot hybridization using the enzymatically biotin-labeled probe Pt10. The results

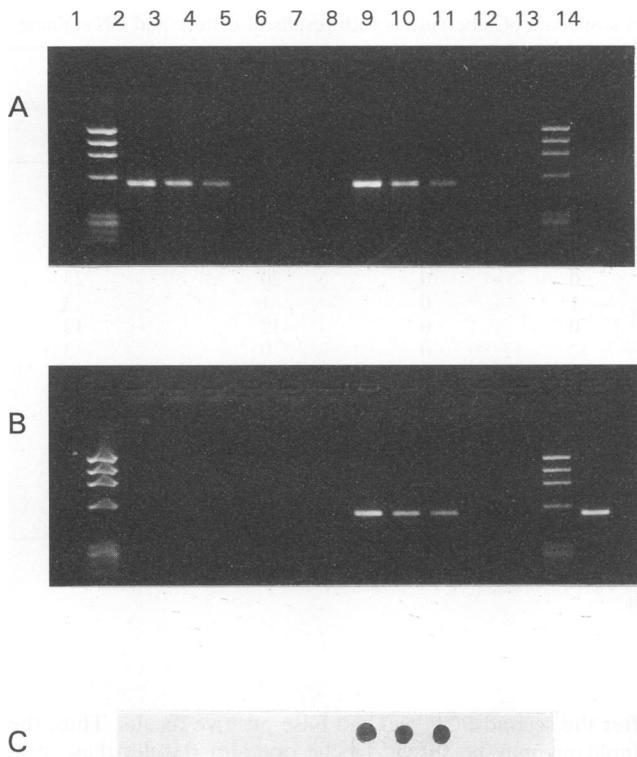


FIG. 1. Ability of UNG to break down dUTP-containing amplicons without changing the efficiency of PCR. dUTP-containing amplicons and *M. tuberculosis* DNA were used as template in PCR in the absence (A) and presence (B and C) of 0.01 U of UNG. Lanes 2 to 6,  $10^3$ ,  $10^6$ ,  $10^9$ ,  $10^{12}$ , and  $10^{15}$ -times-diluted 15-ng/ $\mu$ l amplicon solution; lanes 8 to 11, 1,000, 100, 10, and 1 fg of *M. tuberculosis* DNA; lanes 7 and 12, TE controls; lanes 1 and 13, respectively, 800 and 400 ng of *Hae*III-digested  $\phi$ X174 DNA (replicative form) as a molecular size marker; lane 14, 541-bp fragment as a molecular size marker. Amplification products were detected by agarose gel electrophoresis (A and B) or by dot blot hybridization (C).

with this oligonucleotide were similar to those with the 188-bp digoxigenin-labeled fragment, which we used previously (8). With both probes 100 pg of PCR product could be detected. Similar results were obtained with the chemically biotin-labeled Pt10 probe (data not shown). The oligonucleotide probe has the advantage that the hybridization time can be reduced from the 16 h required for the 188-bp probe to 2 h.

**Pre-PCR amplicon degradation.** The most likely cause of false-positive PCR results is contamination with amplicons from previous PCRs. After using the primer set INS1 and INS2 for a year, we noticed 245-bp amplicons in negative controls. Contamination was due to the presence of amplicons, not *M. tuberculosis* complex bacteria or DNA (8). We replaced the primers INS1 and INS2 with Pt8 and Pt9, and we used dUTP instead of dTTP to make amplicons degradable by UNG. These maneuvers did not affect the overall efficiency of our PCR (data not shown).

To demonstrate the gravity of amplicon contamination we tested 10  $\mu$ l of 1,000-fold serial dilutions of a 15-ng/ $\mu$ l 541-bp amplicon solution in PCR (Fig. 1A). This amplicon solution was prepared by using 1,000 fg of *M. tuberculosis* DNA as a template in a PCR. We chose to start with a 1,000-fold dilution since 10  $\mu$ l of a 100-fold dilution of the 541-bp amplicon solution could contain enough template DNA (2 fg) to give a

positive reaction in PCR, due to the amplification of *M. tuberculosis* DNA. Figure 1A shows that 10  $\mu$ l of a 15-ng/ $\mu$ l amplicon solution can still be amplified in PCR, even following a  $10^9$ -fold dilution. This solution contains 0.15 fg of amplicon.

The minimal amount of UNG necessary to break down contaminating amplicons was investigated by amplification of 10  $\mu$ l of the amplicon dilutions mentioned above in the presence of 0.5, 0.2, 0.1, 0.05, and 0.01 U of UNG. Panels B and C of Fig. 1 show that 0.01 U of UNG was able to break down 150 pg of 541-bp amplicons. The use of UNG did not affect the sensitivity of the PCR, as shown in Fig. 1A and B where the amplicon bands in lanes 8, 9, and 10 are of equal intensity. We performed PCR with 1,000, 100, 10, and 1 fg of *M. tuberculosis* DNA in the absence and presence of UNG. The same sensitivity was seen in both PCRs.

**Stability of the PCR mix.** For routine diagnosis it is necessary to prepare a batch of ready-for-use PCR vials and to test the quality of the batch before using it for clinical samples. We found it convenient to make batches of 200 vials. We consider the batch of sufficient quality if 10 fg of *M. tuberculosis* DNA can be amplified, resulting in a visible band in agarose gel electrophoresis, if 150 pg of 541-bp amplicon can be degraded, and if TE controls are negative.

We tested the stability of the PCR mix at different temperatures. We kept mixes at  $-20$  or  $+4^\circ\text{C}$  for 12 weeks, 18 or  $37^\circ\text{C}$  for up to 1 week and performed a PCR with 1,000, 100, and 10 fg of *M. tuberculosis* DNA. The quality of PCR mixes that had been stored for 12 weeks at  $-20^\circ\text{C}$  was the same as that of freshly prepared mixes. PCR mixes were stable at  $4^\circ\text{C}$  for 1 week, after which the quality of the mix decreased: 10 fg of *M. tuberculosis* DNA could not be detected. The stability of the PCR mixes stored at  $20^\circ\text{C}$  (room temperature) and  $37^\circ\text{C}$  deteriorated within 24 h (results not shown). These experiments show that mixes can be stored for at least 3 months in the freezer and up to 1 week on ice.

The effect of repeated thawing and freezing on the stability of the PCR mix was also tested. Thawing occurred at room temperature over 30 min, during which time the contents of the vial were at  $20^\circ\text{C}$  for about 10 min. After three cycles of thawing and freezing, the quality of the mix was unaffected, but it deteriorated after four or more cycles (results not shown). This means that an accidental thawing is not harmful for the PCR mix.

**Sensitivity of PCR for clinical samples.** To determine the detection limit of the PCR in clinical samples, we divided samples into four portions and spiked three portions with different amounts of *M. tuberculosis* bacteria (1,000, 100, and 10 per portion) before sample treatment. Our samples were four sputum samples (1 ml per portion), one liver biopsy sample (10 mg per portion), and one feces sample (600  $\mu$ l per portion) from patients suffering from diseases other than tuberculosis and one blood sample (600  $\mu$ l per portion) from a healthy individual. PCRs were performed with 10- $\mu$ l portions (1/10 of the volume) of the treated sample. To detect the presence of inhibitors, 10  $\mu$ l of the treated nonspiked sample was tested in the presence of 10 fg of *M. tuberculosis* DNA. In all cases the spiked DNA could be detected, indicating that PCR was not inhibited. In the liver biopsy sample, feces, and two sputum samples the detection limit was 10 *M. tuberculosis* bacteria per reaction vial. In the other two sputum samples and the blood the detection limit was one bacterium per reaction vial (results not shown).

**PCR results compared with those of culture and ZN staining.** Culture provides the "gold standard" for the diagnosis of infections with *M. tuberculosis*. We have tested 218 clinical specimens from patients suspected of having tuberculosis.

TABLE 2. Comparison of the PCR results of clinical samples from patients suspected of tuberculosis with results of culture and ZN staining<sup>a</sup>

Sample source(s)	PCR result	No. of samples with indicated culture result				Total no. tested
		Positive		Negative		
		ZN+	ZN-	ZN+	ZN-	
Sputum from: Thailand	Positive	35	12	2	12	61
	Negative	5 <sup>b</sup>	0	0	20	25
The Netherlands	Positive	26	2	1	1	30
	Negative	3 <sup>c</sup>	0	0	20	23
Cerebrospinal fluid	Positive	1	1	0	1	3
	Negative	0	0	0	12	12
Pleural fluid, fistula fluid, pus	Positive	1	2	0	0	3
	Negative	1 <sup>d</sup>	0	0	11	12
Biopsy	Positive	2	1	2	1	6
	Negative	3 <sup>c</sup>	0	0	15	18
Blood	Positive	0	0	0	1	1
	Negative	0	0	0	13	13
Feces	Positive	0	0	0	0	0
	Negative	0	0	0	6	6

<sup>a</sup> Six sputum samples from Thailand, one biopsy sample, and one feces sample contained inhibiting components.

<sup>b</sup> One *Mycobacterium fortuitum*, one *Mycobacterium chelonae*, and three unidentified mycobacterial strains were cultured.

<sup>c</sup> One *Mycobacterium avium* and two unidentified mycobacterial strains were cultured.

<sup>d</sup> *Mycobacterium gordonae* was cultured.

<sup>e</sup> *M. avium* was cultured in all cases.

Specimens were obtained from a wide variety of sites, and the results are shown in Table 2. One sputum sample from a patient from The Netherlands was positive by PCR but negative by ZN staining and culture. Since bronchoalveolar lavage fluid obtained from this patient was positive both by ZN staining and by culture, it is highly probable that the patient had tuberculosis. The clinical samples other than sputa were obtained from patients suspected of having tuberculosis who had a variety of underlying conditions such as other lung diseases, AIDS, or cancer. When the PCR result was positive and the results of ZN staining and/or culture were negative, the clinical picture corroborated the PCR results and the patients responded to treatment for tuberculosis.

## DISCUSSION

**Amplicon contamination.** False-positive reactions are a major problem with PCR methods for the detection of the *M. tuberculosis* complex. After using the primer set INS1 and INS2 for 1 year, we noticed that negative controls were positive in PCR, because of the presence of amplicons in the reaction vial (8). PCR with 10  $\mu$ l of a 10<sup>9</sup>-times-diluted 15-ng/ $\mu$ l 541-bp amplicon solution, which is about 0.15 fg or 250 amplicon molecules, still gives a clearly visible band in an agarose gel. This means that an aerosol droplet of this solution with a volume of 10<sup>-6</sup>  $\mu$ l can be diluted at least 100 times and can still cause a false-positive reaction. To prevent amplicon contamination, we replaced the primers INS1 and INS2 with Pt8 and PT9 and simultaneously introduced the use of dUTP instead of dTTP to make the amplicons degradable by UNG (10). Neither of these measures affected the efficiency of the PCR. The use of 0.01 U of UNG was sufficient to break down 150 pg of 541-bp amplicon, which is more than enough since this is about 10<sup>6</sup> times more than the amount of amplicon needed in PCR to get an intensity comparable to that of a false-positive negative control when UNG is omitted in the PCR mix.

During a training course five scientists were instructed in the technique of PCR for *M. tuberculosis* and the measures needed to avoid contamination. They were asked to perform PCRs without UNG (but with dUTP instead of dTTP), and already

after the second PCR two had false-positive results. Thus, the amplicons may be spread by the operator despite these precautions. These findings show that false-positive reactions due to the spread of *M. tuberculosis* DNA can also be caused by the investigator. This poses a serious problem and suggests that stringent attention to matters of detail is necessary to prevent contamination with amplicons or DNA carried by laboratory workers and that workers in PCR should not combine their work with isolation of *M. tuberculosis* DNA nor with restriction fragment length polymorphism analysis.

An alternative to the pre-PCR enzymatic amplicon degradation method is the post-PCR photochemical method. We prefer the former since the large concentrations of isopsoralen that are needed in the post-PCR photochemical method for inactivation of small and GC-rich amplicons can reduce the activity of *Taq* polymerase (14); moreover, the isopsoralen is mutagenic.

**Inhibition.** To detect false-negative reactions due to the presence of substances that inhibit *Taq* polymerase, we tested clinical samples in PCR in the presence of 10 fg of *M. tuberculosis* DNA. This approach gives one semiquantitative datum regarding the amount of *M. tuberculosis* in the clinical sample by comparison with a series of amplified *M. tuberculosis* DNA. Quantitation of the number of *M. tuberculosis* bacteria in the clinical sample can be relevant for follow-up of patients on drug therapy to detect drug resistance at an early stage. Inhibition was demonstrated in six sputum samples from Thailand. We are investigating whether the inhibition is caused by the storage vials, the transport of the samples, or storage at -20°C, since the sputum samples from the hospitals in The Netherlands, which were usually tested while fresh, did not show inhibition of the PCR.

**Treatment of clinical samples.** Most or all of the inhibiting substances in clinical samples are effectively removed by the DNA purification method with guanidinium thiocyanate and diatoms (1). This method is also applicable to lymphocyte fractions from blood but not to whole blood, since hemoglobin will inhibit the PCRs. The isolation of the lymphocyte fraction must be done with a sample less than 24 h old. Blood which is

older or is frozen cannot be used because of the lysis of erythrocytes which releases hemoglobin. The method of choice for isolation of DNA from blood is proteinase K treatment followed by phenol-chloroform extraction, since this method gives good results, is simple, and can also be used on frozen blood samples. A further advantage of this DNA isolation procedure over the method using guanidinium thiocyanate and diatoms is that larger volumes of blood can be used, improving the chances of detection of infection, since the DNA binding capacity of diatoms is limiting. We also used proteinase K treatment followed by phenol-chloroform extraction on feces, since this method proved to be superior to the guanidinium thiocyanate method. One of seven feces samples still interfered in the PCR.

**PCR results with clinical samples.** The results with PCR accorded well with the bacteriological data, except for Thai sputa which were negative by ZN staining and culture. At the Tuberculosis Division in Bangkok, ZN staining and culture were done without concentrating the sample during sputum decontamination, leading to a lower sensitivity. This was probably the reason for the high number (12 of 32) of sputum samples that were positive by PCR but negative by ZN staining and culture. The PCR results corresponded well with the culture results, except for one cerebrospinal fluid sample, one tissue biopsy sample, and one blood sample that were positive in PCR but negative in ZN staining and culture. These samples were obtained from patients for whom clinical data supported the diagnosis of tuberculosis and who responded to treatment for tuberculosis. The data suggest that PCR can be a useful tool for the diagnosis of extrapulmonary tuberculosis.

**Mycobacteria other than tuberculosis.** The increased incidence of mycobacterial infections other than tuberculosis emphasizes the need for correct species identification. Twelve samples (6%) contained mycobacteria other than tuberculosis (Table 2). None of these samples were positive in the PCR for *M. tuberculosis* complex, which demonstrates the specificity of our PCR.

**Recommendations.** In conclusion, PCR is the method of choice for the diagnosis of tuberculosis in cases where the suspicion is high but ZN staining is negative. When the sample is positive in ZN staining, PCR permits distinction between *M. tuberculosis* complex and other mycobacterial infections. In comparison with our previously described PCR for the detection of *M. tuberculosis* complex (8), the approach described here has the following advantages: (i) the risk of amplicon contamination is removed by the use of UNG and the incorporation of dUTP instead dTTP, thereby reducing false-positive reactions, resulting in increased specificity of the PCR for clinical samples; (ii) costs are lower since fewer reactions are needed to test one sample, the chemically biotin-labeled probe is cheaper than the digoxigenin labeled probe (8), and the hybridization process takes only 2 h; (iii) by conducting quality control of the PCR procedure and apparatus, the number of failures is reduced; and (iv) contamination with DNA is reduced by limiting the amount of DNA in the sample preparation room. The detection limit for *M. tuberculosis* bacteria in clinical samples has been improved 10-fold by concentrating the samples by centrifugation whenever possible. Stringent attention to matters of detail is necessary to prevent contamination by DNA carried by laboratory workers.

Quality control of the PCR mix and the performance of the amplification itself is mandatory if routine PCR is to replace culture for the diagnosis of tuberculosis.

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