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Utility of PCR in Diagnosing Pulmonary Tuberculosis

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At present, the rapid diagnosis of pulmonary tuberculosis rests with microscopy. However, this technique is insensitive and many cases of pulmonary tuberculosis cannot be initially confirmed. Nucleic acid amplification techniques are extremely sensitive, but when they are applied to tuberculosis diagnosis, they have given variable results. Investigators at six centers in Europe compared a standardized PCR system (Amplicor; Roche) against conventional culture methods. Defined clinical information was collected. Discrepant samples were retested, and inhibition assays and backup amplification with a separate primer pair were performed. *Mycobacterium tuberculosis* complex organisms were recovered from 654 (9.1%) of 7,194 samples and 293 (7.8%) of 3,738 patients. Four hundred fifty-two of the *M. tuberculosis* isolates from 204 patients were smear positive and culture positive. Among the culture-positive specimens, PCR had a sensitivity of 91.4% for smear-positive specimens and 60.9% for smear-negative specimens, with a specificity of 96.1%. Analysis of 254 PCR-positive, culture-negative specimens with discrepant results revealed that 130 were from patients with recently diagnosed tuberculosis and 94 represented a presumed laboratory error. Similar analysis of 118 PCR-negative, culture-positive specimens demonstrated that 27 discrepancies were due to presumed uneven aliquot distribution and 11 were due to presumed laboratory error; PCR inhibitors were detected in 8 specimens. Amplicor enables laboratories with little previous experience with nucleic acid amplification to perform PCR. Disease in more than 60% of the patients with tuberculosis with smear-negative, culture-positive specimens can be diagnosed at the time of admission, and potentially all patients with smear-positive specimens can immediately be confirmed as being infected with *M. tuberculosis*, leading to improved clinical management.

Tuberculosis is on the increase throughout the world (2). Despite this it remains one of the few infections whose diagnosis often relies on clinical acumen. The reasons for this are manifold. Microscopic examination with either Ziehl-Neelsen (ZN)- or fluorochrome-stained smears is insensitive, detecting acid-fast bacilli only when there are $\geq 10^4$ mycobacteria per ml. Culture on solid medium is labor-intensive and too slow for clinical usefulness, taking a minimum of 2 weeks and often longer for microscopy-negative samples. Radiometric liquid (BACTEC) and biphasic (MB Chek) culture systems have improved both the recovery rates and the speed of isolation, but these systems still cannot influence initial bedside decision making (9, 10). Given that half of the estimated 8 million new cases of tuberculosis per year are either smear-negative pulmonary or extrapulmonary infection, many diagnoses cannot be confirmed at the time of presentation (22).

Several amplification techniques (some commercially driven developments) have been evaluated for use in the diagnosis of infections, most commonly PCR (8) but also transcription-mediated amplification (targeting rRNA) (11, 18), branched DNA signal amplification, ligase chain reaction, Q-β replicase amplification, and strand displacement amplification (17, 25). More than 80 reports describing studies that have used in-house PCR assays to detect *Mycobacterium tuberculosis* from clinical samples with one or more of several primer pairs have been published. The primers most commonly target IS*6110*, an insertion sequence which is already preamplified with usually more than 10 copies in *M. tuberculosis* and 1 to 5 copies in *Mycobacterium bovis* (3–6, 14, 19, 20, 23). The lower limit of detection reported by PCR varies between 1 and 100 bacilli. In cases in which clinical sensitivity and specificity have been assessed for studies that analyzed sputa, the results have varied considerably. Clarridge and colleagues (3) reported on the use of an IS*6110*-targeted PCR system with more than 5,000 samples, with a sensitivity of 94% for smear-positive specimens and 62% for smear-negative specimens, yielding *M. tuberculosis* on culture with a 99% specificity. By contrast, in an assessment of reliability and reproducibility between laboratories, all of which routinely used PCR and targeted IS*6110*, Noordhoek and colleagues found that sensitivity and specificity for detecting 10^3 *M. bovis* BCG organisms varied between 2 and 90% and 3 and 77%, respectively (15, 16). Their report emphasized the need for greater standardization of reagents and methodology.

The Amplicor PCR system has been developed and evaluated in many diagnostic areas, including *Chlamydia trachomatis*, hepatitis C virus, and human immunodeficiency virus (13). By standardizing all aspects of extraction, amplification, and detection and including a system that prevents cross-contami-

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TABLE 1. PCR results for specimens defined by investigating site, decontamination procedures, and conventional culture methods

Site	No. of specimens	NALC[a]	Culture medium[b]	Preadjustment		Postadjustment[c]	
				Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
1	2,951	No	L-J, BACTEC	86.1	96.5	86.8	99.8
2	1,229	Yes	L-J, BACTEC, Middlebrook 7H10 and selective 7H11	66.7	97.6	80.6	99.7
3	1,198	Yes	L-J, BACTEC	80.8	90.6	89.1	98.7
4	936	Yes	L-J, BACTEC	90.9	97.1	92.2	99.2
5	538	Yes	L-J, BACTEC	78.9	98.1	78.9	99.8
6a	178	No	L-J, Kirchner	89.7	96.8	89.7	99.4
6b	164	No	L-J, Kirchner				

[a] NALC, *N*-acetyl-L-cysteine. The decontamination method at all sites except site 6b was sodium hydroxide; at site 6b, oxalic acid was also used.
[b] Smear positivity rates before adjustment were as follows: site 1, 61.8%; site 2, 56.9%; site 3, 74%, site 4, 80.5%; site 5, 42.1%; site 6 (combined), 70.0%. L-J, Lowenstein-Jensen medium.
[c] Data for specimens for which there was a presumed laboratory error or aliquoting error were adjusted; data for specimens for which there was a presumed test error or inhibition or which were unresolved remained unadjusted; data for specimens from patients with clinical tuberculosis were removed from the analysis.

nation, the *M. tuberculosis* Amplicor PCR system is more robust than and circumvents many of the problems that bedevil in-house systems. The biotinylated primers used in the assay recognize a highly conserved region of the 16S rRNA gene, resulting in an amplification of a 584-bp sequence (1). Here we report the results of a pan-European study examining the utility of the Amplicor PCR system on respiratory samples, mainly sputa, for rapidly diagnosing pulmonary tuberculosis.

MATERIALS AND METHODS

Clinical specimens and microbiology techniques. From mid-1993 to mid-1994, six sites in Europe compared the Amplicor PCR system against conventional culture by using both liquid medium (BACTEC 12B medium [Becton Dickinson] or Kirchner medium) and/or solid medium (Lowenstein-Jensen medium [glycerol and pyruvate supplemented] and/or Middlebrook 7H10 and selective 7H11 media). No comparative studies of BACTEC and Kirchner media have been performed, but recovery rates on both media have been described as being superior to those on conventional Lowenstein-Jensen medium (9, 12). Unselected respiratory specimens submitted to the laboratories from patients being screened for tuberculosis or being followed up during treatment made up the sample group. Each laboratory continued to use its standard methodology for processing samples for mycobacterial culture. Mostly, this included the use of a mucolytic agent (*N*-acetyl-L-cysteine). All laboratories decontaminated the specimens with sodium hydroxide (Table 1). Two 100-μl aliquots of processed sample (aliquots A and B) were then taken for Amplicor PCR analysis before the remainder was inoculated onto culture medium. Aliquot B was stored at ≤−20°C. Cultures were reviewed at least weekly for growth and were kept for a minimum of 7 weeks. Microscopy was performed by standard procedures with ZN and/or fluorochrome stains. Mycobacteria were identified by the standard methods in routine use in each laboratory (7). Defined clinical information was collected for each patient concerning recent or remote history of tuberculosis and the final diagnosis. In an attempt to gauge the likely error arising from unequal aliquoting of the mycobacteria in specimens with few bacilli, data from site 3, which used three different media (glycerol- and pyruvate-supplemented Lowenstein-Jensen media and BACTEC medium) were analyzed to compare smear and Amplicor PCR results with the number of different culture media which were positive for growth.

PCR testing. Amplicor is a system with a kit-based format, with prepackaged components and extraction, amplification, and detection stages. Following extraction, 50 μl of the prepared sample (sample A1) was amplified in a GeneAmp PCR 9600 system (Perkin-Elmer) by using the following cycle profile: a 2-min hold at 50°C (for AmpErase activity) and 20 s at 98°C, 20 s at 62°C, and 45 s at 72°C (two cycles); this was followed by 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C (35 cycles) and then a 5-min hold at 72°C. The remaining 50 μl (sample A2) was stored at ≤−20°C. The amplified products were then denatured. A total of 100 μl of hybridization buffer was added to microtiter well strips. A total of 25 μl of the denatured amplified product was added to the plate, and the plate was incubated at 37°C for 1.5 h to allow for hybridization. Following washing, 100 μl of avidin-horseradish peroxidase was added to each well and the conjugate was incubated at 37°C for 15 min. A total of 100 μl of substrate was then added, and color was allowed to develop at room temperature for 10 min before stopping the reaction. The optical density was read in an enzyme-linked immunosorbent assay reader at A_{450}. A reading of ≥0.35 was regarded as positive; the positive control had to be ≥3.0 and the negative controls had to be ≤0.25. The system includes AmpErase, which prevents carryover contamination; dUTP replaces dTTP as a nucleotide in the reaction. By using AmpErase, a thermolabile uracil-*N*-glucosylase enzyme, any previously amplified products are cleaved. The enzyme is subsequently inactivated during the first amplification cycle.

The analysis was performed at the time of sample submission; hence, investigators were unaware of the culture results.

Discrepancy analysis. Retesting, inhibition assays, and backup nucleic acid amplification with a separate primer pair were performed on all samples with discrepant results in a single laboratory (27). Two samples were available for retesting: an aliquot of the initial sample preparation (prepared by the site investigator; sample A2) and an aliquot of the original liquefied sputum specimen which was subsequently prepared for PCR testing (sample B). Inhibition assays were performed by spiking samples A2 and B with positive *M. tuberculosis* control DNA (five copies). Samples with false-positive and false-negative results were checked with a second set of primers amplifying nucleic acids encoding the enzyme superoxide dismutase. This has previously been found to be a suitable target for screening for mycobacteria and discriminatory in differentiating species when using species-specific probes; the primers, probes, and PCR conditions used were as described elsewhere (27).

Study analysis. Sensitivity, specificity, and positive and negative predictive values were calculated for PCR compared with standard culture of specimens. Because of the simplicity, availability, and low cost of microscopy and the ease with which repeat sputum samples can usually be obtained, an analysis of the overall results per patient was also performed.

RESULTS

Specimen and patient analysis. (i) Preadjustment. Of 7,431 specimens submitted, 7,194 (96.8%) were analyzed by PCR and standard culture. These were collected from 3,794 patients. The reasons for exclusion from analysis by PCR were insufficient sample volume (<2 ml), persistent contamination of all culture media, and/or persistent viscosity, despite pretreatment with *N*-acetyl-L-cysteine. Mycobacteria were recovered from 833 samples (11.6%) and 418 patients (11.0%). Six hundred fifty-four (9.1%) samples (293 [7.8%] patients) grew *M. tuberculosis* complex and 179 samples (125 patients) grew nontuberculous mycobacteria (NTM). Microscopy was positive for 452 (6.3%) specimens (204 [5.5%] patients) from which *M. tuberculosis* complex was isolated and negative for 202 specimens (89 patients); when samples yielded NTM, 36 (20.1%) had a positive microscopy result. PCR had an overall sensitivity of 82.0% in specimen evaluations and 85.7% in patient evaluations; the overall specificity in specimen evaluations was 96.1% (Table 2). The sensitivity for smear-negative, culture-positive samples was 60.9% (Table 3).

Of 312 positive *M. tuberculosis* complex cultures from site 3, 36 (11.5%) were positive on a single medium, 37 (11.9%) were positive on two media, and 239 (76.6%) were positive on three media. The likelihood of a positive smear or PCR result increased with the number of media that were positive for an individual specimen (Table 4).

(ii) Postadjustment. Any sample for which there was a disagreement between PCR and culture results was considered to

TABLE 2. PCR test results compared with those of culture

Group and culture result	No. of specimens			
	Preadjustment[a]		Postadjustment[b]	
	Positive	Negative	Positive	Negative
Specimens				
Positive	536	118	575	79
Negative	254	6,286	30	6,381
Patients				
Positive	251	42	258	35
Negative	168	3,333	22	3,423

[a] For specimens, the sensitivity was 82.0%, the specificity was 96.1%, the positive predictive value was 67.8%, and the negative predictive value was 98.2%. For patients, the sensitivity was 85.7%, the specificity was 95.2%, the positive predictive value was 59.9%, and the negative predictive value was 98.8%.

[b] Data for specimens for which there was a presumed laboratory error or aliquoting error were adjusted; data for specimens for which there was a presumed test error or inhibition or which were unresolved remained unadjusted. For specimens, the adjusted sensitivity was 87.8%, the adjusted specificity was 99.5%, the adjusted positive predictive value was 95.0%, and the adjusted negative predictive value was 98.8%. For patients, the adjusted sensitivity was 88.1%, the adjusted specificity was 99.4%, the adjusted positive predictive value was 91.8%, and the adjusted negative predictive value was 99.0%. Data for 130 specimens from 56 patients with clinical tuberculosis were removed from the analysis.

have a discrepant result. These specimens were retested by using aliquots A2 and B, inhibition assays, and backup amplification with a separate primer pair; the patient case notes were also reviewed. Clinical tuberculosis was accepted as the cause of a PCR-positive, culture-negative result if *M. tuberculosis* had been isolated or a diagnosis of active tuberculosis requiring treatment had been made in the preceding 12 months.

Adjustment was determined by reclassifying 94 PCR-positive, culture-negative samples presumed to result from a laboratory error as true positives and 38 PCR-negative, culture-positive samples presumed to result from either a laboratory error ($n = 11$) or uneven aliquot distribution ($n = 27$) as true positives (Tables 5 and 6). Thirty PCR-positive, culture-negative samples that resulted from a PCR test error ($n = 6$) or that were unresolvable ($n = 24$) remained false positives and 80 PCR-negative, culture-positive samples that resulted from a PCR test error ($n = 55$) or inhibition ($n = 8$) or that were unresolvable ($n = 17$) remained false negatives (Tables 5 and 6). One hundred thirty false-positive specimens that were found to be from 56 patients with clinical tuberculosis were removed from the analysis. This was necessary because patient case notes were scrutinized only for patients providing samples with discrepant results. The number of PCR-negative, culture-negative patients with clinical tuberculosis was not known. Inclusion of PCR-positive, culture-negative clinical tuberculosis patients as verified positives would have artificially raised the sensitivity of the test.

Following adjustment, the overall sensitivity of PCR on sample analysis improved to 87.9%, with a specificity of 99.5%, and the overall sensitivity of PCR on patient analysis improved to 88.1%, with a specificity of 99.3% (Table 2). Of the smear-negative, culture-positive samples, 66.8% were PCR positive; similarly, 64.0% of patients with persistently smear-negative, culture-positive samples had one or more samples that were PCR positive (Table 3). Among the smear-positive samples, PCR completely discriminated *M. tuberculosis* complex from NTM. One patient with *Mycobacterium gordonae* infection had a false-positive PCR result preadjustment; on retesting, the result was found to be negative with the backup samples A2 and B. The result was therefore reclassified as a true negative. No patient infected with an NTM had a positive PCR result postadjustment.

Site analysis. The results obtained at each site were generally similar. The exception to this were sites 2 (preadjustment sensitivity, 66.7%) and 5 (preadjustment sensitivity, 78.9%), which had the lowest smear positive-to-total positive ratios (0.57 and 0.42, respectively) for *M. tuberculosis* complex isolates (range of ratios for the other sites, 0.65 to 0.81). This will have resulted in an overall lower sensitivity of PCR. Site 6 used Kirchner liquid medium instead of BACTEC medium. The values for site 6 were not different from those for the other sites.

DISCUSSION

In the present study, six collaborating sites in Europe evaluated the Amplicor PCR system in parallel with their routine method of processing respiratory specimens. Despite slight differences in the use of mucolytic agents, decontamination procedures, culture media, and inoculation volumes, the results from all six centers are comparable. The overall preadjusted sensitivity and specificity for 7,194 specimens were 82.0 and 96.1%, respectively, with a positive predictive value of 67.8% and a negative predictive value of 98.2%. Ninety-one percent of smear-positive and 60.9% of smear-negative, culture-positive specimens were positive by PCR. These results are similar to the best possible results previously reported when using in-house PCR (15).

The discrepancy analysis and readjustment of figures are valid because they give a good indication of the true sensitivity and specificity of the test, even though these results are unlikely to be achievable in practice. Of 254 false-positive specimens (168 patients), 130 (56 patients) were from patients who had evidence of recent tuberculosis that had been diagnosed within the preceding 12 months. Thus, positivity presumably resulted from noncultivable *M. tuberculosis* organisms that were either nonviable because of treatment or overzealous decontamination. Knowledge of the clinical status of the patient is available to the clinician at the time of examination, and therefore, for the majority of patients, no difficulty in interpreting this as a true-positive result should arise. A technical laboratory error was presumed to be the cause of the

TABLE 3. Sensitivity of PCR compared with culture

Group and smear result	No. of specimens			
	Preadjustment[a]		Postadjustment[b]	
	Positive	Negative	Positive	Negative
Culture-positive specimens				
Positive	413	39	440	12
Negative	123	79	135	67
Culture-positive patients				
Positive	199	5	201	3
Negative	52	37	57	32

[a] Sensitivity for smear-positive specimens, 91.4%; sensitivity for smear-negative specimens, 60.9%; sensitivity for smear-positive patients, 97.5%; sensitivity for smear-negative patients, 58.4%.

[b] Data for specimens for which there was a presumed laboratory error or aliquoting error were adjusted; data for specimens for which there was presumed test error or inhibition or which were unresolved remained unadjusted. Adjusted results were as follows: sensitivity for smear-positive specimens, 97.3%; sensitivity for smear-negative specimens, 66.8%; sensitivity for smear-positive patients, 98.5%; sensitivity for smear-negative patients, 64.0%.

TABLE 4. Smear and PCR results defined by the number of positive culture media at a single laboratory site

Specimen	Positivity rate (%) for the following no. of *M. tuberculosis*-positive culture media[a]:		
	One medium (n = 36)	Two media (n = 37)	Three media (n = 239)
Smear positive	30.6	59.4	82.8
PCR positive	41.7	67.6	89.1

[a] At site 3, 312 specimens were cultured on pyruvate- and glycerol-supplemented Lowenstein-Jensen and BACTEC media.

false-positive results for 94 specimens (90 patients), but this would only be discernible in clinical practice if all positive results were double-checked. In only six samples was a verified false-positive result identified. The cause is unlikely to relate to carryover contamination of the sample and failure of AmpErase. However, it may result from cross-contamination of preliquefied sputum from another positive sample (21). It is also possible that noncultivable *M. tuberculosis* organisms are being detected in patients with past or current primary pulmonary disease and no signs of clinically active tuberculosis.

Of 118 false-negative specimens (42 patients), 11 (4 patients) resulted from presumed technical laboratory errors. It was also presumed that either inadvertent removal of the centrifuged pellet during preparation or unequal distribution of mycobacteria during aliquoting in patients with paucibacillary disease accounted for 27 false-negatives specimens. Both of these would probably come to light if multiple specimens were sent from the same patient. For eight samples, inhibitors of DNA polymerase were responsible for the false-negative result, although this would not be routinely tested for using a first-generation Amplicor PCR kit; second-generation tests will incorporate an internal inhibition control that is run in parallel with the PCR. Fifty-five specimens (27 patients) with PCR-negative, culture-positive results were verified to be false negatives. Of these, nine specimens were positive by the backup PCR and 46 were negative.

A single primer copy was used, and when there are only a few mycobacteria, it is possible that there has been a base pair deletion or mutation that has interfered with primer attachment. This would explain the false-negative specimens for which the backup PCR result was positive. Occasionally, strains of *M. tuberculosis* have been identified which lack IS*6110* (24) (the most frequently used target in PCRs), but a lack of the rRNA gene should be incompatible with viability, and so this is an unlikely explanation for the majority of false-negative results. The exact explanation for negative rRNA and superoxide dismutase PCRs and negative inhibition assays (46 specimens) remains unresolved, but an uneven distribution of mycobacteria in the specimens and the absence of mycobacterial nucleic acid from the aliquot used for PCR seems to be the most likely explanation. Inoculation volume differences between culture (usually 0.5 to 1.0 ml) and Amplicor PCR (0.1 ml) may partly explain this. The analysis from site 3 demonstrated a nearly linear relationship between the rate of PCR positivity and the number of different inoculated culture media from which *M. tuberculosis* complex organisms subsequently grew, supporting the hypothesis that some PCR-negative, culture-positive specimens result from uneven aliquoting. It is also conceivable that these *M. tuberculosis* complex strains may be particularly sensitive to genome disruption during the extraction phase. With adjustment, the overall sensitivity and specificity for the specimens (n = 7,064) improved to 87.9 and 99.5%, respectively; 97.3% of smear-positive, culture-positive specimens and 66.8% of smear-negative, culture-positive specimens were positive by PCR. The adjusted patient analysis (n = 3,738) demonstrated that 98.5% of patients with smear-positive specimens and 64.0% of patients with persistently smear-negative specimens had one or more samples that were PCR positive.

The availability of a prepackaged kit with instructions, quality-controlled, standardized reagents, and the AmpErase system to prevent carryover contamination reduces the technical demands of diagnosis by PCR, thus enabling standard laboratories with little previous experience with nucleic acid amplification to perform the test. Following sputum decontamination, the results are available within a working day and, therefore, will have a bearing on patient management. The present study of a large number of specimens and patients has demonstrated very good sensitivity and excellent specificity, with a negative predictive value of 98.8% and a positive predictive value of 95.0%, after adjustment. In areas of low endemicity for human immunodeficiency virus infection, microscopy has a specificity of >95% and is an excellent screening test for sputum samples. However, as the present study has confirmed, a significant proportion of respiratory samples and patients with pulmonary tuberculosis who are subsequently culture positive are initially smear negative (nearly one-third in the present study). Pulmonary tuberculosis in two-thirds of these patients can be diagnosed by PCR. Although the Amplicor PCR is relatively costly (approximately $15 per test), this must be balanced by several factors: earlier diagnosis resulting in fewer investigations, reduced hospital stay, earlier initiation of correct therapy (either for tuberculosis or a diagnosis other than tuberculosis), less use of inappropriate empirical antituberculosis therapy, and less risk of continued infectivity in or out of hospital. Moreover, where concomitant human immunodeficiency virus infection is frequent, the specificity of microscopy falls. Rapid distinction from *Mycobacterium avium*, and occasionally other NTM, in this population is vitally important for targeting optimal antimycobacterial therapy, and this would be an appropriate use for the Amplicor PCR system in a smear-positive patient, given the complete discrimination for smear-positive samples shown in the present study. The use of nucleic acid amplification for the diagnosis of mycobacterial infections will almost certainly broaden in the near future. It will be used to determine susceptibility to antituberculosis drugs (26), immediate determination of the infecting mycobacterial species, and maybe the duration of therapy.

TABLE 5. Results for 254 PCR-positive, culture-negative specimens

Presumed cause	Result			No. of specimens	No. of patients
	Clinical tuberculosis[a]	Sample A2 PCR result	Sample B PCR result		
Clinical TB[b]	+	+	+	130	56
Laboratory error[c]	−	+	−	94	90
Test error[d]	−	+	+	6	5
Unresolvable	+	−	+	24	17
	+	+	−		

[a] *M. tuberculosis* isolation or a clinical diagnosis of active tuberculosis requiring treatment in the preceding 12 months.

[b] The likely interpretation was an interpretation error resulting from detection of noncultivable *M. tuberculosis*.

[c] The likely interpretation was a technical error in the initial preparation, amplification, or detection of DNA in the original sample.

[d] The likely interpretation was an unexplained error of the PCR system.

TABLE 6. Results for 118 PCR-negative, culture-positive specimens

Presumed cause	Result			No. of specimens	No. of patients
	Clinical tuberculosis[a]	Sample A2 PCR result	Sample B PCR result		
Uneven aliquoting[b]	+	−	+	27	3
Laboratory error[c]	+	+	+	11	4
Test error[d]	+	−	−	55	22
Inhibited[e]	+	−	−	8	3
Unresolvable	+	+	−	17	10

[a] All *M. tuberculosis* culture-positive patients had clinically active tuberculosis requiring treatment.

[b] The likely interpretation was an error arising from low bacilliary load and an uneven aliquot distribution.

[c] The likely interpretation was a technical error in the initial amplification or detection of DNA.

[d] The likely interpretation was an error of the PCR system; the backup PCR system with superoxide dismutase was positive for nine specimens.

[e] The likely interpretation was an error resulting from DNA polymerase inhibitors, assessed by persistent inhibition, despite the addition of control *M. tuberculosis* DNA to the sample.

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