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Diagnosis of Tuberculosis by Amplicor *Mycobacterium tuberculosis* Test: a Multicenter Study

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The Amplicor *Mycobacterium tuberculosis* test is a new PCR assay for the direct detection of *Mycobacterium tuberculosis* from clinical samples. A multicenter study that included six laboratories was done to evaluate the Amplicor test in comparison with direct microscopy and culture (solid or radiometric media), and the culture method was used as the "gold standard." A total of 2,073 specimens, i.e., 1,749 respiratory specimens and 324 other specimens, were tested. A total of 184 cultures yielded *M. tuberculosis*. Of these 184 cultures, 77 (42%) were smear negative and 23 (12.5%) concerned extrapulmonary specimens. The sensitivity of the Amplicor test for all of the specimens and for extrapulmonary, smear-positive, and smear-negative specimens was 86, 83, 94.5, and 74%, respectively. The sensitivity of direct microscopy in comparison with that of culture was 58%. A total of 95% of patients with culture-proven tuberculosis were diagnosed by the Amplicor test, whereas direct microscopy detected mycobacteria in only 72% of these patients. The Amplicor test exhibited a high degree of specificity (98%). The assay was very rapid and easy to perform.

Because of its increased incidence and the advent of multidrug-resistant strains, tuberculosis represents a great public health problem (18). Early detection of *Mycobacterium tuberculosis* in clinical samples becomes more and more important in the control of tuberculosis both for the clinical treatment of infected individuals and for the identification of exposed individuals. The exact identification of the mycobacterial species involved is also of major importance because of the AIDS epidemic and the consequent increase in the number of associated nontuberculous infections such as those caused by *Mycobacterium avium* complex (8). The diagnosis of tuberculosis is always based on direct microscopy and culture. Despite recent progress in the rapidity of culture (14) and species identification (11), these procedures still require 2 to 6 weeks. Direct microscopy is the most rapid method of detecting mycobacteria in samples, but it lacks sensitivity and specificity. Mycobacteria are not identified, and although epidemiological and clinical information is important, it is not sufficient to make conclusions about the mycobacterial species. Therefore, a positive acid-fast stain is only a presumptive diagnosis of tuberculosis. In principle, these drawbacks could be solved by amplification methods and the detection of mycobacterial nucleic acids. In the last few years, many investigators have described various methods of amplification, especially by PCR, and promising results have been obtained (7, 15, 20). Nevertheless, although simplified procedures were investigated (4, 6, 19), all of these methods remained too complex, too long, and not reliable enough for use in routine clinical practice. The recent development of commercial test systems will perhaps exclude these problems.

In this report, we present the results of a multicenter study of a new PCR method developed by Roche Diagnostic Systems (Amplicor *Mycobacterium tuberculosis* test) for the direct detection of *M. tuberculosis* complex in clinical samples. Results obtained with the Amplicor test and the culture method were compared in order to assess the sensitivity and specificity of the Amplicor test. Our evaluation included 2,173 specimens tested in six different laboratories by direct microscopy, the Amplicor test, and the "gold standard", i.e., culture on Loewenstein-Jensen medium or in radiometric liquid medium. All types of clinical samples except blood were included in the study.

MATERIALS AND METHODS

Clinical specimens. Our study included 2,073 clinical specimens received for mycobacterial culture in six different laboratories, i.e., five hospital laboratories of bacteriology and one private medical analysis laboratory (see Table 1 for details). These specimens were from 1,125 patients and were composed of 1,749 respiratory specimens (sputa, bronchial and tracheal aspirates, and bronchoalveolar lavage specimens) and 324 extrapulmonary specimens (urine, ascitic, pleural, and articular fluid; and tissue biopsy, abcess, and cerebrospinal fluid specimens). The selection of patients and the type of specimens were not based on the same criteria in the six laboratories. Two laboratories (laboratories 1 and 6) included in the research all patients infected with mycobacteria, whereas the four other laboratories included only some of these patients, i.e., those with the highest clinical suspicion of tuberculosis. Therefore, the prevalence of *M. tuberculosis* in the specimens studied was different according to the laboratory. Moreover, most laboratories included all types of specimens except blood, whereas one laboratory (laboratory 1) included only respiratory specimens. Thus, positive and negative values of the Amplicor test were not evaluable, but its sensitivity and specificity were evaluable.

Decontamination procedures. Respiratory specimens were digested and decontamined by the *N*-acetyl-L-cysteine (NALC)–NaOH procedure recommended by the Centers for Disease Control and Prevention. Only one laboratory (laboratory 6) used benzalkonium chloride instead of NALC. After neutralization and centrifugation at 3,000 × *g* for 20 to 30 min, the supernatant was discarded, and the sediment (about 2 ml) was used for direct microscopy, culture,

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TABLE 1. Distribution of the 2,073 specimens studied among the six different laboratories

Laboratory	No. of patients	No. of specimens	No. of respiratory specimens	No. of nonrespiratory specimens
1	249	526	526	0
2	153	199	159	40
3	170	192	159	33
4	83	85	70	15
5	72	92	73	19
6	398	979	762	217
Total	1,125	2,073	1,749	324

and the Amplicor test. Extrapulmonary specimens from closed and normally sterile cavities were not decontamined but were used directly after a single centrifugation or without centrifugation if the quantity was small. The remaining sediments were stored at −20°C until the final results of the amplification and the culture were obtained.

Microscopy. Fixed smears were stained with auramine fluorochrome stain. Positive slides with acid-fast bacilli (AFB) were confirmed to be positive by Ziehl-Neelsen staining (10). One laboratory (laboratory 5) used only Ziehl-Neelsen staining. The results of the microscopic examination were reported on the basis of the following criteria at a ×1,000 magnification: no AFB seen was considered negative, 1 to 9 AFB per 100 fields was considered rare, 10 to 100 AFB per 100 fields was considered moderate, greater than 100 AFB per 100 fields was considered many.

Culture. In two laboratories (laboratories 1 and 2), 0.5 ml of the sediment was cultivated by the radiometric BACTEC technique (14) with the BACTEC 460 TB instrument. Each vial containing BACTEC Middlebrook 7H12 medium (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) was supplemented with 0.1 ml of an antimicrobial mixture (PANTA; Becton-Dickinson) containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin. In the other laboratories, three Lowenstein-Jensen slants were inoculated with 0.2 ml of sediment (10). Mycobacterial cultures were incubated at 37°C for 6 weeks.

Identification of mycobacteria. Conventional biochemical techniques (11) or Accu-Probe culture identification tests (Gen-Probe) (13) were used for the identification of the isolates.

Amplicor test. The Amplicor *Mycobacterium tuberculosis* test was done in accordance with the instructions of the manufacturers. The test is a PCR assay that uses biotinylated genus-specific primers to amplify a 584-bp sequence and then an oligonucleotide probe specific for *M. tuberculosis* complex organisms to bind to the amplicons.

(i) Specimen preparation. A total of 100 μl of sediment was added to a tube containing 500 μl of wash solution, vortexed, and centrifuged at 12,500 × *g* for 10 min. The supernatant was discarded, and 100 μl of specimen lysis reagent was added. The mixture was vortexed and incubated at 60°C in a dry heat block for 45 min. Then, 100 μl of specimen neutralization reagent was added.

(ii) Amplification. A total of 50 μl of the specimen preparation was transferred to a PCR tube containing 50 μl of amplification mixture. The remaining 150 μl of the prepared patient specimen was stored at −20°C. The amplification was performed in a Perkin-Elmer Gene Amp PCR System 9600 thermal cycler.

(iii) Hybridization. After amplification, the amplicons were immediately denatured by adding 100 μl of denaturation solution to each PCR tube. Then, a 25-μl aliquot was transferred to a microwell plate containing the bound oligonucleotide probe.

(iv) Detection. After removing the unbound material by washing, an avidin-horseradish peroxidase conjugate was added to the plate. After the unbound conjugate was removed by a second washing, hydrogen peroxide and tetramethylbenzidine were added as substrates to form a colored complex. The reaction was stopped by the addition of weak acid. The optical density was measured in an automated microwell plate reader at A_{450}.

(v) Results. Specimens with an A_{450} greater than or equal to 0.35 units were considered positive. Specimens with an A_{450} less than 0.35 units were considered negative.

(vi) Controls. Positive and negative controls for amplification and detection were included in each set of reactions. The result for the negative control should be less than 0.25 A_{450} units, and that for the positive control should be greater than 3.0 A_{450} units.

Statistical methods. Statistical comparisons of sensitivities were performed by using chi-squared analysis. For most sensitivities and specificities, 95% confidence intervals were calculated by using Epi Info version 6.

RESULTS

Culture and smear results. Table 2 presents the results obtained for the 2,073 specimens from the 1,125 patients. Cultures revealed 184 *M. tuberculosis* isolates from 100 patients and 44 isolates of nontuberculous mycobacteria (NTM). The species of NTM identified were *Mycobacterium avium* (23 specimens), *Mycobacterium gordonae* (11 specimens), *Mycobacterium fortuitum* (6 specimens), and *Mycobacterium xenopi* (4 specimens). Of the 184 *M. tuberculosis* isolates, 107 (58%) were smear positive and 77 (42%) were smear negative. Of the 44 NTM isolates, 15 (34%) were smear positive.

TABLE 2. Comparison of Amplicor test, culture, and smear results obtained by the various participating laboratories

Test and result	No. of specimens from laboratory:					
	1	2	3/4[a]	5	6	Total
Culture positive for *M. tuberculosis*						
Amplicor test positive						
Smear positive	4	16	21	15	45	101
Smear negative	3	16	13	0	25	57
Amplicor test negative						
Smear positive	0	0	0	0	6	6
Smear negative	3	3	4	1	9	20
Culture positive for NTM						
Amplicor test positive	0	0	0	0	0	
Amplicor test negative						
Smear positive	0	6	0	4	5	15
Smear negative	6	1	2	0	20	29
Culture negative						
Amplicor test positive						
Smear positive	0	0	0	0	6	6
Smear negative	6	4	1	1	16	28
Amplicor test negative						
Smear positive	2	0	0	2	4	8
Smear negative	502	153	236	69	843	1,803
Total for all specimens studied	526	199	277	92	979	2,073

[a] Results from laboratories 3 and 4 are combined.

Amplicor test results. A total of 1,811 specimens were culture and Amplicor test negative. Eight of them were smear positive.

None of the 44 NTM isolates was detected by the Amplicor test. Of the 184 *M. tuberculosis* isolates, 158 (86%) were detected by the Amplicor test, whereas 26 (14%) were missed by the Amplicor test. No significant difference in the sensitivity of the Amplicor test ($P > 0.1$) was observed whether the culture method was the radiometric BACTEC method (sensitivity, 86.6%) or the classical method with Lowenstein-Jensen medium (sensitivity, 85.6%). Of the 26 specimens that were Amplicor test negative and culture positive for *M. tuberculosis*, 20 (77%) were smear negative and 6 (23%) were smear positive. The six specimens that were Amplicor test negative, smear positive, and culture positive for *M. tuberculosis* were composed of three sputum specimens, each containing 10, 100, and <10 AFB per 100 fields by Ziehl-Neelsen staining, two tracheal aspirates each contained 10 AFB, and one tracheal aspirate contained <10 AFB. The 158 specimens were from 95 of the 100 patients in the study with culture-proven tuberculosis.

M. tuberculosis did not grow in culture for 34 of the 192

Amplicor test-positive specimens. Of these 34 specimens, 25 were from patients receiving antituberculous treatment for culture-proven tuberculosis. Thus, these 25 results were considered true positives for the Amplicor test because the Amplicor test detected nonviable bacteria. Six of these 25 specimens were smear positive. The nine other specimens (Amplicor test positive and culture negative) were from seven patients who were receiving no antituberculous medication and for whom there was no suspicion of tuberculosis. In addition, other specimens from these seven patients were smear, culture, and Amplicor test negative. These nine specimens were controlled by a second Amplicor test from a new aliquot of the same sediment. In two cases, the control result was still a positive result, but in seven cases a negative result was obtained, in accordance with a false-positive result by contamination. Concerning these seven specimens, in order to know if contamination had occurred during or after specimen preparation, a second amplification was done from the same prepared specimen, which had been stored at −20°C. Positive results were obtained. Therefore, contamination occurred before the amplification stage, during specimen preparation.

In summary, the sensitivity and the specificity of the Amplicor test versus those of culture as the gold standard were 86% (95% confidence interval, 80 to 90%) and 98% (95% confidence interval, 97.5 to 99%), respectively. Depending on whether the AFB smear was positive or negative, the sensitivity of the Amplicor test was 94.5% (95% confidence interval, 88 to 98%) or 74% (95% confidence interval, 63 to 83%), respectively. The sensitivity of direct microscopy versus that of culture was 58% (95% confidence interval, 51 to 65%). The Amplicor test detected 95 (95%) patients with culture-proven tuberculosis, whereas direct microscopy detected 72 (72%) of patients with culture-proven tuberculosis.

Extrapulmonary specimens. A total of 23 (12.5%) of the 184 *M. tuberculosis* isolates were obtained from extrapulmonary specimens. Of these 23 specimens, 12 were smear positive and 11 were smear negative. The sensitivity of the Amplicor test for extrapulmonary specimens was 83%, i.e., 100 or 64%, depending on whether the AFB smear was positive or negative, respectively. The same values for respiratory specimens were 86, 94, and 76%, respectively. The difference in the sensitivity between respiratory and nonrespiratory specimens was not significant ($P > 0.1$). However, because of the small number of nonrespiratory specimens that were positive by culture, other studies are necessary to confirm these findings.

DISCUSSION

The interest in using molecular biological methods for the diagnosis of tuberculosis is well known, but the implementation of these techniques in routine clinical work was very difficult until the development of a packaged kit. The Gen-Probe Assay (Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test Kit [AMTD]) was the first nucleic acid amplification test which has been developed to detect the rRNAs of *M. tuberculosis* complex organisms (9). The Amplicor *Mycobacterium tuberculosis* test is the first commercialized test that is based on the amplification of mycobacterial DNA by PCR. The kit contains all of the specific reagents needed for specimen lysis, amplification, and product detection, as well as amplification and detection controls.

The Amplicor test was done on 2,073 specimens from 1,125 patients. The assay was easy to perform and could be performed rapidly (just 6 h).

The sensitivity of the Amplicor test was 86% compared with that of culture. The majority (77%) of the false-negative Amplicor test results were obtained with smear-negative specimens, and the six specimens which were smear positive contained quite small numbers of mycobacteria. None of these six specimens contained many AFB (>100 AFB per 100 fields), whereas 50% of specimens which were smear, culture, and Amplicor test positive contained many AFB (data not shown). These six specimens were from four patients. Three of these patients had other specimens with a positive Amplicor test result, but only one specimen from the fourth patient was tested. The six specimens were retested from the same sediment, and the results were still negative, eliminating the possibility of a false-negative result because of technical problems. No internal control of amplification was used in the Amplicor test. Therefore, the false-negative results may be explained by the presence of amplification inhibitors in the sample, similar to those reported in many PCR protocols (3, 5, 17). However, the negative results seem more likely to be due to the small number of mycobacteria in the sample. In fact, the Amplicor test was done with 100 μl of the sediment, but only 25 of the 100 μl was amplified, so the quantity of sediment used for the detection of mycobacteria was 20-fold smaller with the Amplicor test than with culture in BACTEC 12 B vials. The effect of sediment heterogeneity is another factor involved in false-negative results, especially in samples with low-positive results. Analogous studies that used the Gen-Probe test (AMTD) reported sensitivities ranging from 71.4 to 97.2% (1, 2, 9, 13, 16). However, because most difficulties arise with microscopy-negative specimens, comparison of different studies needs to compare the distribution of positive specimens that were from low to high positive. For example, Jonas et al. (9) reported a sensitivity and a rate of smear-negative and culture-positive specimens very comparable to ours (82 and 47% versus 86 and 42%, respectively). Elsewhere, Pfyffer et al. (16) reported a higher degree of sensitivity (97%), but it decreased to 75% with microscopy-negative specimens and so became identical to our (74%). Bodmer et al. (2) reported a lower degree of sensitivity (71.4%), despite a lower rate of smear-negative and culture-positive specimens (33.3%).

The specificity of the Amplicor test was 98%, considering all Amplicor test-positive and culture-negative specimens from patients with culture-proven tuberculosis and that were treated as true positives for the Amplicor test. It must be emphasized that culture is not the ideal gold standard for use in the evaluation of the specificity of a PCR test. It cannot be absolutely proven that culture-negative and Amplicor test-positive results are really false-positive results related to contamination in the Amplicor test. In our study, nine specimens gave false-positive results by the Amplicor test compared with the results obtained by culture. Contamination probably occurred with seven of these nine specimens, because (i) they were from five patients receiving no antituberculous medication and with no suspicion of tuberculosis, (ii) other specimens from these five patients were culture and Amplicor test negative, and (iii) a second Amplicor test done with the same sediment gave a negative result. We are faced with two problems. The first problem is, when did contamination occur? For each of the seven specimens, a second amplification was done from the remaining prepared specimen, giving a positive result. Therefore, contamination arose during the first stage of the Amplicor test, i.e., during specimen preparation. The second problem is, what was the agent of contamination? It was probably either mycobacteria or mycobacterial DNA, since the Amplicor assay includes a process, called AmpErase, that inactivates amplified products. AmpErase contains the enzyme uracil *N*-

glycosylase (UNG), which recognizes and catalyzes the destruction of deoxyuridine-containing DNA. Deoxyuridine is always present in amplicons because of the use of deoxyuridine triphosphate in place of thymidine triphosphate in the amplification reagent. The use of the UNG enzyme excludes a great factor in PCR contamination (12). This is important for the long-term use of a PCR assay in diagnosis.

The two other specimens that were Amplicor test positive but culture negative were from two patients receiving no antituberculous medication and in whom there was no suspicion of tuberculosis. In addition, other specimens from these two patients were culture and Amplicor test negative. However, a second Amplicor test with the same sediment still gave a positive result. Two years previously, one of these two patients had culture-proven tuberculosis that was treated. AMTD was done on this specimen, with a positive result. The second specimen was from a patient with no history of tuberculosis. It was also tested by AMTD, and the result was negative. Are these two positive results false- or true-positive results for the Amplicor test? The patient's future outcome and other laboratory results will perhaps provide us with the answer. True-positive results could be due to the presence in the sample of dead mycobacteria or mycobacteria that are merely unable to grow. Another explanation could be a lack of sensitivity of culture, especially with low-positive and heterogeneous sediments. False-positive results are also possible. Contamination could have occurred during the distribution of the sediment in tubes, before specimen preparation.

The specificity of the Amplicor test in our study (98%) compares with the reported specificities obtained by AMTD (1, 2, 9, 13, 16). Our recommendation is to use Amplicor test-positive results from patients with a low suspicion of tuberculosis, especially patients with a single positive result and no smear-positive specimen, as controls and to consider the control result as the final result. This allows us to exclude the majority of the false-positive results due to contamination. Under this condition, the specificity of the Amplicor test becomes nearly perfect (99.9% in our study).

Among the 100 patients with culture-proven tuberculosis, tuberculosis was diagnosed in 95 patients by the Amplicor test and suspected in 72 patients by direct microscopy. Of the five tuberculous patients not diagnosed by the Amplicor test, three patients had only one specimen tested. It is clear that the sensitivity of the Amplicor test, like those of culture and direct microscopy, is enhanced by the multiplicity of specimens from the same patient. In addition, the multiplicity of specimens makes the interpretation of positive results easier, because the occurrence of several false-positive results because of contamination for the same patient is possible but unlikely.

Another important point of our study is that all types of specimens except blood were included, contrary to all studies reported previously. Of the 184 isolates of *M. tuberculosis*, 23 (12.5%) were extrapulmonary specimens. The sensitivity of the Amplicor test observed with extrapulmonary specimens compared with that observed with respiratory specimens. Nevertheless, the difference between the sensitivity for smear-positive specimens ($n = 11$) and the sensitivity for smear-negative specimens ($n = 12$) was slightly more important (100 and 64% versus 94 and 76%, respectively). These results suggest the validity of the Amplicor test for the detection of *M. tuberculosis* in specimens other than respiratory specimens except blood. However, other studies that include extrapulmonary specimens are necessary to confirm this fact.

The exact role of the Amplicor test and other, similar tests remains to be determined. Since the Amplicor test, like AMTD, is less sensitive than culture, this amplification test could not replace culture. In addition, culture is always necessary to obtain the isolate for antimicrobial testing. The amplification test could not replace direct microscopy, because, unlike direct microscopy, (i) only *M. tuberculosis* complex is detected, (ii) the result is only qualitative, (iii) dead mycobacteria are detected, and (iv) a false-negative result may be obtained with a high-positive specimen because of the presence of amplification inhibitors. Therefore, direct microscopy remains necessary for the evaluation of the contagiousness of the patient at the time of the diagnosis and during the treatment. Nevertheless, there is great interest in the use of the Amplicor test for the rapid diagnosis of tuberculosis: it is much more sensitive than direct microscopy, it is highly specific, and it is much more rapid than culture. Because of the excessive cost, it is not possible at present to do the amplification test for all specimens for mycobacterial research. The present study showed that positive Amplicor test results must be interpreted with some caution. As for negative results, we must keep in mind the lack of an internal control of amplification and the poor sensitivity compared with that of culture.

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