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Detection of *Mycobacterium tuberculosis* Complex in Sputum Specimens by the Automated Roche Cobas Amplicor Mycobacterium Tuberculosis Test

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Three hundred twenty-four sputum specimens from 151 patients with suspected active pulmonary tuberculosis were tested for the presence of the *Mycobacterium tuberculosis* complex with auramine fluorochrome stain and automated PCR assay (Roche Cobas Amplicor Mycobacterium Tuberculosis Test [MTB]). The results were compared with those of the conventional Löwenstein-Jensen tube culture and the BACTEC radiometer liquid culture. A total of 76 specimens from 32 patients were culture positive for *M. tuberculosis*. In addition, 37 specimens from 15 patients were smear and culture positive for other *Mycobacterium* species but negative by the present nucleic acid amplification method and thus were not included in the comparison. Compared with culture, the sensitivities, specificities, and positive and negative predictive values for acid-fast smear were 67, 98, 93, and 91% and those for the Cobas Amplicor MTB were 83, 99, 97, and 95%, respectively. When three consecutive sputum specimens per patient could be obtained, the sensitivity of the Cobas Amplicor MTB improved to 91%, whereas the sensitivity of the acid-fast smear remained unchanged.

One-third of the world's population has been infected by *Mycobacterium tuberculosis*, and there are approximately eight million new cases of tuberculosis annually. The incidence of tuberculosis is constantly increasing, and strains of *M. tuberculosis* resistant to chemotherapeutic agents have been recovered with increasing frequency.

Rapid and accurate diagnosis and effective treatment of tuberculosis are cornerstones in the prevention of this worldwide epidemic. Gene amplification techniques for the diagnosis of *M. tuberculosis* in respiratory specimens, along with traditional smear and culture methods, have attracted considerable interest. The new methods have been expected to improve the speed, sensitivity, and specificity of mycobacterial detection. The first two commercial tests based on amplification of nucleic acids have been the Amplicor Mycobacterium Tuberculosis Test (MTB) PCR (Roche Ltd.), which employs DNA polymerase to amplify *M. tuberculosis*-specific DNA, and the Mycobacterium Tuberculosis Direct Test (MTDT; Gen-Probe, Inc., San Diego, Calif.), which uses transcription-mediated amplification to detect RNA. In several evaluations, these tests have produced comparable results and a sensitivity of 70 to 95% (6, 9, 17). However, recent studies by Noordhoek et al. evaluating interlaboratory comparisons of in-house and commercial amplification methods have demonstrated lack of reproducibility (13, 14). Therefore, the need for standardized, reliable, and automated methods for the detection of *M. tuberculosis* is obvious.

Recently, Roche Ltd. has introduced an automated version of Amplicor MTB PCR referred to as the Cobas Amplicor MTB (F. Hoffmann-La Roche Ltd., Basel, Switzerland). In this test, the amplification, hybridization, and detection steps of the assay are accomplished with the Cobas instrument.

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The purpose of the present study was to evaluate the Cobas Amplicor MTB in the detection of *M. tuberculosis* in sputum specimens. The combined culture result (Löwenstein-Jensen and BACTEC) and the acid-fast smear were used as reference methods. The patients' clinical data were also evaluated. Special emphasis was put on determining the effect of multiple specimen collection on the sensitivity of *M. tuberculosis* detection. The frequency of DNA polymerase inhibition in sputum specimens was monitored with the internal control protocol of the Cobas Amplicor MTB.

MATERIALS AND METHODS

Patients. In Finland, the incidence of tuberculosis is 10/100,000 inhabitants (16). In the Pirkanmaa Hospital district (450,000 inhabitants), there were 38 new cases of pulmonary tuberculosis in 1995. Due to the low prevalence of tuberculosis, sputum specimens were collected from patients with suspected pulmonary tuberculosis during two separate periods of 4 to 6 weeks during 1996 and at the beginning of 1997. One to four specimens from each patient were collected at different outpatient clinics of Tampere University Hospital and the surrounding Pirkanmaa Hospital district. Moreover, all smear-positive sputum specimens detected in routine laboratory work between the collection periods were also included. A total of 367 specimens from 169 patients (55 women and 114 men; mean age ± standard deviation, 67 years ± 15.2 years) were studied. The patient population did not include known human immunodeficiency virus-infected or AIDS patients.

Clinical specimens. Two to five milliliters of expectorated early-morning sputa was transferred in sterile cups to the laboratory, where a direct smear was prepared. All specimens were screened with an auramine fluorescent stain. The status of those deemed positive was confirmed by the Ziehl-Neelsen stain. Specimens for the BACTEC culture and the Roche Cobas Amplicor PCR method were digested and decontaminated by the *N*-acetyl-L-cysteine-NaOH (NALC-NaOH) method (11). An equal volume of NALC-NaOH solution (2% NaOH, 1.45% Na-citrate, 0.5% NALC) was mixed with the specimen and incubated at room temperature for 20 min. A phosphate buffer (67 mM, pH 6.8) was added up to 50 ml, and the mixture was centrifuged (3,500 × *g*) for another 20 min. Excess fluid was poured off, and the sediment was resuspended in 2.0 ml of the phosphate buffer. An aliquot of the suspension was stained as described above. Part of the sediment (0.5 ml) from each specimen was used to inoculate a BACTEC 12B bottle supplemented with PANTA PLUS (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), and the remainder was divided into two parts stored at −20°C. Cobas Amplicor PCR was performed within 2 weeks. Part of the initial specimen was also treated with the Zephiran-trisodium

phosphate method for culture. The sediment (0.1 ml) after treatment was used to inoculate two Löwenstein-Jensen tubes for conventional culture.

Culture identification. Mycobacterial cultures were incubated at 37°C for 6 weeks. Löwenstein-Jensen tubes were examined weekly for positive culture, and BACTEC bottles were read twice a week with a BACTEC 460 apparatus (Becton Dickinson). A growth index of >100 was considered positive. Positive cultures from tubes and bottles were stained with a Ziehl-Neelsen stain to confirm the presence of acid-fast bacilli, which were further identified by AccuProbe RNA-DNA hybridization. AccuProbe reagents (Gen-Probe Inc.) were used according to the manufacturers' instructions and as described earlier (8, 17).

Roche Cobas Amplicor MTB PCR. The Cobas Amplicor MTB was performed according to the manufacturer's instructions. Sediment samples were mixed with a sputum specimen wash solution and centrifuged (13,000 × *g*) for 10 min. Supernatant was discharged, a lysis reagent was added, and the tubes were incubated at 60°C for 45 min. After incubation, a neutralization reagent was added and an aliquot portion of the mixture was transferred to an amplification tube containing the master mix with all necessary components for amplification, including primers for *M. tuberculosis*, nucleotides, the internal control, and DNA polymerase. Amplification was accomplished by the built-in thermocycler. The internal control of amplification in the Cobas Amplicor MTB is a sequence of plasmid DNA with primer-binding regions identical to those of the *M. tuberculosis* target sequence. A unique probe-binding region differentiates the internal control from the target amplicon. The internal control is introduced into each amplification reaction and is coamplified with the possible target DNA from the clinical specimen. If the result was negative, indicating the presence of inhibitory substances, the specimen was prepared again and analyzed both undiluted and diluted 1/10 with the neutralization reagent. After amplification, the amplified nucleotide sequence for *M. tuberculosis* and the internal control were automatically detected with target-specific DNA probes; A_{660} was measured by the built-in spectrophotometer. *M. tuberculosis*-negative and -positive controls were included in each run. The hands-off time for the Cobas Amplicor MTB is about 3.5 h after specimen preparation.

Statistical methods. Sensitivity, specificity, and positive and negative predictive values were calculated to evaluate staining and the Cobas Amplicor MTB in comparison with culture as a "gold standard." The 95% confidence intervals were calculated with the standard normal distribution approximation formula to quantify the random error. If the number of cases was small (<100), the exact binominal formula was applied (12).

RESULTS

A total of 367 sputum specimens from 169 patients were studied. Thirty-seven specimens from 15 patients were culture positive for nontuberculous mycobacterial species, including *Mycobacterium avium* (9 patients), *M. gordonae* (4 patients), and *M. malmoense* (2 patients). These specimens tested negative by Cobas Amplicor MTB and were not included in the final comparison. Moreover, six smear-positive, culture- and Cobas Amplicor MTB-negative specimens were excluded because of prior or subsequent detection of nontuberculous mycobacteria in the patients' specimens without the development of clinical signs of active tuberculous disease during the follow-up.

Altogether, 324 sputum specimens from 151 patients were included in the Cobas Amplicor MTB evaluation. A specimen was considered culture positive for *M. tuberculosis* when it was positive in either the Löwenstein-Jensen tube culture or the BACTEC 12B bottle, confirmed by acid-fast smear, and identified as *M. tuberculosis* complex by AccuProbe RNA-DNA hybridization. In total, 76 specimens from 32 patients were culture positive for *M. tuberculosis*. The diagnosis of tuberculosis was confirmed on the basis of characteristic infiltrations in the chest X-ray. Seven patients had cavitary findings, and four presented with a miliary disease. Of the 76 specimens, 51 were smear positive for acid-fast bacteria and 63 tested positive by Cobas Amplicor MTB. The test performance values of Cobas Amplicor MTB in comparison with culture are presented in Table 1. Two specimens from one patient were positive by both smear and Cobas Amplicor MTB but negative by culture. This patient had active pulmonary tuberculosis in 1993 and had been treated with antituberculous drugs for 12 months. Present clinical symptoms and chest X-ray findings during follow-up did not reveal active disease, and the results by smear and Cobas Amplicor MTB were considered to be false positive.

TABLE 1. Comparison of Cobas Amplicor MTB with culture for detection of *M. tuberculosis* in sputum specimens from 151 patients with suspected active pulmonary tuberculosis

Specimen type (no. of specimens)	Cobas Amplicor MTB[a]			
	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
All specimens (n = 324)	83 (73–91)	99 (98–100)	97 (89–100)	95 (92–98)
Smear positive (n = 55)	90 (79–97)	ND[b]	96 (86–99)	ND
Smear negative (n = 269)	68 (47–85)	ND	100 (100–100)	ND

[a] The 95% confidence intervals are in parentheses.
[b] ND, not determined.

A total of 13 specimens from 12 patients were initially negative by Cobas Amplicor MTB but positive by culture. When the medical records of the patients with discrepant results were reviewed, all 12 patients were confirmed to have active tuberculosis. A summary of the results for these patients is presented in Table 2. Ten of the 13 specimens were reanalyzed by Cobas Amplicor MTB after new sample preparation, and six tested positive. Two specimens that remained negative in the repeated analysis originated from one patient. The internal control of amplification showed the presence of DNA polymerase inhibition in sputum specimens from three patients. When these specimens were reanalyzed, undiluted and diluted, one culture-positive specimen initially negative by Cobas Amplicor MTB turned positive. The two other inhibitory specimens proved to be true negative. Interestingly, 10 of the 14 patients who gave discrepant results or whose specimens were inhibitory were taking antibiotics at the time of specimen collection. In contrast, only 5 of 26 tuberculosis patients who gave positive results with all specimens by Cobas Amplicor MTB were taking antibiotics. The antibiotics were cephalosporins (11 patients), tetracyclines or macrolides (5 patients), ciprofloxacin (2 patients), and co-trimoxazole (2 patients).

The goal of this study was to obtain up to three sequential sputum specimens per patient and to evaluate the effect of multiple specimen collection on the sensitivity of detection of *M. tuberculosis*. All three specimens could be obtained from 69 patients, and two specimens could be obtained from 99 patients. Calculation of the test performance values for smear and Cobas Amplicor MTB was based on the number of patients with one or more specimens culture positive for *M. tuberculosis*. The results for those patients are presented in Table 3. The sensitivity of Cobas Amplicor MTB remained unchanged for the first two specimens but improved from 82 to 91% when the test was performed on three consecutive specimens for one patient. When only the first specimens were considered, Cobas Amplicor MTB missed 5 of 11 (45%) smear-negative and 3 of 22 (14%) smear-positive patients with pulmonary tuberculosis confirmed by culture. When three specimens for one patient were considered, only two of eight (25%) patients who were negative by smear but positive by culture could not be detected by Cobas Amplicor MTB. All 14 patients who were smear and culture positive and from whom three specimens could be obtained were found positive for *M. tuberculosis* by the amplification test.

TABLE 2. Summary of test results for the tuberculous patients with initial false-negative results by Cobas Amplicor MTB

Patient's age (yr)/sex	Results obtained from a single sputum specimen			No. of cumulative positive results obtained from sequential sputum specimens[c]			No. of sputum specimens examined
	Smear	Culture	Repeated Cobas Amplicor MTB[a]	Smear	Culture	Cobas Amplicor MTB	
69/Male	Positive	Positive	Negative	2	2	1	2
24/Male	Positive	Positive	NA	4	4	3	4
78/Female	Positive	Positive	Positive	1	1	0	1
38/Male	Positive	Negative	Negative	3	2	2	3
75/Female	Positive	Positive	Positive	1	1	0	1
63/Male	Positive	Positive	Positive[b]	4	4	3	4
74/Male	Negative	Positive	Positive	0	1	0	3
69/Male	Negative	Positive	NA	0	3	2	3
72/Male	Negative	Positive	NA	0	2	1	3
64/Female	Negative	Positive	Positive	0	2	1	3
76/Female	Negative	Positive	Negative	0	3	1	3
72/Female	Negative	Positive	Positive	0	1	0	2

[a] NA, not available for the repeated Cobas Amplicor MTB.
[b] Specimen containing inhibitory substances. Test was repeated with specimen diluted 1/10.
[c] The results of the repeated Cobas Amplicor MTB were not included in the cumulative results.

DISCUSSION

The present data demonstrate that the novel Cobas Amplicor MTB improves the possibilities for rapid decision making in suspected cases of pulmonary tuberculosis, even in a country with a low prevalence of tuberculosis. In our laboratory routine, the Cobas Amplicor MTB is performed twice a week, providing results within 1 to 4 days of specimen collection. The mean time required for the BACTEC culture to yield positive results in this study was 19 days (range, 6 to 37 days). In previous studies, the mean delay of positive-culture results has varied from 11 to 21 days (7).

The Cobas Amplicor MTB reached a sensitivity of 83% and a specificity of 99%, which are comparable to those reported earlier by the manual Cobas Amplicor PCR (3, 5, 17). When the results were evaluated with three consecutive sputum specimens per patient, the sensitivity of tuberculosis detection increased to 91%.

The role of amplification assays as an adjunct to smear and culture in the diagnosis of tuberculosis has been questioned because of the low sensitivity (43 to 74%) for smear-negative specimens (3, 5). Acid-fast smear also has a relatively good positive predictive value, usually over 90% with culture-positive specimens. Thus, in clinical practice, amplification assays have been considered most beneficial in the rapid differential diagnosis between nontuberculous mycobacteria and *M. tuberculosis*. Yet, the present results strongly indicate that when the smear is positive and the Cobas Amplicor MTB is negative with the patient's first sputum specimen, two additional specimens should be examined to confirm the negative results.

Recently, Yuen et al. compared the Cobas Amplicor MTB, the Abbott LCx, and an in-house nested PCR in the detection of tuberculosis and reported the sensitivity for the Cobas Amplicor MTB to be 84% for all specimens and 58% with smear-negative specimens (18). In the present study, the sensitivity of 68% with smear-negative specimens was only slightly better. However, when performed on three sputum specimens per patient, Cobas Amplicor MTB additionally detected six of eight (75%) smear-negative, culture-positive patients. Two smear-positive, culture-negative specimens from a patient who had been adequately treated for active tuberculosis 3 years earlier tested positive by the Cobas Amplicor MTB. This discrepancy might be explained by previous findings that PCR can detect nonviable mycobacteria (2, 5). Thus, a patient's medical history should be carefully evaluated together with PCR results.

Six of 10 initially false-negative specimens by the Cobas Amplicor MTB turned positive in repeated analysis, indicating that duplicate analysis of specimens might be useful if PCR test results and clinical findings are contradictory.

The nature of PCR inhibition is still unclear. In studies in which inhibition has been systematically sought, an inhibition rate of at least 3 to 5% has been found (1, 2, 4, 10, 15). In this study, the presence of inhibitory substances was detected in only 3 of 376 specimens. Thus the prevalence of PCR inhibition was less than 1% of all specimens, explaining 8% of the false-negative Cobas Amplicor MTB results. This finding stresses the importance of monitoring inhibitory substances in clinical specimens.

Of the 14 patients with false-negative or inhibitory Cobas Amplicor MTB results, 10 (71%) were receiving antibiotics for bacteria other than *M. tuberculosis* at the time of sputum col-

TABLE 3. Sensitivities, specificities, and positive and negative predictive values for Cobas Amplicor MTB and smear compared with culture results from one to three sputum specimens collected within a week from each patient[a]

No. of specimens (sample size)	Cobas Amplicor MTB				Smear			
	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
One specimen (n = 151)	82 (60–95)	98 (89–100)	95 (74–100)	92 (81–97)	59 (36–79)	98 (89–100)	93 (66–100)	84 (71–92)
Two specimens (n = 99)	82 (60–95)	98 (89–100)	95 (74–100)	92 (81–97)	59 (36–79)	98 (89–100)	93 (66–100)	84 (71–92)
Three specimens (n = 69)	91 (71–99)	98 (89–100)	95 (76–100)	96 (86–99)	64 (41–83)	98 (89–100)	93 (68–100)	85 (73–93)

[a] The 95% confidence intervals are in parentheses.

lection. In contrast, only five (19%) of the 26 patients who gave positive Cobas Amplicor MTB results with all specimens tested were receiving antibiotics. The effect of antibiotics on the results is unclear and needs further evaluation. Unequal distribution of mycobacteria in specimens with a low target number and the small volume of specimen used in a PCR assay may offer another explanation for the false-negative results.

In conclusion, the automated Cobas Amplicor MTB is suitable for routine use in clinical microbiology laboratories. Its internal control protocol can be used for monitoring inhibitory specimens. The Cobas Amplicor MTB proved to be sensitive and specific; its sensitivity was 91% for all specimens and 75% for smear-negative specimens when three sputum specimens per patient were tested. Cobas Amplicor MTB can be used with smear-negative specimens from patients with a clear clinical suspicion of tuberculosis as well as with smear-positive specimens. Three consequent specimens should be examined before the exclusion or confirmation of the diagnosis of tuberculosis.

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