Automated PCR evaluation of Mycobacterium tuberculosis complex in respiratory and nonrespiratory specimens

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Abstract
In this study, Mycobacterium tuberculosis complex isolates recovered from respiratory and nonrespiratory specimens with culture were evaluated using an automated PCR method. Specimens with suspected tuberculous disease were decontaminated and concentrated using the standard N-acetyl-L-cysteine NaOH method and were inoculated onto glycerol-supplemented Löwenstein–Jensen media and BACTEC B12 vials. Forty-one specimens with typical colonies on solid media and 127 specimens identified as M. tuberculosis complex in a BACTEC system were selected as the study group. As the control group, 46 specimens without growth on either culture media were selected. The PCR results were positive in 33 (80.5%) and 87 (68.5%) samples that were culture-positive on solid and liquid media, respectively. All (100%) culture-negative specimens within the control group were also negative in the COBAS AMPLICOR Mycobacterium tuberculosis (MTB) PCR method. In conclusion, although it is a fast method for identifying M. tuberculosis complex isolates from clinical specimens, the COBAS AMPLICOR MTB PCR method is found to be less sensitive than culture techniques, we propose therefore that it should only be used in combination with culture results in the clinical diagnosis of tuberculosis.

Introduction

Tuberculosis, which is caused mainly by a group of mycobacteria called Mycobacterium tuberculosis complex (Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti), is one of the leading health problems worldwide. In 1993, the World Health Organisation (WHO) declared tuberculosis a global public health emergency (Haas, 2000).

The traditional detection of mycobacteria is based on microscopic examination and culture of the microorganisms. Examination of direct smears for acid-fast bacilli is a rapid method, but it lacks sensitivity and specificity. Although considered to be the ‘gold standard’ method, isolation of micro-organisms on conventional culture media prior to identification is very time consuming. Recently, the introduction of molecular biology methods, such as PCR, has attracted considerable interest. These new methods have been expected to improve the speed, sensitivity and specificity of assays for the detection of M. tuberculosis complex in clinical specimens (D’amato et al., 1995; Ninet et al., 1999).

The COBAS AMPLICOR Mycobacterium tuberculosis (MTB) PCR test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA) is an automated standardized commercial test for the detection of M. tuberculosis, based on the amplification of nucleic acids (Kim et al., 2003). The purpose of this study was to evaluate the diagnostic value of the COBAS AMPLICOR MTB PCR test in the identification of M. tuberculosis complex in respiratory and nonrespiratory clinical specimens, using the Löwenstein–Jensen and BACTEC culture techniques as reference methods.

Materials and methods

Specimen collection

This study was performed with respiratory and nonrespiratory specimens, collected from patients suspected of having tuberculosis. From the beginning of 2002 until the end of 2003, a total of 2920 clinical specimens from 2452 patients were collected at the mycobacteriology laboratory of Gaziantep University Hospital and all of them were processed on receipt.

Processing of the specimens

Specimens were screened by microscopy for acid-fast bacilli after using the Ehrlich–Ziehl–Neelsen stain and cultured on
glycerol-supplemented Löwenstein–Jensen media or BACTEC B12 vials. Specimens such as sputum, gastric aspiration fluid, bronchoalveolar lavage, urine, wound, biopsy and pus were inoculated after being digested and decontaminated with N-acetyl-l-cysteine- NaOH (NALC–NaOH) method. Cerebrospinal, pleural and pericardial fluids were centrifuged at 3000 g for 15 min, the supernatant was aspirated and discarded, and the pellet was resuspended in 2 mL of sterile water. An aliquot of 0.5 mL of sediment was used for inoculation onto the solid or liquid medium, the remaining sediment being aliquoted and stored at −80 °C until evaluation by PCR (Nolte & Metchock, 1995).

Culture identification

Of the 2920 specimens, 900 were cultured on glycerol-supplemented Löwenstein–Jensen medium and 2020 were cultured in BACTEC B12 vials (Middlebrook 7H12 medium) supplemented with PANTA PLUS (Becton Dickinson Diagnostic Instruments Systems, Sparks, MD, USA) and incubated at 37 °C for 6 weeks. Löwenstein–Jensen tubes were examined weekly, and BACTEC B12 vials were read three times a week for the first 3 weeks and once a week for the remaining period using a BACTEC 460 instrument for positive culture. A growth index (GI) = 10 in the system was considered positive. Positive cultures were stained using the Ehrlich–Ziehl–Neelsen method to confirm the presence of acid-fast bacilli. Cultures without any visual growth on solid media, or whose GIs remained <10 in the BACTEC system for 6 weeks were considered as negative (Siddiqi, 1989).

Cultures that were positive for Mycobacterium tuberculosis complex either in Löwenstein–Jensen medium or in BACTEC system were selected for the study group. The sediments of those cultures were stored at −80 °C and further identified using the COBAS AMPLICOR MTB PCR test within a 2-month period. Forty-six specimens which were culture-negative both in the Löwenstein–Jensen medium and in the BACTEC B12 medium were selected as the control group.

COBAS AMPLICOR MTB PCR test

The procedure consisted of three steps: the first step was specimen preparation and was combined with the fully automated amplification and detection steps. The preparation of the specimens and amplification were performed according to the manufacturer’s instructions. Amplification was accomplished using the built-in thermal cycler. After amplification, the nucleotide sequence for M. tuberculosis and the internal control were detected with specific DNA probes and the absorbances were measured using the built-in spectrophotometer. An absorbance value above 0.350 (OD450) was considered positive. Specimens showing inter-

Statistical methods

The sensitivity, specificity, positive and negative predictive values of the COBAS AMPLICOR MTB PCR test were calculated by comparing the PCR results with the smear and culture results, which were considered as the references. The statistical analysis of the results were calculated by using the McNemar test.

Results

In this study, a total of 2920 specimens from 2452 patients were investigated. In total, 168 (5.75%) specimens from 124 patients yielded Mycobacterium tuberculosis complex in culture; 41 (4.55%) strains were isolated from 900 cultures on Löwenstein–Jensen media and 127 (6.28%) strains from 2020 cultures in BACTEC B12 vials. Four strains (0.13%) were identified as nontuberculous species, i.e. mycobacteria other than M. tuberculosis (MOTT). The distribution of the 168 culture-positive clinical specimens were as follows: 114 (67.9%) sputa, 29 (17.3%) bronchoalveolar lavages, 11 (6.5%) biopsies, seven (4.25) gastric aspiration fluids, five (2.9%) pleural/pericardial fluids and two (1.2%) urine samples. Of the culture-positive specimens, 143 (85.1%) were from respiratory and 25 (14.9%) were from nonrespiratory origins. Three (75%) of the four nontuberculous mycobacteria were isolated from sputa and one (25%) from bronchoalveolar lavage.

Eighty-six (51.2%) of the culture-positive samples were positive for acid-fast bacilli, and 67 (77.9%) of them were positive by the COBAS AMPLICOR MTB PCR test. Among the smear-negative cases, PCR was positive for 49 (59.7%) samples (Table 1). All culture-negative patients were also smear-negative. Of the 46 samples from the control group that were smear- and culture-negative for acid-fast bacilli, none was found to be positive using the COBAS AMPLICOR MTB PCR test.

A total of 120 (71.4%) culture-positive samples were found to be positive by the COBAS AMPLICOR MTB PCR test; 33 (80.5%) of which were from 41 positive cultures on Löwenstein–Jensen media and 87 (68.5%) were from 127

| ARB positive | 68 | 18 | 86 |
|ARB negative | 52 | 30 | 82 |
|No. of specimens | 120 | 48 | 168 |

Table 1. COBAS AMPLICOR MTB PCR test results compared with those of smear results in culture-positive specimens

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positive cultures on BACTEC B12 media. Eight (19.5%) samples that were culture positive on Löwenstein–Jensen media and 40 (31.5%) samples that were positive in the BACTEC system were found to be negative by the COBAS AMPLICOR MTB PCR test. A total of 104 (72.7%) of 143 culture-positive respiratory samples and 15 (60%) of 25 culture-positive nonrespiratory samples were found to be positive with PCR. Of the 46 samples from the control group that were smear- and culture-negative for acid-fast bacilli, none was positive using the COBAS AMPLICOR MTB PCR test. Four nontuberculous mycobacteria were also found to be negative using PCR. Three (1.4%) out of 214 specimens tested by the AMPLICOR assay showed inhibition and were found to be positive after dilution, therefore the results of these specimens were added to the study calculations.

Compared with culture on Löwenstein–Jensen medium, the specificity, sensitivity, positive and negative predictive values and reliability of the COBAS AMPLICOR MTB PCR test in identification of M. tuberculosis complex were 80.5%, 100%, 100%, 85.1% and 90.8%, respectively (Table 2).

Compared with culture in the BACTEC system, the specificity, sensitivity, positive and negative predictive values and reliability of the COBAS AMPLICOR MTB PCR test in identification of M. tuberculosis complex were 68.5%, 100%, 100%, 53.5% and 76.8%, respectively (Table 3).

Eighty-six (51.2%) of the culture-positive samples were positive for acid fast-bacilli, and 67 (77.9%) were found to be positive by the COBAS AMPLICOR MTB PCR test. Among the smear-negative cases, PCR was positive for 49 (59.7%) samples (Table 1). All of the culture-negative patients were smear- and PCR-negative also. The sensitivity of the COBAS AMPLICOR MTB test was 77.9% for smear- and culture-positive samples, and 59.7% for smear-negative, culture-positive specimens.

There was no significant difference between the COBAS AMPLICOR MTB PCR test and the culture methods in the identification of M. tuberculosis complex from clinical samples (P = 0.1).

### Discussion

The first two commercial tests used for the identification of *Mycobacterium tuberculosis* were the Amplicor Mycobacterium tuberculosis test (MTB; Roche Diagnostic Systems, Rotkreuz, Switzerland) and the *Mycobacterium tuberculosis* direct test (MTDT; Gen-Probe, Inc., San Diego, CA, USA). The average sensitivity and specificity of these assays were 70%–95% in clinical studies (Vuorinen et al., 1995; Dalovich et al., 1996; Ichiyama et al., 1996). In a multicenter study, Noordhoek et al. (1996) reported that results obtained with both methods were not reproducible, and mycobacterial detection from clinical specimens therefore required the development of reliable, standardized and automatized new methods were required.

Based on the culture results, the diagnostic sensitivity of the COBAS AMPLICOR MTB PCR assay on the specimens positive in Löwenstein–Jensen and BACTEC B12 media were determined to be 80.5% and 68.5%, respectively. The specificity of the assay was 100% with each culture method. Both the liquid and solid medium cultures are considered today as 'gold standard' procedures in the identification of *M. tuberculosis*. However, the lack of a generally accepted standard procedure for mycobacterial cultures when evaluating culture-independent assays renders the direct comparison of different studies very difficult, because an improved sensitivity of the respective gold standard procedure in a laboratory necessarily lowers the sensitivity of the evaluated assay.

Previously published data on respiratory specimens cultured onto Löwenstein–Jensen and BACTEC media are in agreement with our findings: the sensitivity and specificity of AMPLICOR system were 92.6% and 95.6%, respectively (Bodmer et al., 1997). Some authors reported that the sensitivity of this system was improved when PCR was performed on two or three consecutive specimens (Eing et al., 1998; Rajalahti et al., 1998).

In this study, PCR results were negative in eight (19.5%) and 40 (31.5%) (total 48) specimens which were culture-positive on Löwenstein–Jensen and BACTEC B12 media, respectively. Forty-one of the PCR-negative patients were under antitubercular therapy and 30 of the 48 specimens were smear-negative. The failure of PCR to detect mycobacteria in the specimens mentioned above was probably due to the low numbers of the micro-organisms present in the corresponding specimens. For the rest of the false-negative PCR results, we presume that inadvertent removal of the centrifuged pellet during specimen preparation or unequal distribution of mycobacteria during aliquoting of the sediments could be the responsible factors. Inoculation volume differences between culture (usually 0.5–1.0 mL) and the

### Table 2. COBAS AMPLICOR MTB PCR test results compared with those of culture on Löwenstein–Jensen medium

<table>
<thead>
<tr>
<th>Culture positive</th>
<th>Culture negative</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Löwenstein–Jensen</td>
<td>PCR positive</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>PCR negative</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>No. of tests</td>
<td>41</td>
</tr>
<tr>
<td>BACTEC B12</td>
<td>PCR positive</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>PCR negative</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>No. of tests</td>
<td>46</td>
</tr>
</tbody>
</table>

### Table 3. COBAS AMPLICOR MTB PCR test results compared with those of culture on BACTEC B12 medium

<table>
<thead>
<tr>
<th>Culture positive</th>
<th>Culture negative</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>BACTEC B12</td>
<td>PCR positive</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>PCR negative</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>No. of tests</td>
<td>173</td>
</tr>
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COBAS AMPLICOR MTB test (0.1 mL) may be the most likely explanation (Bennedsen et al., 1996).

Although the nature of PCR inhibition is still unclear, the variable inhibition rate for *M. tuberculosis*-yielding samples of 1–19% in different studies stresses the importance of monitoring inhibitory substances in clinical specimens (Roche Diagnostic Systems Inc., 1995; Bodmer et al., 1997; Yuen et al., 1997; Rajalalhti et al., 1998; Ninet et al., 1999). In our study, inhibition of the PCR amplification was observed in three (1.4%) of 214 specimens and all were positive after 1 : 10 dilution. Although the COBAS AMPLICOR MTB test is designed to detect *M. tuberculosis* in respiratory specimens, several studies evaluated the diagnostic value of this test in nonrespiratory samples. These studies stated that results obtained by the PCR with nonrespiratory specimens were in good agreement with those obtained with respiratory specimens (Bodmer et al., 1997; Rajalalhti et al., 1998; Reischl et al., 1998; Shah et al., 1998). In our study, the sensitivities of PCR in respiratory and nonrespiratory specimens were 72.7% and 60%, respectively. The difference between these two types of specimens was not significant (P = 0.1).

Although amplification assays have been found to have low sensitivity (43–74%) for smear-negative specimens, their use implies the detection of an important portion of smear-negative tuberculosis cases. Given the high sensitivity of PCR, mycobacterial DNA could be detected in patients without evident clinical signs of infection, or those under antibiotic treatment. Thus, in clinical practice amplification assays have been considered most beneficial in smear-negative cases.

In a number of studies it was stressed that testing with multiple specimens from one patient had increased the sensitivity of the COBAS AMPLICOR MTB PCR assay in smear-negative tuberculosis (Wobeser et al., 1996; Bodmer et al., 1997; Yuen et al., 1997; Rajalalhti et al., 1998; Lim et al., 2000; Johnsson & Ridell, 2003; Levidiotou et al., 2003; Kim et al., 2004). By contrast, in smear-positive specimens the rapid diagnosis and/or confirmation of the clinical diagnosis and differentiation between *M. tuberculosis* and nontuberculous mycobacteria is emphasized (Yuen et al., 1997; Eing et al., 1998).

In our study, the sensitivity of the COBAS AMPLICOR MTB test was 77.9% for smear-positive and 59.7% for smear-negative, culture-positive specimens. We can conclude that samples that are negative by the smear test may still prove positive by culture, and a negative PCR test result should not be interpreted as ruling out tuberculosis.

In conclusion, the automated COBAS AMPLICOR MTB PCR test is rapid, easy to perform and suitable for routine use in clinical microbiology laboratories. The system has standardized procedures with prepackaged kit contents for specimen processing and amplification, and an internal control for monitoring the presence of PCR inhibitors, but it cannot replace conventional culture techniques due to its low sensitivity. Another undesired effect of the PCR test is the high costs per result per patient, which would hinder the implementation of this method in a resource-poor country.

References


