
Rapid Diagnosis of Active Pulmonary Tuberculosis In Children By AMPLICOR *Mycobacterium tuberculosis* PCR Test

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Abstract

Conventional diagnosis of Mycobacterium tuberculosis by culture generally takes 3 to 8 weeks. Acid fast smears lack sensitivity and can not distinguish M. tuberculosis from other mycobacteria. Rapid differentiation of M. tuberculosis from other mycobacteria species is therefore of great potential benefit. The PCR can provide a rapid and specific identification of M. tuberculosis complex organisms. The reliability of the Roche AMPLICOR Mycobacterium tuberculosis test (AMPLICOR MTB) for the diagnosis of active pulmonary tuberculosis in children was evaluated by testing 204 specimens (sputum, early morning gastric aspirates, and tracheobronchial lavage) which employs a fast and simplified sample preparation method appropriate for routine diagnostic testing. The specimens were taken from 86 children who were suspected of having active pulmonary MTB on the basis of the presence of one or more of the following criteria: (1) positive tuberculin skin test, (2) abnormal chest radiograph consistent with tuberculosis and/or (3) history of exposure to an adult with infectious tuberculosis. In order to evaluate the accuracy of the PCR assay, PCR results were compared with culture, staining techniques and medical history. Of these 204 specimens, 35 were culture positive for M. tuberculosis from 24 patients. 27 specimens were smear positive for acid fast bacteria (AFB). On initial testing, the sensitivity and specificity of the AMPLICOR MTB assay, compared with culture, were 88.6% and 98.2% respectively. After resolution of discrepancies (by review of medical history), the sensitivity, specificity, and positive and negative predictive values of the AMPLICOR MTB assay were 89.2%, 99.4%, 97.1%, and 97.6%, respectively. One specimen was AMPLICOR MTB positive and culture positive for Mycobacterium avium complex. For AFB smear-positive specimens, the sensitivity, specificity, and positive and negative predictive values of AMPLICOR MTB were 96.4%, 100%, 100%, and 50%, respectively. For AFB smear-negative specimens, the sensitivity, specificity, and positive and negative predictive values of AMPLICOR MTB were 66.7%, 99.4%, 85.7% and 97.6%, respectively. Our results support the use of AMPLICOR MTB for rapid diagnosis of tuberculosis in children whose respiratory specimens are AFB smear positive. Further studies are needed to determine the most clinically relevant and cost-effective use of this assay with AFB smear-negative specimens.

Introduction

The rapid diagnosis (within 24 h) of infectious disease, particularly those which represent a public health problem due to their communicability, presents one of the most challenging problems to the clinical microbiologist.

Acid-fast staining of smears is a rapid technique, but it has a low sensitivity (approximately 10^4 bacteria per ml of specimen are necessary for a positive result), and it does not differentiate between species of Mycobacterium.⁽¹⁾ Currently, diagnosis of tuberculosis requires growth of the organism on solid or in a liquid medium, which can take up to 6 to 8 weeks, followed by the use of nucleic acid probes, high performance liquid chromatography, or conventional biochemical tests for identification. Because optimal patient management requires early initiation of drug therapy and isolation of infectious individuals as soon as

possible, a technique which provides rapid, reliable detection of Mycobacterium tuberculosis is needed.⁽²⁾

The most promising diagnostic modality extend to address this problem is PCR. PCR permits the exponential amplification of target DNA or RNA molecules, In the clinical microbiology laboratory its application to the detection and identification of fastidious or slowly growing organisms such as mycobacteria, directly in clinical specimens, has the potential to provide a truly rapid laboratory diagnosis of tuberculosis, The major benefits of this rapid laboratory diagnostic test are improved patient care, reduced medical costs. To accomplish this, Roche Molecular Systems Inc. has developed a nucleic acid amplification (PCR) test, AMPLICOR MTB, for evaluation of respiratory specimens.^(3,4)

The aim of this study was to evaluate the commercial PCR system, the AMPLICOR Mycobacterium tuberculosis for the detection of M. tuberculosis, in the respiratory specimens of

children who were suspected to have active pulmonary tuberculosis, and compare AMPLICOR MTB results with culture and staining techniques.

Subjects and Methods

This prospective study was conducted from August 1994 to November 1997 at the pulmonary departments of HAI AL-JAMEA HOSPITAL, ERFFAN HOSPITAL, and EL-SALAMA HOSPITAL, JEADDAH, K.S.A. We consecutively investigated 86 children aged 2 to 14 years (56 males, and 30 females) who were suspected of having active pulmonary MTB on the basis of the presence of one or more of the following criteria: (1) positive tuberculin skin test, (2) abnormal chest radiograph consistent with tuberculosis, and/or (3) history of exposure to an adult with infectious tuberculosis. Non of these children suffered from AIDS, of immunosuppression due to medications, or radiotherapy.

204 specimens [96 sputa, 71 early morning gastric aspirates, 30 tracheal aspirate specimens and 7 bronchoalveolar lavage (BAL) fluid specimens] from 86 children with detailed history and clinical examination.

Specimens were decontaminated and digested by N-acetyl-cysteine 2% sodium hydroxide and concentrated by centrifugation according to standard laboratory protocol.⁽⁵⁾ A smear of the sediment was stained with Auramine O and examined for acid-fast bacilli (AFB). Middlebrook 7H10/7H11 biplates (Remed, Lenexa, Kans.) and BACTEC 12B bottles (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.) were inoculated with several drops and 500 µl of sediment from each specimen, respectively. Two aliquots, each consisting of 200 µl of sediment, were frozen at -20 °C for batch analysis by the AMPLICOR MTB test. Middlebrook plates were incubated in an atmosphere of 7 to 10% CO₂ at 37 °C for up to 8 weeks and examined for mycobacteria weekly. BACTEC 12B bottles were incubated at 37°C and monitored for growth by the BACTEC 460 (Becton Dickinson) every 3 days for the first 2 weeks and then weekly for an additional 3 weeks . When growth index (GI) reached 100 or greater, a smear of broth was stained with Kinyoun, and if AFB were present, the BACTEC vial was reincubated and monitored daily until a GI of 999 was reached, at which time a 100 µl aliquot was removed to perform DNA-RNA probe analysis for M. tuberculosis complex (AccuProbe, Gene-Probe Inc, San Diego,

Calif.). A Lowenstein-Jensen tube was also inoculated with several drops of broth from the BACTEC vial. If the probe was negative, tests for identification were performed on colonies recovered on solid media. On the basis of colony morphology, appropriate RNA-DNA nucleic acid probes were selected for identification of M. tuberculosis, M. avium complex, M. Kansasii, or M. gordonae. If the mycobacterium was a rapid grower, biochemical tests were used to identify the organism.

Detection of M. tuberculosis by AMPLICOR MTB. The AMPLICOR MTB procedure consists of three steps: specimen preparation, amplification, and detection.

Specimens are prepared by addition of 100µl of concentrated digested-decontaminated specimen to 0.5 ml of wash buffer and then centrifugation at $\geq 12,500 \times g$ for 10 min. The supernatant is aspirated, and 100µl of lysis reagent is added to the sediment. After vortexing, the suspension is incubated for 45 min at 60°C to complete lysis of the mycobacteria. The lysed material is then neutralized by the addition of 100 µl of neutralization reagent.

AMPLICOR MTB amplifies a 584-bp region of the 16S rRNA gene sequence common to all mycobacteria. Carryover contamination is prevented by incorporation of dUTP in place of dTTP in the amplification reaction and utilization of uracil-N-glycosylase (AmpErase) to enzymatically cleave any contaminating amplicon carried over from previous reactions. AmpErase is subsequently inactivated at the temperatures used for thermal cycling. For amplification, 50 µl of master mix. The tray containing specimens and controls is then placed in a TC-9600 thermal cycler (Perkin-Elmer, Norwalk, Conn.) and amplified according to the following program: hold at 50°C for 2 mi: 2 cycles of 98°C for 20 s, and 72°C for 45 s, 35 cycles of 94°C for 20 s, 62°C for 20 s, and 72°C for 45 s, hold at 72°C for 5 min, and hold at 72°C indefinitely.

Detection of M. tuberculosis complex organisms is accomplished by hybridization of the amplified product to a DNA probe specific for organisms of the M.tuberculosis complex. Following amplification, 100µl of denaturation solution is added to all tubes, this is followed by a 10-min room

temperature incubation to allow complete denaturation of the double-stranded products. One hundred microliters of hybridization buffer is added to a microwell plate coated with a DNA probe specific for members of the *M. tuberculosis* complex. Twenty-five microliters of denatured amplicon is then added, and hybridization is carried

out at 37°C for 90 min. Detection of hybridized duplex is accomplished with an avidin-horseradish peroxidase conjugate-tetramethylbenzidine substrate system. The reaction is stopped by addition of dilute hydrosulfuric acid, and the results are read at 450 nm. A result is considered positive if the absorbance is greater than or equal to 0.35. ⁽⁶⁾

Results

Of the 204 specimens examined, 44 were culture positive, for mycobacteria, 35 specimen from 24 patients grew *M. tuberculosis*. Isolates from the remaining 8 cultures were *M. avium* complex (7 specimens), and *M. Kansasii* (1 specimen). Thirty one specimens were AFB smear positive, *M. tuberculosis* was eventually isolated from 29 specimens, and *M. avium* complex was isolated from 1.

The data in table I shows that, the *M. tuberculosis* PCR assay was positive for 34 specimens from 25 patients. Of these 34, 31 were culture positive for *M. tuberculosis* (22 patients) and 26 were AFB smear positive (17 patients). Of the remaining 3, one was culture positive for *M. avium* complex and 2 were culture negative for mycobacteria. Compared with culture, the overall sensitivity, specificity, positive predictive value (PPV), and negative predicted value (NPV) of PCR were 88.6%, 98.2%, 91.2%, and 97.6% respectively. For AFB smear-positive specimens, PCR sensitivity, specificity, PPV, and NPV were 96.3%, 100%, 96.3%, and 50% respectively, whereas the PCR sensitivity, specificity, PPV, and NPV for AFB smear-negative specimens were 62.5%, 98.8%, 71.4%, and 98.2% respectively.

Of the three specimens from three patients that were culture negative but PCR positive, one patient whose sputum culture was positive for *M. avium* had an abnormal chest radiograph consistent with tuberculosis but negative tuberculin skin test. Both remaining patients had a previous diagnosis of pulmonary tuberculosis. One of these had been diagnosed 4 months ago, when antituberculous

drugs were started on the basis of smear-positive sputum specimen that was subsequently PCR positive and *M. tuberculosis* culture positive. The last patient, had been diagnosed with pulmonary tuberculosis 6 months earlier on the basis of *M. tuberculosis* culture positive smear-negative gastric aspirate specimen and had been on antituberculosis therapy for 3 months and stopped. Of the initial 3 PCR-positive, *M. tuberculosis* culture negative specimens, one remained false positive i.e., PCR positive, tuberculosis negative.

As shown in table II, 27 of the 28 AFB smear positive specimens from children with tuberculosis were PCR positive, and only 1 of these specimens (from a patient previously diagnosed with tuberculosis) was PCR negative. Of 166 AFB smear-negative specimens, tuberculosis negative. 1 was PCR positive,. The resolved overall sensitivity, specificity, PPV, and NPV of PCR analysis (compared with culture and the patients clinical history) were 89.2%, 99.4%, 97.1% and 97.6%, respectively. For AFB smear-positive specimens, the sensitivity, specificity , PPV, and NPV of AMPLICOR MTB were 96.4%, 100%, 100%, and 50%, respectively. For AFB smear-negative specimens, the sensitivity, specificity, PPV, and NPV of AMPLICOR MTB were 66.7%, 99.4%, 85.7%, and 97.6%, respectively.

As shown in table III, 22 of 24 patients with culture positive are PCR positive. The AMPLICOR MTB results compared with culture sensitivity, specificity, PPV, and NPV were 91.6, 98.3, 95.65, and 96.8 respectively.

Table I: Initial comparison of AMPLICOR MTB test with culture and acid fast smear for detection of *M. tuberculosis* in respiratory specimens

Specimen Type (n°)	N° of specimens (n° of patients)				Sens-itivity (%)	Spec-ificity (%)	PPV (%)	NPV (%)
	Culture positive*		Culture negative*					
	PCR+	PCR-	PCR+	PCR-				
All (204)	31(22)	4(2)	3(3)	166(59)	88.6	98.2	91.2	97.6
Smear positive(29)	26(17)	1(1)	1(1)	1(1)	96.3	100	96.3	50
Smear negative(175)	5(5)	3(1)	2(2)	165(58)	62.5	98.8	71.4	98.2

*Culture positive or negative for *M. tuberculosis*.

Table II: Comparison after discrepant analysis of AMPLICOR MTB test and results of acid-fast smear and patients' clinical histories for tuberculosis

Specimens Types (n ^o)	N ^o . of specimens (n ^o . of patients)				Sensitivity (%)	Specificity (%)	Predictive values (%)	
	Tuberculosis positive ^a		Tuberculosis negative ^a				positive	negative
	PCR+	PCR-	PCR+	PCR-				
All (204)	33(24)	4(2)	1(1)	166(59)	89.2	99.4	97.1	97.6
Smear positive(29)	27(18)	1(1)	0	1(1)	96.4	100	100	50
Smear negative(175)	6(6)	3(1)	1(1)	165(58)	66.7	99.4	85.7	97.6

^a Tuberculosis positive or negative for *M. tuberculosis* as detected by culture or clinical history.

Table III: Comparison of AMPLICOR MTB and culture results from 86 children

PCR	N ^o . of patients		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Positive**	Negative**				
Positive	22	1	91.6	98.3	95.65	96.8
Negative	2	61				

** A positive result indicates that one or more specimens for a patient were PCR or culture positive.

Discussion

The definitive and rapid diagnosis of tuberculosis in routine clinical work is difficult since culturing takes about 3 to 6 weeks and usually requires at least 3 samples.⁽⁵⁾

Our study demonstrates the application of the AMPLICOR MTB test is similar to those of other investigators.^(7,8,9,10) The sensitivity of the PCR test has been 96.3% for AFB smear-positive samples but much lower 62.5% for AFB-smear negative specimens. Our overall sensitivity of 89.2% (after resolution of discrepancies) is close to 83% reported by Moore and Curry, higher than the 67% reported by D'Amato and colleagues, but lower than 95% reported by Beavis et al.^(7,8,9) The results of Beavis et al, however, reflect the fact that 93% of the specimens in their evaluation that were culture positive for *M. tuberculosis* were AFB smear positive.⁽⁹⁾ In contrast, the sensitivity of the AFB smear for diagnosing of tuberculosis was 75% in our evaluation and only 50% to 51% in the studies by Moore and Curry and D'Amato et al.^(7,8)

The specificity of the AMPLICOR MTB test appears to be excellent: greater than 99%.^(7,8,11,12) In our experience, after initial testing, the specificity was 98.2%, there were 3 possible false positive results. Two of these, potentially false positive specimens were collected from children who had been previously diagnosed with tuberculosis (4 and 6 months earlier) and who were receiving antituberculosis therapy, both were reclassified as true positives. This same scenario (i.e. MTB PCR-positive, culture negative specimens from persons known to have tuberculosis) has been reported by

others.^(13,14,15) Exactly what these results mean in regard to patient care, however is not clear at this time. False-positive specimens in our study, were probably due to the presence of nonviable organisms in patients while on therapy. Published experience with PCR supports the concept that a patient can remain PCR positive after cultures become negative.^(16,17) In one study, PCR remained positive 1 to 2 months after cultures became negative, and occasionally results can be positive at 6 months after the initiation of therapy.⁽¹⁴⁾ The PCR result is a true positive, but how this information should be used in the clinical setting has not yet been established. Further investigation of this issues is needed. The final one false-positive specimen was culture positive for *M. avium* complex. The optical density of this specimen tended to be lower than those of specimens containing *M. tuberculosis* but was above the assay cutoff. It is unlikely that this patient was dually infected with *M. tuberculosis*. The biological basis for this potential cross-reactivity, is unclear.

In the present work, to determine the potential value of AMPLICOR MTB for diagnosis of tuberculosis in patients whose specimen are negative, we found one child with 3 culture positive, smear negative and PCR negative results and one child with culture positive, smear positive, and PCR negative results. The cause of false negative result has not been definitively determined, however, a technical error possibly occurring during pipetting, is strongly suspected. We did not test for the presence of inhibitors, which have been reported to occur in 5

to 13% of specimens and could have been responsible for some of the false-negative results.⁽¹⁸⁾ The sensitivity, specificity, PPV, and NPV for AFB negative smears were 66.7%, 99.4%, 85.7%, and 97.6% respectively. These results were consistent with data reported by others.^(7,8,9,19,20)

AMPLICOR MTB compares very favorably to culture in sensitivity 91.6%, specificity 98.3%, PPV 95.65%, and NPV 96.8%. It has the decided advantage over culture because of its ability to provide a diagnosis of tuberculosis the day of specimen receipt by the laboratory. A reliable, positive result, provided this rapidly, will have a significant impact on patient care and the cost of providing that care. This was consistent with data reported by others.^(7,8,9,10,11,12)

In conclusion, our findings support that AMPLICOR MTB provides the clinician, laboratorian, and infection control practitioner with very valuable, rapid, and clinically relevant

information for the diagnosis of tuberculosis. Not all children with AFB smear-positive sputum samples have tuberculosis (18 of 24 patients [75%] in our study); therefore, a rapid yes or no answer would allow optimal patient management by APPLICOR MTB test. Data from our study and others also indicate that, at this time, PCR cannot replace mycobacterial culture. The sensitivity of the PCR assay for AFB smear-negative specimens is approximately 66.7%, and an isolate is necessary for susceptibility testing. Therefore, because PCR must be a supplemental test, requiring additional resources, recommendations for its appropriate use are needed. Issues that should be addressed regarding the role of PCR in diagnosis as well as management of tuberculosis include optimal use of PCR in testing AFB smear-negative specimens, because a rapid diagnosis clearly will benefit certain patients whose sputum smears are AFB negative, and its role in evaluation of response to therapy.

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