Rapid and Specific Detection of *Mycobacterium tuberculosis* by Using the Smart Cycler Instrument and a Specific Fluorogenic Probe

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Received 19 February 2003/Returned for modification 31 March 2003/Accepted 22 July 2003

A procedure using the Smart Cycler instrument and a fluorescence quencher (FQ) probe for the specific identification of *Mycobacterium tuberculosis* complex (MTB) was used to detect organisms in 366 acid-fast bacillus smear-positive respiratory specimens. It was compared to culture and the AMPLICOR *M. tuberculosis* PCR test. MTB was isolated from 198 of these samples. The FQ PCR assay was sensitive (197 of 198, 99.5%) and specific (165 of 168, 98.2%); no significant difference was observed between the two PCR protocols. After DNA extraction, a final result was available within 1.5 h with the real-time PCR protocol.

Because of the high risk of person-to-person transmission, rapid detection of *Mycobacterium tuberculosis* directly in patient specimens is recognized as an important step in the control of infection due to this organism (9, 12). The detection of acid-fast bacilli (AFB) in smears prepared from respiratory secretions has been the mainstay of rapid identification of potentially infectious individuals. Direct staining of prepared smears is generally performed with Ziehl-Neelsen stain or auramine fluorescent dye (8). Because the AFB smear lacks specificity, there is a need for a laboratory test for specific detection of the *M. tuberculosis* complex (MTB) that can be performed within a short period of time. Currently, nucleic acid amplification tests offer a rapid, specific, and sensitive approach to the detection and characterization of MTB (2, 3, 6, 14, 16, 20, 21). However, many of these assays require multiple user-dependent steps for amplification and detection and have the potential for error and sample contamination. Real-time PCR techniques (22), involving fluorescent dyes or fluorophores with a spectrofluorometric thermal cycler, have been developed and evaluated for the detection of mycobacteria and for the detection of drug resistance (5, 11, 18, 19). The ABI 7700 TaqMan system (Applied Biosystems, Foster City, Calif.) has been used to quantitate MTB DNA in sputum during the treatment of tuberculosis patients (4). The probe used in this assay was specific for the IS6110 gene region (7).

In addition, we recently reported a rapid and sensitive method for the identification of MTB by amplification of the internal transcribed spacer and the use of a specific fluorogenic probes for MTB in the LightCycler system (10, 15).

In this study, we compared the fluorescence quencher (FQ) PCR assay to the AMPLICOR PCR test and conventional culture techniques. We examined AFB smear-positive respiratory specimens that were submitted for culture from January 2001 through December 2002. All specimens were liquefied and decontaminated with *N*-acetyl cysteine–2.5% NaOH and

concentrated by centrifugation (17). The sediment was used to inoculate a selective 7H11 agar plate and a supplemented BacT/ALERT MP (MP; Organon Teknika, Durham, N.C.) culture bottle. Also, two smears were prepared and stained with an auramine fluorochrome dye (8). Isolates of mycobacteria growing on solid media were identified by DNA probes (Accuprobe; Gen-Probe, Inc., San Diego, Calif.) for *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. gordonae*, and *M. kansasii* or by conventional biochemical tests performed in accordance with standard protocols (13).

DNA extraction of AFB-positive specimens was performed with the Roche sputum preparation kit (Roche Diagnostics, Indianapolis, Ind.). All manipulations of specimens were performed in a biological safety cabinet with unidirectional work flow for all procedures. The remaining lysate were frozen at –20°C and used for further analyses. The AMPLICOR *M. tuberculosis* PCR test was performed in accordance with the manufacturer's (Roche Diagnostics) instructions, as previously described (15).

The FQ PCR assay was performed with the IS6 forward primer (5'-GGCTGTGGGTAGCAGACC-3') and the IS7 reverse primer (5'-CGGGTCCAGATGGCTTGC-3'), which are directed at a 163-bp region of the IS6110 gene sequence (4). The internal oligonucleotide probe was labeled with the fluorescent dyes 5-carboxyfluorescein (FAM) on the 5' end and *N,N,N',N'*-tetramethyl-6-carboxyrhodamine (TAMRA) on the 3' end (5'-[FAM]-TGTCGACCTGGGCAGGGTTCG-[TAMRA]-3'). The primers and hybridization probe were synthesized by Synthegen LLC (Houston, Tex.). The PCR fragment was inserted into the pGEM cloning plasmid with the pGEM T-Easy Vector Cloning System I kit (Promega, Madison, Wis.). The pGEM TB IS6110 plasmid DNA was used to optimize the real-time PCR assay for primer concentration, probe concentration, MgCl₂ concentration, and annealing and extension temperature conditions. The optimized FQ PCR protocol included an initial denaturation step of 95°C for 240 s, followed by 40 cycles of 95°C for 15 s and 68°C for 30 s. Each 25-μl PCR chamber contained a single puRe TaqReady-To-Go PCR Bead (Amersham Bioscience, Piscataway, N.J.), 0.5 μM primers IS6 and IS7, 0.5 μM probe, 3.5 mM MgCl₂, and water to bring the volume to 20 μl. In a separate work area

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TABLE 1. Detection of *M. tuberculosis* DNA from acid-fast positive smears of respiratory specimens

Organism cultured	No. of isolates	No. positive by amplification		No. of patients
		Amplior	FQ PCR	
<i>M. tuberculosis</i>	198	190 ^a	197 ^b	97
<i>M. avium-intracellulare</i>	72	0	1 ^c	40
<i>M. kansasii</i>	40	0	1 ^c	16
<i>M. chelonae</i>	19	0	0	8
<i>M. fortuitum</i>	3	0	0	2
<i>M. goodii</i>	1	0	0	1
<i>M. simiae</i>	1	0	0	1
None (no growth)	31	0	1 ^c	29

^a New lysates prepared from the original specimen of the AMPLICOR-negative, TB culture-positive subset; five of seven were positive on repeat testing.

^b New lysate prepared from the original specimen of the Smart Cycler-negative, TB culture-positive subset; it was negative on repeat testing.

^c New lysates prepared from the original specimen of the Smart Cycler-positive, TB culture-negative subset; two of three were positive on repeat testing.

and with a CleanSpot PCR/UV workstation (Coy Laboratory Products, Grass Lake, Mich.), 5 µl of sample was added to the reaction chamber. The total time of amplification, detection, and analysis with this protocol is approximately 42 min for 16 samples. Fluorescence measurements are made in every cycle. The threshold cycle (Ct) is the cycle at which there is a significant increase in fluorescence, and this value is associated with exponential growth of the PCR product during the log-linear phase. Negative and positive controls were included in every run. The negative control consisted of the amplification master mixture and a water blank used to prepare the reagents. The positive control was prepared from pooled 1+ smear-positive sputum samples and treated exactly as a patient sample. This pooled 1+ smear-positive sample had a Ct value of 34.6 ± 0.5 cycles during the test period.

We tested a total of 366 smear-positive specimens (Table 1). There were 198 AFB smear-positive specimens tested that grew MTB; 136 grew mycobacteria other than *M. tuberculosis*, and 31 yielded no growth. Of the MTB group, 197 specimens were positive by FQ PCR (99.5% sensitivity) and 190 specimens were positive by the AMPLICOR PCR assay (96% sensitivity). There was no significant difference between the two protocols ($P = 0.249$, Fisher's exact test). Within these subsets, new lysates of the original negative specimens were prepared and tested in each system. The negative FQ sample was negative upon retesting, whereas five of seven AMPLICOR PCR samples were positive. One sample was not available for further examination. These data suggested that the initial result may have been due to PCR inhibition or to a sampling error because of a low number of AFB in the patient sample. Negative PCR sample lysates were spiked with MTB DNA; all were positive in their respective systems, indicating that there was no evidence of PCR inhibition in these specimens. The majority of negative or equivocal results were obtained with 1+ smear-positive samples. To address the question of low numbers of AFB in the patient sample, we performed triplicate testing on 1+ smear-positive specimens obtained from 40 patient samples. A sufficient sample volume for all 56 patient samples for triplicate testing was not available. The FQ PCR assay was positive in 118 of 120 cases (98.3% sensitivity), while

TABLE 2. PCR detection of *M. tuberculosis* based on the quantity of organisms detected in acid-fast smears

Parameter	No. with an acid-fast smear result ^a of:				Total no.
	1+	2+	3+	4+	
FQ PCR/AMPLICOR PCR results					
Positive/positive	49	45	35	59	188
Positive/negative	5	1	1	0	7
Positive/equivocal ^b	1	1	0	0	2
Negative/negative	1	0	0	0	1
Ct ^c value (FQ PCR)	33.2	28.8	26.1	23.9	

^a Fluorescence microscopy (×400 magnification). 1+, one to three per slide; 2+, one to nine per 10 fields; 3+, one to nine per field; 4+, greater than nine per field.

^b Both specimens were positive on repeat testing of the same lysate by the Amplior method.

^c Threshold cycle where there is a significant increase in fluorescence; value is associated with exponential growth of the PCR product during the log-linear phase.

the AMPLICOR PCR test was positive 115 of 120 cases (95.8% sensitivity) and 5 samples gave an equivocal result. These results were highly concordant, and no significant difference between these assays was observed (Fisher exact test, $P = 0.46$). There was a single patient in the FQ PCR group who had two negative results and one positive result. For the AMPLICOR PCR assay, three patients had a combination of equivocal and positive results; none of the determinations were negative. These data support the conclusion that a small number of organisms in the patient sample resulted in the original negative or equivocal result.

There were 136 AFB smear-positive specimens that grew mycobacteria other than *M. tuberculosis*. All were negative in the AMPLICOR PCR assay, and two were positive in the FQ PCR assay. One patient sample that grew *M. avium-intracellulare* remained positive in the FQ PCR assay after a new lysate was prepared from the original specimen. The one patient sample that grew *M. kansasii* was negative on repeat testing of a new lysate. The initial result probably represents contamination of the original sample lysate. Two additional specimens from this patient grew *M. kansasii*, and all were negative in the FQ PCR assay. An additional 31 specimens were negative for mycobacterial growth and AMPLICOR PCR. One patient sample was positive by the FQ PCR assay, and a new lysate prepared from the original specimen was likewise positive. This patient had five AFB smear-negative specimens available for testing, and all were negative by FQ PCR assay and AMPLICOR PCR. The overall specificity of the AMPLICOR PCR assay was 100% (168 of 168), while the specificity of the FQ assay was 98.2% (165 of 168).

A closer examination of the MTB culture specimens revealed that the FQ PCR assay was able to detect MTB DNA when the AFB smear contained few organisms (Table 2). The one specimen that was negative by the FQ PCR assay contained a rare number of organisms. The eight samples that were negative in the AMPLICOR PCR assay contained rare and moderate organisms by acid-fast smear. The Ct value correlated with the acid-fast smear result. When these data were examined by degree of smear positivity, it was evident that the specimens with small numbers of organisms were more likely

to give a longer time of detection (Table 2). The average Ct values for 4+, 3+, 2+, and 1+ samples were 23.9, 26.1, 28.8, and 33.2 cycles, respectively.

We are concerned about the potential false-positive results that were found with this survey of AFB smear-positive samples. Two patient specimens failed to grow MTB but were positive by FQ PCR. A review of the patient charts could not rule out the diagnosis of tuberculosis. Both were human immunodeficiency virus-infected patients who had multiple admissions to the hospital, and they were treated for tuberculosis during their hospitalization. As with any amplification protocol, there are always concerns about contamination with extraneous nucleic acid. This may occur through improper handling of the original specimen during the initial processing for culture or at the stage of processing for amplification. Normally, our specimens are batch processed daily for culture and smear preparation. If a specimen is AFB smear positive, the concentrated specimen is taken to a separate biosafety cabinet in a laboratory area designated for DNA extraction. All work flow was unidirectional, and separate laboratory areas were used for all reagent preparation and addition of the specimen to the reaction chamber. In the FQ PCR assay, amplification and detection are accomplished in a closed system; the reaction vessels are never opened after the cycling process has started. Therefore, there is no opportunity for carryover contamination to occur postamplification.

This study did not address the ability of the FQ system to detect MTB in smear-negative specimens. Since Ct values are inversely proportional to the number of organisms present in the sample, it is likely that a larger specimen volume or methods that yield greater amounts of DNA are necessary to detect MTB in these specimens. For the FQ PCR assay, a volume of 5 μ l is used. It is unlikely that this volume could be increased without running the risk of inhibition of the PCR. Therefore, it seems reasonable to try to concentrate the crude lysates for use in PCR assays. One system that we would like to explore involves the use of a silica membrane, the QIAamp DNA mini kit (Qiagen, Valencia, Calif.). This method has been used to remove inhibitors in clinical specimens extracted with the AMPLICOR protocol (1). If the laboratory could reliably detect those specimens that contained MTB, regardless of the AFB smear result, we could more effectively use respiratory precautions for those patient who are positive by amplification. The hospital cost benefit of this protocol would be significant.

The Smart Cycler instrument and similar real-time PCR instruments offer a technology that is a significant breakthrough in the clinical diagnostic laboratory. In this study, the FQ PCR assay demonstrated a sensitivity of 99.5% (197 of 198) and a specificity of 98.2% (165 of 168). This assay proved to be very quick and to offer potential labor savings in the laboratory. The DNA extraction procedure, the reagent master mix preparation, and sample inoculation were common to both procedures, and it took approximately 2 h to process 16 samples. For the FQ PCR assay, amplification and detection were completed within 45 min. No other manual manipulations were necessary after the reaction cuvette was placed in the Smart Cycler instrument. The total time for this protocol was less than 3 h; hands-on time was approximately 1.5 h. For the AMPLICOR PCR, 1.5 h was required for amplification and then 1.5 h was required for detection by colorimetric microwell

plate probe hybridization. Additional manual steps were needed after the thermocycling process to complete the assay. The total time for this protocol was greater than 6 h; hands-on time was approximately 3 h. Another benefit of the Smart Cycler system is that the reaction vessel with the amplified product is never opened in the laboratory after the initiation of amplification.

We are indebted to Octavio V. Martinez for thoughtful review of the manuscript and Lisa W. Plano for construction of the pGEM IS6110 plasmid.

This work was funded by the Clinical Microbiology Research Fund and the Department of Pathology.

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