

Ability of PCR Assay To Identify *Mycobacterium tuberculosis* in BACTEC 12B Vials

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Introduction of PCR to directly detect *Mycobacterium tuberculosis* in clinical specimens has shown promise; however, interfering substances in clinical material have contributed to lowered assay sensitivities. We evaluated the ability of a PCR assay to detect *M. tuberculosis* in BACTEC 12B broth cultures. Clinical specimens were processed and inoculated into BACTEC 12B vials. Evaluation was approached in two phases, starting with an initial evaluation in which an aliquot of 12B broth was removed when the growth index (GI) was ≥ 10 and stored at 4°C until assayed by PCR. Of the 290 specimens initially assayed, 129 were culture negative for mycobacteria as well as PCR negative for *M. tuberculosis*. Except for one, cultures ($n = 102$) which grew mycobacteria other than *M. tuberculosis* were all PCR negative. The remaining 59 broths were all culture and PCR positive for *M. tuberculosis*; 39% ($n = 23$) of these cultures when assayed by PCR had GIs of ≤ 50 . Following initial evaluation, 200 12B BACTEC vials with GIs of ≥ 10 were assayed in a similar manner except that specimens were amplified twice weekly to determine PCR's impact on the length of time to identification of *M. tuberculosis* as compared with standard laboratory practices. Utilization of PCR resulted in a mean time to detection of *M. tuberculosis* of 14 days, compared with 29 days by using commercially available nucleic acid probes to identify *M. tuberculosis* complex from growth of BACTEC 12B subcultures on solid media. In light of an overall sensitivity and specificity of 100 and 99.7%, respectively, coupled with the ability to identify *M. tuberculosis* days or weeks before other methods can be applied, we conclude that PCR might prove to be a rapid alternative for identification of *M. tuberculosis* in culture and allow for earlier setup of susceptibility testing.

The emergence of epidemic multiple-drug-resistant strains of *Mycobacterium tuberculosis* in conjunction with the ending of the downward trend of reported cases of tuberculosis in the United States represents a major public health problem. Implicit in this increased prevalence of tuberculosis is the demand for more rapid and reliable laboratory methods to diagnose *M. tuberculosis* infections. The introduction of the BACTEC 460 system (Becton Dickinson Diagnostic Instruments, Sparks, Md.) and, more recently, the biphasic Septi-Chek AFB System (Roche Diagnostics Systems, Nutley, N.J.) have made major improvements in mycobacterial cultures by providing more rapid detection and better recovery of mycobacteria (10, 11). DNA probes have also significantly decreased the length of time required for the identification of *M. tuberculosis* and other mycobacterial species. Several studies have evaluated nucleic acid probes for their ability to identify the *M. tuberculosis* complex and *Mycobacterium avium* complex (MAC) directly from BACTEC broth cultures in attempts to further decrease turnaround time. Although identification by nucleic acid probes of MAC directly from BACTEC broth cultures is highly sensitive and specific, several studies have reported lowered sensitivity with *M. tuberculosis* complex probes (5, 7, 13). Thus, days to weeks may still be required for sufficient growth for identification.

Because of the obvious demand for a reliable and rapid means of diagnosing tuberculosis for public health and therapeutic reasons, we recently developed and evaluated a PCR assay for its ability to directly detect *M. tuberculosis* in respiratory specimens (9); this assay was also easily incorporated into a clinical laboratory routine. Although our assay had an

overall sensitivity and specificity of 87.2 and 97.7%, respectively, at least 16% of specimens contained interfering substances which contributed to lowered sensitivity. Inhibition of PCR by interfering substances in clinical specimens has been reported by several investigators (2, 18, 20); the elimination of inhibitory factors remains a challenge in the use and acceptance of PCR assays in the clinical diagnostic setting.

In light of the inherent problems with the use of PCR to directly detect *M. tuberculosis* in clinical specimens, we evaluated the ability of our PCR assay to detect *M. tuberculosis* directly in BACTEC 12B broth cultures. We approached this study in two phases: (i) to determine the ability of PCR to detect *M. tuberculosis* directly in BACTEC 12B vials and (ii) to assess the impact of the PCR assay on the length of time required to report findings of *M. tuberculosis* in culture.

MATERIALS AND METHODS

Culture protocol. All clinical specimens submitted for routine mycobacteriological smear and culture were processed by standard procedures. In brief, contaminated specimens were digested with 2% NaOH. Following concentration by centrifugation, sediments were resuspended in 3 to 5 ml of phosphate-buffered saline. Smears were prepared, stained with auramine O, and examined for acid-fast bacilli (AFB) by fluorescence microscopy. Lowenstein-Jensen (LJ; Becton Dickinson Microbiology Systems, Cockeysville, Md.) medium was inoculated with processed specimens and incubated for 8 weeks; solid medium was examined at 1, 3, 5, and 8 weeks for growth. BACTEC 12B vials (Becton Dickinson Diagnostic Instruments) were inoculated with 0.5 ml of the appropriately processed specimen according to the manufacturer's instructions. 12B vials were monitored by using the BACTEC 460 reader on a regular basis for 6 weeks: vials were read twice weekly for the first 2 weeks and weekly thereafter for 4 weeks.

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When the growth index (GI) of a BACTEC 12B vial reached ≥ 10 , the vial was tested daily until the vial attained a GI of ≥ 100 , at which time an AFB stain was performed to confirm the presence of AFB.

Once the presence of AFB was confirmed by staining, two LJ tubes were inoculated with 0.5 ml from the BACTEC 12B vial; one tube was wrapped in foil, and both were incubated at 37°C. A Middlebrook 7H11 (Becton Dickinson Microbiology Systems) plate was also inoculated with 0.1 ml from the positive 12B vial. Because of inconsistent results with a commercially available nucleic acid probe for *M. tuberculosis* complex (Gen-Probe, San Diego, Calif.), DNA hybridization assays were not performed directly on growth from the BACTEC 12B vials (13). As soon as growth appeared on the LJ slant, mycobacteria were identified as *M. tuberculosis* complex or MAC with commercially available nucleic acid probes (Gen-Probe). If growth was detected only on the LJ slant or before growth of the BACTEC 12B vial's subculture, probes were performed on the growth from solid media. Probes were performed weekly. All other species of mycobacteria were sent to the New York State Department of Health for complete identification.

During the first phase of our study, a 0.5-ml aliquot of 12B broth was removed when the GI was greater than or equal to 10 and then stored at 4°C until assayed by PCR. On the basis of results from the first phase of our study, the PCR assay was then incorporated into the laboratory routine. Laboratory routine practices were not changed; specimens were processed 7 days/week, and BACTEC 12B bottles were run twice weekly for the first 2 weeks and weekly thereafter for 4 weeks. However, once a 12B vial attained a GI of ≥ 10 , a 0.5-ml aliquot was removed and stored at 4°C until subsequently assayed by PCR. PCR assays were performed twice weekly. The vial was then handled as previously described by monitoring daily until the GI achieved a value of ≥ 100 , at which time the presence of AFB was confirmed by staining. Similarly, cultures growing AFB were handled in a routine manner for identification.

PCR assay. (i) Sample preparation. In preparation for PCR, the aliquot from the 12B vial(s) with a GI of ≥ 10 was boiled for 10 min. With a Poppette positive-displacement pipette (Lab Industries, Berkeley, Calif.), 10 μ l of the boiled suspension was then used for the amplification assay.

(ii) Primers. Two sets of primers, one (6) based on the IS6110 repeated sequences of *M. tuberculosis* (IS6110) and the other (17) based on the 38-kDa protein antigen b (PAB) of *M. tuberculosis*, were employed. The expected sizes of the amplification products of the IS6110 and PAB primers were 123 and 419 bp, respectively.

(iii) DNA amplification and product detection. Reaction conditions and product detection were as previously described (9). In brief, 10 μ l of the boiled suspension was added to 85 μ l of the amplification cocktail containing 12 pmol of IS6110 primers, 20 pmol of PAB primers, 2 mM MgCl₂, and 200 μ M deoxynucleoside triphosphate in PCR buffer II (Perkin-Elmer Corp., Norwalk, Conn.). The reaction mix was overlaid with 100 μ l of sterile, light mineral oil, boiled for 8 min, and chilled on ice, and then 2.5 U of *Taq* polymerase (Perkin-Elmer) was added. Multiple controls, including reagent and positive controls, were included in each amplification run as previously described (9). Similarly, all previously described practices to minimize false-positive results were employed.

Reaction mixtures were then subjected to 40 cycles of amplification. Polyacrylamide gels (8%) were stained with ethidium bromide, and products were then visualized with UV light. The presence of the 123-bp IS6110 product and/or the

TABLE 1. Results of the initial evaluation of PCR for the detection of *M. tuberculosis* in 12B vials (phase 1) and of incorporation of the assay into the laboratory's routine (phase 2)

Phase	No. of specimens	Culture result	PCR results ^a
1 (n = 290)	129	No growth	-
	71	MAC	-
	18	<i>M. xenopi</i>	-
	8	<i>M. gordonae</i>	-
	4	<i>M. fortuitum</i>	-
	1	<i>M. kansasii</i>	-
	1	MAC	+
	59	<i>M. tuberculosis</i>	+
	2 (n = 200)	108	No growth
43		MAC	-
8		<i>M. gordonae</i>	-
5		<i>M. xenopi</i>	-
36		<i>M. tuberculosis</i>	+

^a All BACTEC 12B vials with GIs of ≥ 10 were assayed by PCR.

419-bp PAB product was considered a positive PCR result for *M. tuberculosis*.

RESULTS

Initial evaluation. Two hundred ninety 12B cultures with GIs that were ≥ 10 were assayed by PCR; these data are summarized in Table 1. Of the 102 12B cultures which grew mycobacteria other than *M. tuberculosis*, 71 grew MAC, 18 grew *M. xenopi*, 8 grew *M. gordonae*, 4 grew *M. fortuitum*, and 1 grew *M. kansasii*. All of these cultures except one were PCR negative for *M. tuberculosis*; the exception was culture positive for MAC. Fifty-nine specimens were culture and PCR positive for *M. tuberculosis*. Of note, 39% (23 of 59) of these cultures were assayed by PCR with GIs between 10 and 50, 30.5% (18 of 59) of cultures had GIs between 51 and 200, and the remainder had GIs of >200 .

Routine laboratory evaluation. On the basis of initial findings, the PCR assay was then introduced into the mycobacteriology laboratory routine in order to assess its impact on the length of time to reporting of *M. tuberculosis*. Two hundred BACTEC 12B vials attained GIs of ≥ 10 and were assayed by PCR; the results are summarized in Table 1. Of the 36 12B broth vials which were culture and PCR positive for *M. tuberculosis*, 23% (n = 8) were assayed by PCR with GIs of ≤ 50 while 39% (n = 14) were assayed with GIs between 51 and 200; the remaining vials were assayed with GIs of >200 .

Of particular interest was the determination of the impact of PCR with regard to decreasing the time to reporting of *M. tuberculosis*. We compared the times from specimen submission to the identification of *M. tuberculosis* by PCR and DNA hybridization. Utilization of PCR to identify *M. tuberculosis* in BACTEC vials resulted in a mean time to identification of 14 days, compared with 29 days by our present laboratory routine of confirming the presence of AFB in positive 12B vials, subculturing to solid media, and identifying growth by nucleic acid hybridization; the ranges for PCR identification and routine practices were 4 to 23 and 13 to 61 days, respectively.

Finally, analysis of culture data demonstrated the significantly increased recovery of *M. tuberculosis* from clinical specimens by the BACTEC system compared with that with solid media (Table 2). *M. tuberculosis* was isolated from 36 BACTEC 12B cultures and only 25 LJ medium cultures. In only one instance was *M. tuberculosis* isolated on solid medium

TABLE 2. Comparison of culture and identification results of specimens culture positive for *M. tuberculosis* ($n = 36$) following introduction of PCR analysis into the laboratory's routine

Specimen no.	Time (days) to detection of growth by:		Time (days) to identification		GI of 12B vial at time of PCR assay
	LJ culture	BACTEC	Without PCR	With PCR	
1	No growth	15	24	15	129
2	25	13	27	12	61
3	28	17	30	18	115
4	25	19	27	14	52
5	12	5	19	5	999
6	23	14	29	16	58
7	18	8	33	12	178
8	11	4	13	4	897
9	31	11	26	10	69
10	No growth	13	21	14	498
11	32	10	25	10	154
12	No growth	13	28	13	297
13	No growth	11	28	14	29
14	No growth	10	26	5	70
15	18	8	33	8	11
16	No growth	6	21	6	66
17	28	10	22	14	506
18	27	10	21	7	10
19	38	18	33	17	14
20	No growth	10	22	14	209
21	31	13	26	18	406
22	27	10	22	14	371
23	No growth	15	32	16	13
24	20	10	22	13	575
25	No growth	14	30	14	21
26	29	22	30	23	109
27	26	14	26	18	278
28	31	17	17	17	242
29	No growth	38	61	19	144
30	21	17	29	16	15
31	No growth	26	41	20	570
32	60	25	41	21	18
33	40	24	40	20	28
34	No growth	38	61	19	38
35	28	10	22	14	118
36	21	10	26	13	70

and not from broth. Moreover, the average time to detection of *M. tuberculosis* by the BACTEC system was 13 days, compared with 27 days with solid media; the range for the BACTEC was 4 to 38 days, while the range for solid media was 11 to 60 days.

DISCUSSION

Despite promising results of numerous published reports, the introduction of PCR into the routine clinical laboratory for the direct detection of *M. tuberculosis* in clinical specimens has been hampered for a variety of reasons. Problems of contamination, expense, and lack of sensitivity and/or specificity have been reported (2, 3, 6, 9, 14, 17, 18, 20, 21); as previously mentioned, clinical specimens can often contain substances which interfere with PCR. In addition, acceptance of PCR into the clinical laboratory setting is further impeded by complex procedures required for amplification, such as cumbersome sample preparation methods.

On the basis an overall sensitivity and specificity of 100 and 99.7%, respectively, PCR proved to be a useful and rapid means for the detection and identification of *M. tuberculosis* in 12B broth cultures. Of particular interest was the one BACTEC 12B vial which was PCR positive for *M. tuberculosis*

but was culture positive for MAC only; the LJ culture also yielded MAC. Because of these discrepant results, another aliquot from the 12B vial was assayed by PCR and was again positive for *M. tuberculosis* sequences. The original and subsequent subcultures of the BACTEC 12B vial showed growth of MAC only, despite careful examination of growth; subsequent hybridization with the *M. tuberculosis* probe of all subcultures and reprobing of the original subculture were negative. This specimen was obtained from a human immunodeficiency virus-infected patient who was admitted with fever and pulmonary infiltrates; the patient was anergic, and his purified protein derivative status was unknown. During his hospital admission, eight sputum specimens were submitted for mycobacterial culture; of these, four yielded growth of MAC. Since these specimens were submitted during the first phase of our study, only the last specimen submitted for mycobacterial culture was analyzed by PCR. MAC was also recovered from four of four blood specimens. Unfortunately, no further specimens were obtained since the patient died within days of the last specimen submission and no autopsy was performed. Although considered a false positive in our analysis, we feel that the PCR result cannot be totally ruled out as a possible true positive.

Cormican et al. (4) assayed 45 BACTEC cultures by PCR using primers to the gene encoding the immunogenic protein MPB64 from *Mycobacterium bovis* BCG. Rather than being assayed at or above a particular GI, vials were inoculated onto blood agar at the earliest detectable rise in GI and incubated at 37°C overnight. GIs of 12B vials ranged from 6 to 999. Samples with no growth upon subculture were then examined by PCR. Of 45 broth vials, 15 were culture and PCR positive for *M. tuberculosis*. Data on the methods of routine identification of *M. tuberculosis* and a comparison of routine results and those obtained by PCR in terms of time to detection were not presented.

Rather than assaying at a detectable rise in GI, we assayed vials when a GI threshold of 10 or greater was achieved in order to facilitate the use of PCR in the laboratory's routine. One-third of 12B broths which were culture and PCR positive for *M. tuberculosis* had GIs of ≤ 50 at the time of PCR testing. Morris et al. (12) reported similar findings in which 1 ml of fluid was removed from all BACTEC 12B vials with GIs of ≥ 20 during a 3-month period and stored at -70°C . Following extraction, supernatants were used for PCR according to previously published methods (6). In this retrospective study, detection of *M. tuberculosis* in BACTEC 12B by PCR was 100% sensitive and specific. Twenty-four specimens with *M. tuberculosis* came from 10 patients. Of the 24 original specimens containing *M. tuberculosis*, 15 (72%) were smear positive. Of particular interest, the clinical utility of PCR was assessed by examining the medical records of all 10 patients infected with *M. tuberculosis*; only one patient with a smear-negative, culture-positive specimen might have benefited from the PCR result. According to the authors, if direct detection of *M. tuberculosis* in the original specimen had been done by PCR, a positive PCR result would have been helpful for two of their five smear-negative patients. However, the authors raise two concerns in this regard: (i) the sensitivity of PCR for the direct detection of *M. tuberculosis* in clinical specimens is considerably lower in smear-negative specimens, and (ii) clinical management may not necessarily be dramatically improved by using PCR.

Although PCR may or may not offer significant advantages for the clinical management of patients, PCR may have significant potential to streamline mycobacteriology laboratory practice. The impact of the PCR assay for our laboratory was significant in that the time to identification of *M. tuberculosis*

was halved compared with that obtained by performing nucleic acid probes weekly on growth from the subculture of BACTEC 12B vials. Since many laboratories centrifuge and then probe an aliquot from 12B vials once the GI is in the range of 500 to 999, the impact of the PCR assay in regard to the time to identification of *M. tuberculosis* for these laboratories may not be as great. Studies are presently under way to determine the shortest incubation of 12B vials required prior to assay by PCR, such that a regular schedule for assay by PCR which easily incorporates into a laboratory's routine yet is sensitive and specific may be established. If *M. tuberculosis* can subsequently be detected in 12B vials by PCR assay after a minimal period of incubation such that vials are assayed at GIs of ≤ 10 , all laboratories may realize a significant decrease in the time to identification.

Although larger studies will be required, on the basis of its exquisite sensitivity and reliable specificity, PCR assay of BACTEC 12B vials may obviate the need to use other methods for identification of *M. tuberculosis* such as a nucleic acid probe for *M. tuberculosis* complex. Moreover, if amplification-based methods used in conjunction with either hybridization to species-specific probes (1, 8), sequencing of the amplicon (16), or restriction enzyme analysis of product for subsequent identification to the species level (15, 19) prove sensitive and specific in detecting and identifying all mycobacteria, greater advantages in terms of further decreasing turnaround times, costs, and streamlining of laboratory practices may be realized. Regardless, it should be noted that the more frequently that PCR is performed, the greater its impact on the time to identification of *M. tuberculosis* will be. Another advantage of using PCR to identify *M. tuberculosis* in BACTEC 12B broth, which became apparent during our evaluation, was the ability to begin corresponding primary drug susceptibility testing by the BACTEC system earlier than can be done when routine laboratory practices are used. In conclusion, PCR appears to be an easy and reliable means to detect and identify *M. tuberculosis* in 12B BACTEC broth cultures, on the basis of our findings from this large study.

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