Strand Displacement Amplification and the Polymerase Chain Reaction for Monitoring Response to Treatment in Patients with Pulmonary Tuberculosis


Specific amplification of Mycobacterium tuberculosis DNA was investigated as an alternative to conventional microbiologic follow-up in 31 cases of smear- and culture-positive pulmonary tuberculosis. Strand displacement amplification (SDA) and the polymerase chain reaction (PCR) were applied to 438 sequential sputum specimens: 67 (15%) were positive by culture, 248 (57%) by SDA, and 231 (53%) by PCR ($\chi^2 = 3.94, P = .05$). Of 200 specimens collected >180 days after treatment started, none yielded positive cultures, while 50 (25%), representing 16 patients, were positive by both DNA assays. A weak correlation was demonstrated between DNA persistence in sputum and duration of culture positivity ($r = 0.45, P = .01$), although no correlation was found with the radiographic extent of disease. The inability to distinguish live and dead organisms precludes DNA amplification from use in therapeutic monitoring. For this purpose, quantitative RNA assays are needed if such techniques are to supplant conventional microbiology.

The enormity of the global increase in tuberculosis case rates [1] has focused attention on the need for more rapid and reliable methods of diagnosis. Microscopic examination of sputum smears requires $\sim 10^4$ organisms/mL for detection, and although it is more sensitive, culture of Mycobacterium tuberculosis takes 2–6 weeks because of the slow growth rate of the organism. Recently, several techniques have been described for the amplification of nucleic acid sequences that are specific for species within the M. tuberculosis complex [2–7]. Despite widespread application of such technology to diagnosis of the disease, there remains little information regarding its use to monitor the response of patients to treatment [8].

Patients with pulmonary tuberculosis typically undergo smear and culture conversion within 3–4 months of the start of effective treatment. Yuen et al. [9] reported that among 41 cases of culture-documented pulmonary tuberculosis, 71% remained positive by the polymerase chain reaction (PCR) after 4 weeks of treatment compared with 39% by acid-fast smear and 32% by culture. Other limited studies have shown that sputum may remain positive by PCR for up to 6 months after the start of treatment and between 1 and 6 months after microbiologic conversion [10, 11].

We studied 31 patients to determine whether DNA amplification will provide a viable alternative to conventional microbiologic follow-up in the treatment of tuberculosis. We compared two independent DNA amplification systems, which were applied to sequential sputum specimens from patients who had received chemotherapy for smear- and culture-positive pulmonary disease. Both the strand displacement amplification (SDA)– and PCR-based assays use the IS6110 insertion element [12] as a specific target for organisms of the M. tuberculosis complex (M. tuberculosis, Mycobacterium bovis, M. bovis bacille Calmette-Guérin [BCG], Mycobacterium africanum, and Mycobacterium microti) and have analytical sensitivities equivalent to 1 genome (<5 fg) of purified DNA. Data were analyzed to determine whether DNA amplification correlated with smear, culture, and clinical outcome.

Materials and Methods

Patients and specimens. Sputum samples were obtained from the Arkansas Department of Health microbiology laboratory between July 1992 and January 1995. Each specimen was treated with the standard N-acetyl-L-cysteine (NALC)–NaOH method [13]. Sediments were examined by microscopy and $\sim 50\%$ used to inoculate commercial and BACTEC culture media. The remaining sediments were stored at $-20^\circ$C before further processing.

Patient records were reviewed, and all patients who had completed their course of therapy or had died and who met the following criteria were included in the study: a diagnosis of pulmonary tuberculosis confirmed by both microscopy and culture, the provision of at least 9 sputum specimens collected at discrete intervals over a period of $\geqslant 9$ months, and the availability of demographic information and details regarding the course of chemotherapy.
Patients whose follow-up was irregular or intermittent were excluded from analysis. In Arkansas, the standard treatment regimen for patients with pulmonary tuberculosis is a 9-month course of isoniazid and rifampicin, and sputum is collected for 2 years after the start of treatment.

The radiographic extent of disease was assessed by examination of chest radiographs taken at intervals throughout the course of treatment using a classification system adapted from that developed by the US Veterans Administration [14, 15]. An arbitrary scoring system was applied, based on the number and diameter of the cavity wall(s) (score 1–9), thickness of the cavity wall(s) (score 1–5), and the anatomic extent of disease apart from cavitation (score 1–10). According to this scheme, the higher the patient’s total score, the more severe the radiographic extent of disease. Films were examined by three experienced pulmonologists without prior knowledge of microbiologic or DNA amplification results.

Specimen processing for SDA and PCR. Specimens from each patient were randomized before sample processing to avoid bias in the interpretation of results. NALC pellets were thawed and centrifuged at 12,000 g for 5 min. The supernatant was decanted to leave ~100 μL of liquid, to which was added an equal volume of 1 M NaOH, 2% Triton X-100. Samples were boiled for 5 min and neutralized with 100 μL of 1 M TRIS-EDTA. DNA was recovered using the GeneClean procedure (Bio101, La Jolla, CA) and eluted in 30 μL of water. Five-microliter aliquots of purified DNA were amplified by SDA and PCR.

SDA. SDA was done in 50-μL volumes as described by Walker et al. [3]. Target DNA was added to buffer containing (final concentrations) 50 mM KPO₄, pH 7.6; 0.5 μM primers S1 and S2; 0.05 μM primers B1 and B2; 1 μM Mtb15 primer (figure 1); 0.2 mM dCTP, dGTP, and 2'-deoxyadenosine 5'-O-(1-thiotriphosphate); 0.5 mM dUTP; 0.1 mg/mL acetylated bovine serum albumin; 10% dimethyl sulfoxide; and 1 ng/μL human placental DNA (Sigma, St. Louis). Primers were boiled for 2.5 min and cooled to 41°C, and 1 U of uracil DNA glycosylase (UDG; Life Technologies GIBCO BRL, Gaithersburg, MD) was added to remove any contaminating amplifiers from previous reactions [16]. Incubation was continued for 30 min before addition of 150 U of HincII (New England Biolabs, Beverly, MA); 4 U of exo- Klenow polymerase (New England Biolabs); 2 U of UDG inhibitor (Life Technologies); 7.8 mM magnesium acetate (final concentration); and 25 copies of the amplification control Stg15U (figure 1). Amplification was done at 41°C for 2 h, and reactions were terminated by boiling for 2 min. Tubes were stored at −20°C before detection.

Detection of SDA products. Amplified IS6110 DNA and signature sequence were detected by primer extension with 32P-labeled Mtb15 probe (figure 1) and T4 DNA polymerase. Oligonucleotides of 35 and 56 bases were generated for IS6110 and of 46 and 67 bases for signature. These products were visualized by autoradiography after electrophoresis through 8% denaturing polyacrylamide gels. Autoradiographs were exposed for 1 h at −70°C using Fuji RX film with intensifying screens. Results were confirmed by reexposure for 12 h.

PCR. PCR was done as described by Rish et al. [17] with the exception that 0.36 mM dUTP was used in place of dTTP and 1 U of UDG (Perkin-Elmer Cetus, Foster City, CA) was included in the reaction mixture. Primer sequences for PCR are given in figure 1. Before amplification, complete mixtures containing target DNA were incubated at 25°C for 30 min to facilitate cleavage of contaminating dU-containing PCR products by the UDG enzyme. After amplification, tubes were incubated at 72°C for 30 min to ensure complete inactivation of the UDG enzyme and stored at −20°C.

Detection of PCR products. IS6110-specific PCR products were detected in a liquid hybridization assay using 32P-labeled DNA to Monitor Tuberculosis Therapy

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**Figure 1.** Sequences of oligonucleotide primers, listed in 5' to 3' direction. SDA requires 4 primers: 2 bumper primers, B1 and B2, which bind to IS6110 at nts 954–966 and 1032–1044, outside the 2 SDA primers S1 and S2, corresponding to positions 972–984 and 1011–1123, respectively [3]. Regions of homology to IS6110 within S1 and S2 are underlined, and HincII recognition sites are lower-case. PCR primers T4 and T5 hybridize to IS6110 at nts 865–884 and 762–781. Mtb15 and LK229 are detector probes for SDA and PCR, respectively, and bind to IS6110 at nts 992–1006 and 812–851.

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**Polymerase Chain Reaction**

| T4  | CCT GCG AGC GTA GGC GTC GG  |
| T5  | CTC GTC CAG CCG CCG TTC GG  |
| LK229 | GTA GGC GAA CCC TGC CCA GGT CGA CAC ATA GGT GAG GTG T |

JID 1996; 173 (April) DNA to Monitor Tuberculosis Therapy 935
L. K229 (figure 1) [17]. The resulting partially double-stranded hybrid molecules were analyzed by electrophoresis through 12% non-denaturing polyacrylamide gels, which were exposed to Kodak X-Omat film for 1 h at room temperature. Results were confirmed by reexposure for 5 h under the same conditions. The 600-bp PCR product generated by amplification of the internal control sequence has no homology with LK229 and was detected by ethidium bromide staining of gels.

Control procedures. Control procedures were adopted as recommended in the proposed guidelines for molecular diagnostic methods [18], with unidirectional workflow and physical separation of reagent preparation, sample preparation, amplification, and product detection. Each laboratory was equipped with dedicated equipment, and standard good laboratory practices were enforced.

As controls for cell lysis and contamination during sample preparation, tubes containing 10^5 cfu of M. bovis BCG and sterile distilled water were processed with each batch of sputum samples. Reactions containing 10 and 1 genome equivalents of purified M. tuberculosis H37R, DNA with 14 copies of the IS6110 element per genome were included as positive amplification controls in each series. Negative controls were provided by reactions containing water in place of target DNA or reagent blanks containing all of the reaction components without addition of template. Controls for UDG activity were as follows: for SDA, 10 genomes of M. tuberculosis H37R, DNA were amplified without UDG inhibitor; for PCR, 10^5 dU-containing PCR amplicons were included as target in reaction mixtures with and without addition of UDG.

IS6110 copy number of M. tuberculosis isolates. The copy number of IS6110 in each strain of M. tuberculosis was determined by analysis of restriction fragment length polymorphisms obtained by PvuI digestion of chromosomal DNA and hybridization with a PCR-derived fragment of the insertion element [19].

Statistical analysis. Linear regression and correlation analysis were done using Microsoft (Redmond, WA) Excel version 5.0. Statistical significance was determined using the McNemar test for proportions and the Mann-Whitney test for comparison of means, with P ≤ .05 considered significant [20].

Results

Patients. A total of 31 patients were enrolled in the study, representing ~15% of all those with culture-positive pulmonary tuberculosis who submitted sputum samples to the Arkansas Department of Health during the period of the study. Ten patients were black and 21 were white; all had a diagnosis of tuberculosis confirmed by positive smears and cultures. Eighteen patients (58%) were men and 13 (42%) were women; mean age was 64.3 years (SD, ± 15.6). Fifteen patients (48%) had one or more predisposing risk factors for tuberculosis, including 4 with previous episodes of the disease, 5 with a history of alcohol abuse, 4 with diabetes mellitus, 3 with cancer, 2 who received steroid therapy, and 1 with antibodies to human immunodeficiency virus. Five patients had isolates of M. tuberculosis that were resistant to isoniazid, 2 of which were also resistant to rifampicin. Nineteen patients received therapy with isoniazid and rifampicin, while the remaining 12 received various combinations of isoniazid, rifampicin, pyrazinamide, streptomycin, ethambutol, ciprofloxacin, and capreomycin. Patients were followed for a median of 412 days after the start of treatment (range, 245–888). Twenty-six patients provided specimens after the completion of therapy. Two died of other causes while still receiving tuberculosis medication, and in the 3 remaining cases, treatment was stopped within 6 weeks of collection of the last sample. Seventeen patients were followed for >3 months after the end of treatment, and in 12 cases, specimens were provided >6 months after completion of therapy.

The 4 patients with a previous diagnosis of pulmonary tuberculosis had all received therapy with isoniazid and rifampicin with a good clinical and microbiologic response. All 4 relapsed to a culture-positive state after completing their course of treatment, at which point they entered the present study. At this time, 2 of the patients harbored isoniazid- and rifampicin-resistant organisms, but with retreatment, all 4 became culture-negative.

Comparison of SDA and PCR. Parallel analysis by SDA and PCR was done on 438 sputum specimens collected from these patients at intervals throughout their course of treatment (table 1). Detection of internal control DNA or products specific for IS6110 (or both) indicated that no inhibition of amplification was observed with any of these samples. Of the specimens, 248 (57%) were positive by SDA, 231 (53%) by PCR (χ² = 3.94, P = .05), and 207 (47%) by both assays; 166 specimens (38%) were negative by both SDA and PCR. Of 67 M. tuberculosis culture–positive specimens, 66 (99%) were positive by either SDA or PCR, with 64 (96%) positive by both. For PCR, 4 (2%) of 178 negative control reactions yielded false-positive results compared with none of 185 SDA controls.

Clinical and microbiologic correlations. All 31 patients responded to therapy as determined by conversion of smears and cultures to negative. Figure 2 compares the percentage of specimens tested that were positive by acid-fast smear, culture, and DNA amplification during 3-month intervals after the start of treatment. There was a rapid decline in the number of culture-positive specimens, from 49% in the first 3 months to 5% in the second 3. Of 200 specimens collected after >180 days of treatment, none yielded M. tuberculosis on culture, while 50 (25%), representing 16 patients, were positive by both SDA and PCR. Of 112 specimens collected within the first 3 months of treatment, 81 (72%) were positive by both SDA and PCR compared with 69 (58%) of 119, 32 (36%) of 89, and 11 (22%) of 49 collected 4–6, 7–9, and 10–12 months, respectively, after initiation of therapy. In addition, among 23 patients who had specimens collected >1 year after the start of chemotherapy, 5 (22%) yielded samples that were both SDA- and PCR-positive. Two of these patients had received therapy with corticosteroids and 1 was a diabetic who harbored an isoniazid-resistant strain. Four of the 5 were no longer receiving treatment at the time of the last SDA- and PCR-positive specimen, although none have subsequently relapsed.
Of 29 surviving patients who completed therapy between April 1993 and June 1994, only 1 has relapsed. This patient was an alcoholic who had been treated for tuberculosis previously and entered the present study with an isoniazid- and rifampicin-resistant strain on his first relapse. On retreatment, he remained culture-positive for 94 days, and the last SDA- and PCR-positive specimen was obtained after 270 days. Three DNA amplification-negative specimens were obtained over the following 5 months. The patient relapsed a second time 11 months after retreatment was stopped and 13 months after the last DNA-positive sample.

Autoradiographs depicting the results of DNA analysis from a single patient are shown in figure 3. No attempt was made to quantify the yield of IS6110-specific product from each specimen, although, as expected, weaker signals were generally obtained from samples collected later in the course of treatment. In some cases DNA-positive and -negative specimens were interspersed throughout the course of follow-up.

In the present study there was a median of 31 days (range, −7 to 171) from the onset of treatment to the last positive culture and a median of 247 days (range, 1–504) to the last sample positive by SDA and PCR. Linear regression analysis demonstrated a correlation between the number of days of treatment until the last positive culture and the persistence of DNA in sputum (r = 0.45, P = .01). Similar levels of correlation were found between DNA persistence and the duration of smear positivity by both fluorochrome (r = 0.38, P = .04) and Ziehl-Neelsen staining (r = 0.47, P = .01). Cultures that were negative for M. tuberculosis or that became contaminated were obtained from 51 fluorochrome- or Ziehl-Neelsen-positive specimens. Of these, 42 were SDA- and PCR-positive. These data, together with the isolation of nontuberculous mycobacteria from 11 (35%) of 31 patients, indicate that the correlation between smear and DNA amplification results must be interpreted with caution.

Patients with coexisting disease placing them at increased risk for tuberculosis, who harbored drug-resistant organisms, or who relapsed after prior treatment, did not demonstrate a greater duration of culture positivity (median, 25 days; range, −7 to 171) than did patients with no known risk (median, 33 days; range, 0–110; P = .81). Similarly, this group of patients had a median of 270 days (range, 51–504) from the start of treatment to the last SDA- and PCR-positive specimen compared with 158 days (range, 1–460) for patients with no risk (P = .12). Four of the 5 patients with drug-resistant isolates of M. tuberculosis received combinations of three or more antibiotics. In these 4 cases, the last SDA- and PCR-positive samples were collected respectively at 176, 270, 273, and 439 days after the start of treatment. The last M. tuberculosis-positive cultures from these patients were obtained from 1 patient 7 days before treatment began and from the remaining 3 on days 94, 10, and 171 after treatment was started. Patients who had less radiographic lung involvement as reflected by a low initial chest radiograph score did not exhibit more rapid clearance of DNA than did those with higher scores (r = 0.11, P = .55).

**IS6110 copy number.** The number of copies of the IS6110 target sequence found in each strain of M. tuberculosis was determined for 27 of 31 patients. Each carried between 2 and 15 copies, but no correlation was observed between the number of IS6110 copies and DNA persistence (r = 0.05, P = .81).

**Discussion**

The purpose of this study was to determine whether detection of M. tuberculosis by amplification of a specific DNA target sequence provides an alternative to conventional microbiologic follow-up for patients receiving chemotherapy. We also examined whether the duration of M. tuberculosis DNA persistence in the sputum after the start of chemotherapy could be correlated with the radiographic extent of disease.

Application of PCR-based assays for the diagnosis of tuberculosis has been widely reported in the literature. Unique to the present study is a comparison between a well-established PCR assay for detection of M. tuberculosis in clinical specimens [2, 17] with the novel SDA procedure of Walker and colleagues [3, 21], which has not previously been used in a large study. Although the SDA and PCR assays both use the
IS6110 repetitive element as an *M. tuberculosis* complex–specific target, different regions are amplified in each system. As a consequence, there is no possibility of cross-contamination of amplified products between the two assays. We performed a combined total of 363 negative control reactions with either SDA or PCR. Incorporation of dUTP and UDG decontamination of reaction mixtures, together with physical separation of sample preparation, amplification, and detection, effectively eliminated problems of contamination with amplicons from previous amplification reactions. The 4 false-positive results with PCR probably arose as a result of contamination with *M. tuberculosis* DNA during addition of target to the reaction mixtures. SDA yielded a significantly greater number of positive results than PCR, although there was agreement between the two assays for 366 (84%) of 438 specimens tested. Only weak positive signals were obtained from the 72 specimens that gave discrepant results, perhaps indicating the presence of low concentrations of *M. tuberculosis* DNA that was heterogeneously distributed throughout the sample.

We have demonstrated the persistence of *M. tuberculosis* DNA in sputum >12 months after the start of treatment and >6 months after culture conversion in some patients. These...
Figure 3. Representative autoradiographs showing results of SDA (A) and PCR (B) on sputum samples from single patient treated with isoniazid and rifampicin for 284 days. Last culture-positive specimen was on day 33 of treatment. Lanes (days after start of treatment): 1, 18; 2, 33; 3, 80; 4, 94; 5, 109; 6, 122; 7, 137; 8, 165; 9, 193; 10, 221; 11, 236; 12, 256; 13, 291; and 14, 382. Control lanes: 15, lysis control (10^3 cfu of Mycobacterium bovis bacille Calmette-Guérin); 16, processed water; 17, water; 18–20, water in A, no target in B; 21, 10 genomes of Mycobacterium tuberculosis H37Rv; 22, 1 genome of M. tuberculosis H37Rv; 23, 10 genomes of M. tuberculosis H37Rv without uracil DNA glycosylase (UDG) inhibitor in A, 10^5 deoxyuracil-containing amplicons in B; and 24 (B), 10^5 deoxyuracil-containing amplicons without UDG. In A, primer extension with Mtb15 generated products of 46 and 67 bases for signature molecule and 35 and 56 for IS6110; 67-mer product is not visible with this length of exposure. M. tuberculosis was detected in lanes 1-6, 8, 9, and 11; 35-mer products in lanes 6 and 9 and 46-mer product in lane 21 are clearly visible on original autoradiograph. In B, position of partially double-stranded hybrid formed between 123-bp IS6110-specific PCR product and LK229 probe is indicated; M. tuberculosis was detected in lanes 1–8 and 13.

results are consistent with another smaller study by Levee et al. [11] in which PCR was used to monitor therapy in 13 tuberculosis patients who received a 6-month course of treatment. All became smear- and culture-negative after 2 months, while 23% remained PCR-positive, with 8% remaining positive at 6 months. Kennedy et al. [10] reported that in successfully treated patients, a PCR assay was able to detect conversion from positive to negative 1–2 months after culture. The presence of amplifiable DNA over such prolonged periods among patients who have become culture-negative is not surprising in view of the exquisite sensitivity of DNA amplification systems for detection of low numbers of organisms. Microscopy requires ~10^4 bacilli/mL of sputum for reliable detection, while culture has been estimated to detect no fewer than 10^2 viable organisms/mL of sputum [13]. Both the assays used in the present study have analytical sensitivities of 1 genome of purified M. tuberculosis H37Rv DNA (14 copies of IS6110). In addition, we used a method of sample preparation that concentrated the DNA from 5–10 mL of sputum in a 30-μL volume, further enhancing the sensitivity of detection.

There are three possible sources of amplifiable M. tuberculosis DNA in these sputum specimens: viable bacilli, dead bacilli, and free DNA liberated into the sputum from degenerated organisms. Because of the low-speed centrifugation step in the routine NALC-NaOH sample processing procedure, it is unlikely that free DNA would be contained within the final NALC pellet. The principal source of DNA before the start of chemotherapy is viable M. tuberculosis bacilli, although within 2 weeks, >99% of the organisms are killed when a patient harboring an isoniazid-sensitive organism is treated with an isoniazid-containing regimen [22]. The irregular pattern of DNA-positive and -negative specimens from individ-
rial patients can be explained by the intermittent release of dormant or dead organisms from pulmonary lesions as well as variation in specimen quality. Alternatively, the sputum might contain long-lived macrophages that have escaped from healed, sterile, granulomatous foci and that carry DNA fragments from incompletely digested tubercle bacilli that were engulfed months or years earlier.

We have demonstrated a correlation between the clearance of DNA from sputum and the duration of smear and culture positivity, although it is unlikely that these findings are of clinical significance. Interpretation of the correlation between smear and DNA positivity was hampered by the detection of *M. tuberculosis* DNA in smear-positive culture-negative specimens and by the isolation of other acid-fast organisms from more than one-third of patients in the study. We were also unable to establish a relationship between the clinical extent of disease or host risk factors for tuberculosis and the duration of DNA persistence. Patients with minimal radiographic abnormality had no greater ability to clear *M. tuberculosis* DNA from their sputum than did those with much more extensive lesions. The absence of a correlation between *IS6110* copy number and the persistence of *M. tuberculosis* DNA is interesting in that the variable number of target elements present in different strains could be construed as a weakness in the assay systems. These data suggest that the presence of strains of *M. tuberculosis* with very few copies of *IS6110* is not a limiting factor in the sensitivity of these assays.

Important limitations of the present study are widespread variation among patients in the length of follow-up, provision of specimens at nonuniform intervals, and administration of a variety of different treatment regimens. Such weaknesses can be overcome only by prospective analysis over a period of years of a large cohort of patients matched with respect to demographics, treatment, and risk factors. However, in view of the data presented here, such an undertaking may be of little relevance in terms of patient management. The inability of DNA amplification to distinguish between viable and dead organisms, together with the absence of a quantitative methodology, makes it most unlikely that simple DNA amplification procedures will be useful in monitoring the response of patients to treatment. A recent study by Moore et al. [23] reported that the median time to last positive test was 23 days for culture and 40 days for the Gen-Probe Amplified Mycobacterium Tuberculosis Direct Detection Test. That test is based on amplification of rRNA but has a sensitivity for diagnosis relative to culture as low as 71% [24]. The highly labile nature of RNA makes it attractive as a potential indicator of bacterial viability [25], although this has not been applied to *M. tuberculosis* in a clinical setting. There is an urgent need to develop quantitative reverse transcription—mediated amplification systems for detection of rRNA and mRNA with sensitivities equivalent to those of current DNA assays if amplification technology is to substitute for conventional microbiology in monitoring treatment efficacy.

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**References**