Monitoring Therapeutic Efficacy by Real-Time Detection of *Mycobacterium tuberculosis* mRNA in Sputum

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**BACKGROUND:** Current laboratory methods for monitoring the response to therapy for tuberculosis (TB) rely on mycobacterial culture. Their clinical usefulness is therefore limited by the slow growth rate of *Mycobacterium tuberculosis*. Rapid methods to reliably quantify the response to anti-TB drugs are desirable.

**METHODS:** We developed 2 real-time PCR assays that use hydrolysis probes to target DNA of the IS6110 insertion element and mRNA for antigen 85B. The nucleic acids are extracted directly from concentrated sputum samples decontaminated with sodium hydroxide and N-acetyl-L-cysteine. We prospectively compared these assays with results obtained by sputum mycobacterial culture for patients receiving anti-TB therapy.

**RESULTS:** Sixty-five patients with newly diagnosed TB and receiving a standardized first-line anti-TB drug regimen were evaluated at week 2 and at months 1, 2, and 4 after therapy initiation. Both the DNA PCR assay (98.5% positive) and the mRNA reverse-transcription PCR (RT-PCR) assay (95.4% positive) were better than standard Ziehl–Neelsen staining techniques (83.1%) for detecting *M. tuberculosis* in culture-positive sputum samples. The overall agreement between culture and mRNA RT-PCR results for all 286 sputum samples was 87.1%, and compared with culture, the mRNA RT-PCR assay’s diagnostic sensitivity and specificity were 85.2% and 88.6%, respectively. For monitoring efficacy of therapy, mRNA RT-PCR results paralleled those of culture at the follow-up time points.

**CONCLUSIONS:** The continued presence of viable *M. tuberculosis* according to culture and results obtained by RT-PCR analysis of antigen 85B mRNA correlated clinically with resistance to anti-TB drugs, whereas the DNA PCR assay showed a high false-positive rate. This mRNA RT-PCR assay may allow rapid monitoring of the response to anti-TB therapy.

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*Mycobacterium tuberculosis* grows slowly, and its detection in clinical samples requires several weeks with standard culture techniques. This period may delay the microbiologic diagnosis of tuberculosis (TB), compared with the culture times required for most other bacterial infections. The slow growth of *M. tuberculosis* also delays the availability of results for drug-susceptibility assays, which may be necessary to guide therapy. The conversion of sputum culture results from positive to negative within the initial weeks to months of therapy is correlated with the sterilizing activity (i.e., killing of *M. tuberculosis* in tissues) of the anti-TB drug regimen and is considered the best predictor of treatment success (1). Effective treatment regimens rapidly decrease the number of viable *M. tuberculosis* organisms in sputum, with the number of cultivatable bacilli typically reduced by approximately 10-fold within the first 1–2 weeks (2). Because of the slow growth rate of *M. tuberculosis*, however, the results of cultures for samples obtained early in treatment, if such cultures were obtained, would not be available in a timely manner.

The selection of an anti-TB therapy is largely empirical. Patients are generally prescribed a standard first-line anti-TB regimen consisting of 4 antimicrobial agents. Treatment may be modified weeks or months later as the results of tests of antimicrobial-treatment become available. A rapid, reliable method that reflects effective anti-TB drug activity is extremely desirable. In recent years, tests based on nucleic acid amplification techniques have been developed for the direct detection of *M. tuberculosis* in clinical samples (3–9). Mo-

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5. Nonstandard abbreviations: TB, tuberculosis; MDR, multidrug-resistant; NCTLD, National Center of Tuberculosis and Lung Diseases; AFB, acid-fast bacilli; NaOH-NALC, sodium hydroxide and N-acetyl-L-cysteine; FAM, 6-carboxyfluorescein; MGB, minor-groove binder; OR, odds ratio; RT-PCR, reverse-transcription PCR.
Laboratory Monitoring of Tuberculosis Therapy

Molecular techniques have the potential to improve clinical care by dramatically reducing the time required for detection and may provide substantial savings in the overall costs of patient care (4,10). Because the half-life of bacterial mRNA is extremely short compared with rRNA or genomic DNA, assays that target mycobacterial mRNA better reflect mycobacterial viability (11). The ability of mRNA-based assays to distinguish viable from nonviable organisms suggests that such assays should also be useful in monitoring the efficacy of anti-TB therapy (12), and others have described their use in this context (12–16).

In 2006, a TB rate of 142 cases per 100 000 individuals was reported in the Republic of Georgia, an incidence that is among the highest rates in the WHO European region. Although the case-detection rate for new smear-positive TB cases has increased in recent years, the rate of treatment success remains low: only 73% for new smear-positive cases in the 2005 cohort (17). A major challenge for TB control is the high rate of multidrug-resistant (MDR) TB. In 2006, MDR TB strains caused 6.8% of new cases of active TB, whereas 26.4% of cases in previously treated individuals were caused by MDR strains (18–20). Approximately 25% of all TB cases are managed in Tbilisi, the capital city.

We have developed 2 real-time PCR assays that use hydrolysis probes to detect M. tuberculosis–specific DNA and mRNA directly in sputum samples. In a prospective study, we evaluated the performance of the assays for monitoring the response to anti-TB therapy and compared the results with those obtained by mycobacterial culture for patients referred to the National Center of Tuberculosis and Lung Diseases (NCTLD).

Materials and Methods

STUDY COHORT
The study cohort included new cases of pulmonary TB managed at the inpatient and outpatient departments of the NCTLD. Study participants were $\geq 15$ years of age and resided in Tbilisi. In accordance with the WHO definition, a new case was defined as a patient who had never had treatment for TB or who had taken anti-TB drugs for $<1$ month (21). All patients in this study were enrolled within the first or second day of treatment initiation. Each new TB case was diagnosed and recruited initially from clinical signs, symptoms, and a chest radiograph; all cases were confirmed by the growth of M. tuberculosis in sputum culture. Patients were enrolled in the study from January 2006 through June 2007. The study was approved by the Georgian NCTLD Ethics Committee, and all participants provided written informed consent before study entry. A questionnaire was used in accordance with current standard practices at NCTLD to assess clinical symp-
PHENOTYPIC TESTING FOR ANTIMYCOBACTERIAL SUSCEPTIBILITY

Antimicrobial testing for susceptibility to antimycobacterial drugs was performed using the absolute-concentration method on Lowenstein–Jensen agar slants (24). In brief, a mycobacterial suspension was prepared from the primary culture, and the turbidity was adjusted with sterile saline to 1 McFarland standard. A series of 10-fold dilutions were prepared, and 0.2 mL was inoculated onto media containing the first-line TB drugs streptomycin (4 mg/L), rifampin (40 mg/L), and ethambutol (2 mg/L). Media containing isoniazid (0.2 mg/L) were inoculated with 0.2 mL of a 100-fold dilution of the suspension. All inoculated sets were incubated at 37 °C for 28–42 days. Isolates resistant to both isoniazid and rifampin were defined as MDR TB.

NUCLEIC ACID EXTRACTION

Total nucleic acids were extracted from NaOH-NALC–decontaminated and concentrated sputum samples with the NucliSENS easyMAG system (bioMérieux). In brief, 200 μL of the mixture of NaOH-NALC–treated sputum sample and Lysis Buffer was placed in the instrument according to the default extraction protocol (25). Total nucleic acids were eluted in 55 μL of Elution Buffer (bioMérieux), and 5 μL of each extract was used for nucleic acid amplification. The recombinant plasmids were serially diluted in triplicate with pooled samples of M. tuberculosis–negative sputum before nucleic acid extraction. The human β-actin gene (ACTB) was amplified as an internal amplification control. The sequences of primers and fluorophore hydrolysis probes for ACTB and the real-time PCR protocol have previously been published (22).

REAL-TIME DNA PCR ASSAY

We used a real-time assay that targets the M. tuberculosis–specific IS6110 insertion element (26). In brief, 5 μL of extracted nucleic acid was added to 20 μL of a reaction mixture containing 0.8 μmol/L of each primer and 0.4 μmol/L fluorophore hydrolysis probe (final concentrations) and mixed with 25 μL of TaqMan Universal PCR Master Mix (Applied Biosystems). The thermocycling conditions were a 2-min degradation of the preamplified templates at 50 °C and then 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 60 s (22). Primers (MTB-IS6110-791F, 5′-TAA CCG GCT GTG GGT AGC A-3′; MTB-IS6110-864R, 5′-CGG TGA CAA AGG CCA GTG A-3′) and the fluorophore hydrolysis probe (MTB-IS6110-830MGB, 5′-CTG GGC AGG GGT TTT C-3′) were modified from those previously described (5, 27). Probes were dual-labeled with the reporter dye FAM (6-carboxyfluorescein) at the 5′ end and the quencher MGB (minor-groove binder) at the 3′ end. The assay was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

STATISTICAL ANALYSIS

Qualitative results were obtained for each sample tested, and PCR efficiencies were calculated as previously described (23). Assays of samples with quantification cycle values between 38 and 40 were repeated; results were considered positive if the repeat quantification cycle value was ≤38. Groups were compared with the χ², Fisher exact, and McNemar tests with the aid of Epi Info™ software (version 3.4; CDC, Atlanta, GA) or SAS (version 9.1; SAS Institute). Odds ratios (ORs), 95% CIs, and P values were calculated; P values ≤0.05 were considered statistically significant.

Results

Experiments were first performed to determine the assay limit of detection. The recombinant plasmid calibrators were spiked with pooled samples of M. tuberculosis–negative sputum. Plasmids covering the range of 0–1024 copies/reaction at 2-fold dilutions were included in the experiment, and assays of each dilution were performed in triplicate. The limits of detection for the 2 PCR assays for the IS6110- and 85B-specific fragments were the same: 4 copies/reaction (1000 copies/mL of sputum). Calibration curves are shown in Figs. 1 and 2 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol55/issue9. PCR efficiencies were 1.91 and 1.66 for IS6110–specific and 85B–specific targets, respectively. Both PCR assays were specific for M. tuberculosis strains, and samples spiked with mycobacteria
other than *M. tuberculosis* (including *M. avium-intracellularare*, *M. kansasii*, *M. scrofulaceum*, *M. paratuberculosis*, and *M. marinum*) were not detected by the PCR.

During the 1.5-year study period, 65 individuals with culture-confirmed TB were enrolled in the study. The study group included 36 males and 29 females, with a mean (SD) age of 36.5 (13.7) years. All enrolled patients tested seronegative for HIV, had newly diagnosed TB, and either had never previously taken TB drugs or had started TB drugs only within 2 days before recruitment into the study. Follow-up sputum samples were available at week 2 and months 1, 2, and 4 of recruitment into the study. Follow-up sputum samples collected at the time of patient recruitment.

ACTB was amplified in all nucleic acid extracts prepared from NaOH-NALC–decontaminated and concentrated sputum and spiked plasmid samples, indicating that no complete inhibition had occurred in the nucleic acid amplification reactions. For sputum samples collected before treatment and at each follow-up visit, total nucleic acids were tested by both the DNA PCR and the mRNA reverse-transcription PCR (RT-PCR) assays with hydrolysis probes. Of the 65 sputum samples collected at the recruitment visit, 54 were AFB smear positive, whereas 64 and 62 samples were positive by the DNA PCR assay and the mRNA RT-PCR assay, respectively. The diagnostic sensitivities of the DNA PCR assay (98.5%; OR, 13.04; 95% CI, 1.03–6.81; *P* = 0.025) and a lower diagnostic specificity (51.3% vs 88.6%; OR, 7.39; 95% CI, 3.99–13.82; *P* < 0.0001) than the mRNA RT-PCR assay, when results of mycobacterial culture were used as the standard (Table 2). The overall agreement between the culture and RT-PCR results was 87.1%, which was significantly better than between the culture and DNA PCR results (87.1% vs 70.3%; OR, 2.85; 95% CI, 1.82–4.47; *P* < 0.0001) (Table 2).

Conversion of assay results from positive to negative during therapy was used to indicate effective anti-TB therapy. At 2 weeks of therapy, culture and mRNA RT-PCR results were negative in 21 (32.3%) and 29 (44.6%) of the cases, respectively. After 1, 2, and 4 months of therapy, culture results were negative in 46 (63.0%), 55 (98.2%), and 36 (97.3%) of the cases, respectively. By comparison, the mRNA RT-PCR results were negative at the corresponding therapy times in 45 (72.3%), 48 (86.7%), and 34 (91.9%) of the cases. Results for the mRNA RT-PCR assay agreed with culture results at follow-up time points in 36 (55.4%) of the cases and converted to negative earlier than culture results in 13 cases (20.0%) and later than culture results in 13 cases (20.0%) (Fig. 1). Although these differences from the culture results were not statistically significant (*P* > 0.05), results for the mRNA RT-PCR assay appeared to be more diagnostically sensitive for monitoring treatment response at the 2-week follow-up but to be less sensitive thereafter. A higher false-positive rate was noticed in the late follow-up stage when the DNA PCR assay was used for therapy monitoring (Fig. 1).

### Table 1. Performance of AFB, DNA PCR, and mRNA RT-PCR tests for detecting *Mycobacterium tuberculosis* in culture-positive sputum samples collected at the time of patient recruitment.

<table>
<thead>
<tr>
<th>Test</th>
<th>Tested, n</th>
<th>Positive, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB (carbol fuschin stain)</td>
<td>65</td>
<td>54 (83.1)</td>
</tr>
<tr>
<td>DNA PCR</td>
<td>65</td>
<td>64 (98.5)</td>
</tr>
<tr>
<td>mRNA RT-PCR</td>
<td>65</td>
<td>62 (95.4)</td>
</tr>
</tbody>
</table>

### Table 2. Performance of DNA and mRNA real-time assays with hydrolysis probes for detecting *Mycobacterium tuberculosis*-specific DNA and mRNA in sputum samples, compared with results for mycobacterial culture.

<table>
<thead>
<tr>
<th>Real-time assay</th>
<th>C+ P+ (true positives), n</th>
<th>C+ P− (false negatives), n</th>
<th>C− P+ (false positives), n</th>
<th>C− P− (true negatives), n</th>
<th>Diagnostic sensitivity, %</th>
<th>Diagnostic specificity, %</th>
<th>Agreement with culture, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA PCR</td>
<td>120</td>
<td>8</td>
<td>81</td>
<td>81</td>
<td>93.8</td>
<td>51.3</td>
<td>70.3</td>
</tr>
<tr>
<td>mRNA RT-PCR</td>
<td>109</td>
<td>19</td>
<td>18</td>
<td>140</td>
<td>85.2</td>
<td>88.6</td>
<td>87.1</td>
</tr>
</tbody>
</table>

* C, culture; P, DNA or mRNA real-time PCR assay.
We then explored the relationships between time to conversion to negative during anti-TB therapy and antimicrobial-susceptibility patterns at treatment initiation. Of 59 non-MDR TB cases, 47 (79.7%) of the cultures turned negative within 1 month of therapy, whereas 5 (83.4%) of 6 MDR TB cases did not turn negative until after at least 2 months of therapy (OR, 19.58; 95% CI, 1.88 – 488.24; \( P < 0.0037 \), Fisher exact test) (Table 3). The trend was similar when an RT-PCR assay was used to follow the conversion on anti-TB therapy (OR, 15.00; 95% CI, 1.46 –370.50; \( P < 0.0086 \), Fisher exact test). These results, as determined by both culture and mRNA assays, indicated that patients with non-MDR TB tended to convert to negative more rapidly than those with TB caused by MDR isolates. The results of culture and the RT-PCR assay were not significantly different with respect to the time to conversion to negative (\( P > 0.5 \), Fisher exact test; Table 3).

**Discussion**

In this prospective study, we used *M. tuberculosis*–specific DNA PCR and mRNA RT-PCR assays to monitor the response to anti-TB therapy. Conversion of assay results from positive to negative during follow-up was considered an indicator of effective therapy. Results with the mRNA RT-PCR assay agreed with culture results at every time point after the initiation of therapy in 55.4% of the cases and predicted conversion earlier in 20.0% of the cases and later in another 20.0% of the cases. Validation of the performance of molecular assays by comparison with the results of a conventional culture may be associated with an artificially decreased diagnostic specificity. Nevertheless, the mRNA RT-PCR assay we have described may provide a rapid real-time tool for monitoring the efficacy of anti-TB therapy.

The emergence and spread of MDR and extensively drug-resistant strains of *M. tuberculosis* pose a serious threat to current anti-TB regimens (30). Current therapy involves the initial administration of 4 first-line drugs. The initial regimen is subsequently modified as necessary according to the results of antimicrobial-resistance assays, which may not become available for several months. Current laboratory methods for monitoring the efficacy of TB therapy rely on mycobacterial culture, and the slow growth of *M. tuberculosis* causes delays in obtaining results. Our data obtained with both culture and mRNA assays indicated that patients with resistance to single drugs tended to convert to negative more rapidly than those with TB caused by MDR isolates. This finding makes the mRNA assay attractive, especially in a population with a higher prevalence of MDR disease. DNA target–based molecular methods have not yet met the need for a more rapid test (10). Although these assays can shorten the time to verify the presence of *M. tuberculosis* in a clinical sample, they are not specific enough to be used as an index of “test of cure” because bacterial DNA may persist long after the bacteria have been killed (27).

Several groups of investigators have described efforts to use mRNA target–based assays to rapidly monitor treatment efficacy (12–16). The concentrations of *M. tuberculosis* mRNA decline after the initiation of therapy, as do counts of viable *M. tuberculosis* colonies, with 90% of patients becoming negative for both markers within 2 months of treatment (13, 14). The rapid disappearance of *M. tuberculosis* mRNA from sputum suggests that it is a good index of microbial viability and a useful marker for assessing the response to ther-

| Table 3. Relationship between time to conversion to negative and drug-resistance patterns. |
|------------------|----------|------------|---------------|--------------|
| Assay            | Drug resistance | Cases, n  | ≤1 Month, n (%) | ≥2 Months, n (%) |
| Culture          | MDR       | 6          | 1 (16.7)        | 5 (83.4)     |
|                  | Non-MDR   | 59         | 47 (79.7)       | 12 (20.3)    |
| RT-PCR           | MDR       | 6          | 1 (16.7)        | 5 (83.4)     |
|                  | Non-MDR   | 56*        | 42 (75.0)       | 14 (25.0)    |

* Three cases were excluded because they were culture positive but negative in the mRNA RT-PCR assay at the time of patient recruitment.
apy. In our study, we used a high concentration of gua-
 dine isothiocyanate in Lysis Buffer to stabilize M. tu-
 berculus mRNA in sputum samples immediately after
 they had been decontaminated and concentrated with
 NaOH-NALC. Our evaluation of 65 new TB cases
 showed that the results for the mRNA RT-PCR assay
 agreed well with culture results at most follow-up time
 points. Furthermore, the mRNA result turned negative
 in 13 cases (20.0%) at the first follow-up time point at
 week 2, while the culture result remained positive. Si-
lar to the culture results, the time to the conversion
 of the mRNA result to negative correlated with suscepti-
bilities to anti-TB drugs. Cases involving strains with
 only isoniazid resistance responded more quickly to
 anti-TB therapy than cases of MDR TB. The mRNA
 RT-PCR assay can be used as a rapid and real-time tool
 for assessing the clinical response to anti-TB therapy,
 replacing more time-consuming phenotypic assays of
 antimicrobial susceptibility.

Our RT-PCR protocol was designed to amplify
and detect mRNA only from concentrated and decon-
taminated sputum samples. Because the NucliSENS
easyMAG extraction system recovers total nucleic ac-
ids, we could not avoid residual background amplifica-
tion of DNA encoding the gene for the 85B antigen.
This result may explain a slightly delayed TB conver-
sion at later follow-up time points, compared with the
culture results. Techniques that can either specifically
recover mRNA or destroy residual DNA during extract-
tion may enhance the performance of the mRNA RT-
PCR assay (31, 32). Another potential way to enhance
performance is based on emerging evidence that cer-
tain host cytokine responses are associated with effec-
tive therapy (33–35). Combining assays to measure
both M. tuberculosis transcripts and changes in the host
response may ultimately prove even better for moni-
toring the response to anti-TB therapy (36–38).

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the intellectual content of this paper and have met the following 3 re-
quirements: (a) significant contributions to the conception and design,
acquisition of data, or analysis and interpretation of data; (b) drafting
or revising the article for intellectual content; and (c) final approval of
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