Direct Detection of Rifampin- and Isoniazid-Resistant *Mycobacterium tuberculosis* in Auramine-Rhodamine-Positive Sputum Specimens by Real-Time PCR

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Our objective was to evaluate the feasibility of a molecular assay based on a real-time PCR technique, carried out with a LightCycler instrument (Roche Biochemicals), to identify *Mycobacterium tuberculosis* bacilli and to detect rifampin and isoniazid resistance in DNA extracts from sputum samples. We studied three genes: *rpoB*, which is associated with rifampin resistance, and *katG* and *inhA*, which are associated with isoniazid resistance. A total of 205 sputum samples collected from 108 patients diagnosed with pulmonary tuberculosis with positive auramine-rhodamine-staining (AR) sputum samples, were tested. The sensitivities of the Light-Cycler PCR assay for the positive AR specimens was 97.5% (200 of 205) for *rpoB* and *inhA* genes and 96.5% (198 of 205) for the *katG* gene. For the total number of patients tested, the sensitivity was 100% (108 of 108 patients) for rifampin, whereas the sensitivity was 98.1% (106 of 108 patients) for isoniazid. Full agreement was found with the Bactec MGIT 960 method and the genotype inferred from the LightCycler data for rifampin. The phenotypic method for isoniazid reported 13 resistant strains (≥0.1 μg/ml). In seven (53.8%) strains there was a concordance between both methods, but we found that six (46.2%) strains reported as resistant by the phenotypic method were determined to be susceptible by real-time PCR. For the 75 strains reported as susceptible by the phenotypic method, the concordance with the LightCycler data was 100%. Our results demonstrate that rifampin-resistant *M. tuberculosis* could be detected in DNA extracted from auramine-rhodamine-positive sputum samples in a single-tube assay that took less than 3 h to perform for a collection of auramine-rhodamine-positive specimens obtained from patients with culture-documented pulmonary tuberculosis. Similarly, this occurs in half of the isoniazid-resistant *M. tuberculosis* DNA extracted from auramine-rhodamine-positive specimens.

Mycobacterium tuberculosis remains a serious public health threat. According to the most recent data reported by the World Health Organization, one-third of the world's population is infected with tuberculosis, and each year nearly two million people dye from this disease. If current control efforts are not massively expanded, tuberculosis will kill more than 40 million people over the next 25 years (27, 28). Early diagnosis, effective treatment, and successful cessation of transmission are major strategies in the control of tuberculosis.

Current treatment for tuberculosis is a multidrug regimen based on rifampin and isoniazid, the drugs most efficient against *M. tuberculosis* infection. Although use of the appropriate drug with full patient compliance is highly effective in curing pulmonary tuberculosis, the emergence of *M. tuberculosis* strains that are resistant to rifampin and isoniazid reduces the efficacy of standard treatment (9). This fact, and the association of tuberculosis with outbreaks (4, 6, 16), shows that rapid diagnosis of active tuberculosis and early detection of resistant strains are essential for effective patient management and implementation of infection control measures.

Due to the slow-growth of *M. tuberculosis* bacilli, delays in the detection of resistance strains can occur when conven-

tional phenotypic assays are used. Nucleic acid amplification-based techniques are potentially the most rapid and sensitive methods for detection, identification, and susceptibility testing and are theoretically able to provide a same-day diagnosis from clinical samples (10, 15, 17, 19). These methods can potentially reduce the diagnostic time from weeks to days (20).

The molecular basis of antitubercular drug resistance in *M. tuberculosis* is becoming clearer. More than 96% of rifampin-resistant strains have mutations in an 81-bp "core region" of the *rpoB* gene, which encodes the β subunit of the RNA polymerase (10, 21), and the majority of isoniazid-resistant strains have been found to contain mutations in codon 315 of the *katG* gene, which encodes the catalase-peroxidase (30), or mutations in the *inhA* ribosomal binding site (1). Different genotypic approaches have been developed for the detection of resistance in *M. tuberculosis* (5, 7, 18, 22).

In the present study we evaluate the use of a real-time PCR technique, using the LightCycler system (Roche Biochemicals), to identify *M. tuberculosis* bacilli and to detect rifampin and isoniazid resistance in DNA extracts from auramine-rhodamine-positive sputum samples obtained from tuberculous patients. We studied three genes—*rpoB*, which is associated with rifampin resistance, and *katG* and *inhA*, which are associated with isoniazid resistance—because previous studies demonstrated that the most frequent mutations found in ri-

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fampin- and/or isoniazid-resistant strains of *M. tuberculosis* in our region were in those genes (8).

MATERIALS AND METHODS

Clinical samples collection and processing. We processed 205 sputum samples obtained from 108 patients diagnosed with pulmonary tuberculosis according to the radiological and clinical criteria described by Catanzaro et al. (3), with documented positive auramine-rhodamine slides, attended at the HH UU Virgen del Rocío in Seville, Spain, between 2000 and 2001. A total of 85 samples had an acid-fast bacillus count of one to nine per ten fields in the smear, 97 samples had one to nine bacilli per field, and 23 samples had more than nine bacilli per field. The specimens were liquefied and decontaminated with an equal volume of *N*-acetyl-ʟ-cysteine and 2% NaOH, homogenized by centrifugal swirling, and then incubated for 15 min (14). The reaction was neutralized by adding 0.067 M phosphate-buffered saline (pH 6.8), which resulted in a final volume of 50 ml. The specimens were concentrated by centrifugation at $3,000 \times g$ for 15 min. The supernatant was discarded, and the sediment was resuspended in 5 ml of sterile water. Part of the sediment from each specimen was used to inoculate a Löwenstein-Jensen solid medium and to prepare a smear for auramine-rhodamine staining (AR), whereas the remaining portion was stored at −20°C until use for DNA extraction.

Löwenstein-Jensen slants were incubated at 37°C for 6 weeks and inspected weekly for growth. When growth was detected, a smear was prepared to confirm the presence of acid-fast bacilli from suspect colonies by Ziehl-Neelsen staining.

M. tuberculosis strains and drug susceptibility testing. We studied 88 clinical isolates retrieved from the processed sputum samples from 88 different patients. Isolates were identified by DNA probes (AccuProbe; Gen-Probe, Inc., San Diego, Calif.) for *M. tuberculosis* complex, *M. avium*, *M. gordonae*, and *M. kansasii*. The drug susceptibilities of all isolates were determined by the nonradiometric dilution method Bactec MGIT 960 (29).

Sample preparation. Purified DNA of *M. tuberculosis* H37Rv and from other seven *Mycobacterium* species were also used in the assay. The species were as follows: *M. bovis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. chelonae*, *M. marinum*. The different strains were grown on Löwenstein-Jensen slants and the DNA was prepared from one or two colonies by chloroform-isoamyl extraction as previously described (25). Between 0.1 and 1 ng of DNA was used as the template for each PCR assay, to mimic the concentration expected to occur in sputum samples.

The sputum samples were inactivated by heating at 80°C for 30 min, and 1-ml sputum samples were taken. The samples were extracted by DNA IV (Unipath S.A., Oxoid, Spain) method according to the instructions of the manufacturer.

After decontamination and before the DNA extraction, we added human chromosomal DNA (final concentration, 10 ng/μl) in all of the sputum samples tested to monitor the nucleic acid recovery during specimen preparation and the presence of PCR inhibitors by amplification with the β-globin primers GH20 and PC04. A sputum sample, smear and culture negative, was processed along with the rest of the sputa to control contamination during sample processing. No contamination was detected.

LightCycler real-time PCR. All DNA extracts were amplified with the primers used in conventional PCR, with the addition of the fluorescein and Red 640-labeled probes to the amplification mix. Four different pairs of the probes were used, rpo1 anchor-sensor that covered the region containing codons 526 and 531 of the *rpoB* gene (22), rpo2 anchor-sensor that covered the region containing codons 510 to 528 of the *rpoB* gene (23), kat1 anchor-sensor that covered the region containing codon 315 of the *katG* gene (22), and inh1 anchor-sensor that detected nucleotide substitution C209T in the regulatory region of the *inhA* gene (23). All primers and hybridization probes were synthesized by TIB MOLBIOL (DNA Synthesis Service; Roche Diagnostics, Berlin, Germany). The probes were designed to detect certain mutations based on previous studies (8, 24).

The components for PCR in a final volume of 20 μl included 2 μl of a commercial ready-to-use reaction mix for PCR (LightCycler-DNA master hybridization probes; Roche Diagnostics) that contains *Taq* DNA polymerase, reaction buffer, a deoxynucleoside triphosphate mix, and 10 mM MgCl₂. We added MgCl₂ to a final concentration of 4 mM. The primers and probes were added to final concentrations of 0.5 and 0.2 μM, respectively. Finally, we used 5 μl of the corresponding template DNA. The 20 μl (final volume) reaction mix was placed in glass capillary cuvettes, which were filled by pulse centrifugation in a microcentrifuge. In each set of experiments we always included negative as well as positive controls. The negative control sample was prepared by replacing the template DNA with PCR grade water. The positive control sample was prepared by adding 2 μl of genomic DNA from *M. tuberculosis* H37Rv (susceptible strain) to the reaction mix. Conditions for cycling were 95°C for 45 s, followed by 45

TABLE 1. Cycling numbers (Ct values) for Light Cycler amplification with *katG* gene assay versus auramine-rhodamine counts

Auramine-rhodamine count[a]	No. of samples	Ct					
		Mean	Median	Minimum	Maximum	SD	SE
1 to 9/10 fields	83	30.11	30.26	26.26	33.83	1.61	0.21
1 to 9/field	93	27.11	27.71	19.75	34.55	3.44	0.40
>9/field	22	23.91	22.62	19.79	29.64	3.51	0.85

[a] As determined by fluorescence microscopy (magnification, × 400).

cycles of 95°C for 1 s, 55°C (annealing temperature for the *rpoB* gene) and 60°C (annealing temperature for the *katG* and *inhA* genes) for 2 s, and 72°C for 6 s, with fluorescence monitoring during the annealing phase; this, in turn, was followed by a melting program of 50 to 85°C at 0.1°C/s, with continuous monitoring of the fluorescence.

For β-globulin gene amplification we used LC-Sybrgreen I method that included, in a final volume of 20 μl, 2 μl of a ready-to-use reaction mix for PCR (LightCycler-DNA Master Sybrgreen), with the same components as before and Sybrgreen I. We added MgCl₂ until a final concentration of 4 mM and primers GH20 and PC04 to a final concentration of 0.5 μM. The conditions for cycling were also the same except for the annealing temperature (55°C) and the fluorescence monitoring that was done at the end of the amplification phase. The melting program started at 50°C and ended at 95°C.

RESULTS

Analytical sensitivity and specificity. The sensitivity of the assay in water with mycobacterial DNA from the wild-type *M. tuberculosis* H37Rv or from a mutant isolate was 1 pg (corresponding to 200 mycobacterial genomes) for the *rpoB* primers and probes or 0.1 pg (corresponding to 20 mycobacterial genomes) for the *katG* and *inhA* primers and probes.

To determine whether the assay is specific for *M. tuberculosis*, we tested DNA that was isolated from seven different mycobacterial species. The results show that only *M. tuberculosis* and the closely related *M. bovis* (a member of the *M. tuberculosis* group, all of which cause tuberculosis) elicited a positive response for the *katG* and *inhA* probes. Although the *rpoB* probes gave positive results with all mycobacterial species, the melting temperature (T_m) were low enough to differentiate *M. tuberculosis* and *M. bovis*, with identical T_m values, from the remaining species (data not shown).

Genotypic assays by real-time PCR. Fluorescence measurements were 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 the exponential growth of the PCR product during the log-linear phase. Table 1 shows the CT values for the *katG* gene versus auramine-rhodamine counts to provide an idea of the analytical sensitivity of the assay.

Table 2 shows the detection of *M. tuberculosis* DNA by real-time PCR according to the auramine-rhodamine bacillus count in the smear. A total of 205 sputum samples collected from 108 patients diagnosed with pulmonary tuberculosis and with positive AR smears were tested. Two hundred samples (97.5%) were determined to be positive by LightCycler PCR assays when rpo1, rpo2, and the inh pair of probes were used, whereas with the kat1 pair of probes 198 (96.5%) specimens were determined to be positive by LightCycler PCR. Five samples from five different patients were determined to be nega-

TABLE 2. Detection of *M. tuberculosis* DNA from auramine-rhodamine-positive smears from sputum samples by real-time PCR

Acid-fast bacillus count[a]	No. of samples	*rpoB* PCR		*katG* PCR		*inhA* PCR	
		Positive	Negative	Positive	Negative	Positive	Negative
1 to 9/10 fields	85	84	1	83	2	84	1
1 to 9/field	97	94	3	93	4	94	3
>9/field	23	22	1	22	1	22	1
Total	205	200	5	198	7	200	5

[a] See Table 1, footnote *a*.

tive by LightCycler PCR when all pair of probes were used. These samples were tested a second time and remained negative. We believe this result was due to a failed DNA extraction, since each patient provided other specimens that were positive in other PCR assays, and the AR counts in the smear were one to nine per field (at a magnification of ×400) or more in four of them and one to nine per 10 fields in the other. Furthermore, positive results with β-globulin primers have been found in all of the samples, indicating the absence of PCR inhibitors in the specimens. Two culture-negative samples from two different patients that were determined to be negative by LightCycler PCR when the kat1 pair of probes were used were determined to be positive when rpo1, rpo2, and the inh1 pair of probes were used; this could be explained by a partial deletion of the *katG* gene in these two strains. These two patients had auramine-rhodamine bacillus counts of one to nine per 10 fields and one to nine per field in the smear (magnification, ×400), respectively, and no more specimens to be tested.

A comparison of amplification assays and culture results is shown in Table 2. *M. tuberculosis* was isolated from 159 AR-positive sputa; *M. kansasii* was isolated from 3 AR-positive sputa from the same patient and excluded from the study. In these three sputa, very low Tm values for the *rpoB* gene were found (55.1°C for the rpo1 probes and 55.9°C for the rpo2 probes) and no amplification with the *katG and inhA* genes was observed. These data are not included in Table 3. A total of 192 (96%) samples were found to contain strains susceptible to

TABLE 4. Results of susceptibility testing of 88 clinical isolates: comparison of PCR amplification assays with the Bactec MGIT 960 method

Drug and method	No. of isolates (%) that were:	
	Susceptible	Resistant
Rifampin		
MGIT	84 (95.5)	4 (4.5)
PCR with:		
rpo1 probe	85 (96.6)	3 (3.4)
rpo2 probe	87 (98.9)	1 (1.1)
Isoniazid		
MGIT	75 (85.2)	13 (14.8)
PCR with:		
kat1 probe	81 (92)	7 (8.0)
inh1 probe	88 (100)	0 (0)

rifampin, and 185 (93.4%) samples were found to contain strains susceptible to isoniazid (<0.1 μg/ml), according to its T_m as previously described (24). Eight (4%) samples were found to contain strains resistant to rifampin. A mutation at codon 516 of the *rpoB* gene was found in four of them, whereas a mutation at the region containing codons 526 and 531 of the *rpoB* gene was found in the remaining four samples. These eight rifampin-resistant samples were obtained from four patients. Resistance to isoniazid was directly detected in 13 (6.6%) samples collected from seven patients. All of them showed a mutation at the codon 315 of the *katG* gene.

Phenotypic assays by the nonradiometric dilution method. Of the 108 patients studied, 88 clinical isolates retrieved from the processed sputum samples of 88 different patients were tested (Table 4). Full agreement was found with the Bactec MGIT 960 method and the genotype inferred from the Light-Cycler data when the rpo1 and rpo2 pair of probes was used. The phenotypic method for isoniazid reported 13 resistant strains (≥0.1 μg/ml). In seven (53.8%) strains there was a concordance between both methods, but we found that six (46.2%) strains reported as resistant by the phenotypic method were determined to be susceptible by real-time PCR when the TB pair of probes and the inh pair of probes were used. For the

TABLE 3. Results for 205 respiratory specimens from 108 patients: comparison of PCR amplification assays with culture results

Patient status[a]	No. of samples	*rpoB* PCR			*katG* PCR			*inhA* PCR		
		Positive		Negative	Positive		Negative	Positive		Negative
		Resistant	Susceptible		Resistant	Susceptible		Resistant	Susceptible	
Smear positive, culture positive	159	8	146	5	13	141	5	0	154	5
Smear positive, culture negative (final diagnosis: TB)	31	0	31	0	0	29	2	0	31	0
Smear positive, contaminated culture (final diagnosis: TB)	15	0	15	0	0	15	0	0	15	0
Total	205	8	192	5	13	185	7	0	200	0

[a] TB, tuberculosis.

75 strains determined to be susceptible by the phenotypic method, the concordance with the LightCycler data was 100%.

Sensitivity and specificity of LightCycler real-time PCR. The sensitivity of the LightCycler PCR assay for the positive AR specimens was 97.5% (200 of 205) when the rpo1 and rpo2 pair of probes and the inh probes were used and 96.5% (198 of 205) when the TB pair of probes was used. For all 108 patients tested, the sensitivity was 100% with rpo1, rpo2, and the inh pair of probes, whereas the sensitivity was 98.1% (106 of 108) with the TB pair of probes.

The ability to detect true resistance or susceptibility and the specificity of the rifampin genotypic method were 100%, since this approach classified correctly all of the 88 strains tested by the Bactec MGIT 960 method. The ability to detect the true resistance and specificity of the isoniazid genotypic method were 53.8 and 100%, respectively. The positive predictive value and the negative predictive value of the isoniazid genotypic method were 100 and 92.6%, respectively.

DISCUSSION

Our results demonstrate that rifampin-resistant *M. tuberculosis* can be detected in DNA extracted from positive AR sputum samples in a single-tube assay that takes less than 3 h to perform. Similarly, this occurs in half of the isoniazid-resistant *M. tuberculosis* DNA extracted from auramine-rhodamine-positive sputum samples by using the *katG* and *inhA* probes. A major drawback of direct detection of rifampin-resistant and/or isoniazid-resistant *M. tuberculosis* in sputa by other molecular methods is the lack of sensitivity (11), but we have not found such problems because we extracted *M. tuberculosis* DNA after decontamination of the sputa by the *N*-acetyl-NaOH method, although this could be a problem with other types of clinical samples such as pleural fluid. For isoniazid resistance, the sensitivity of the method is only 53.8%. As with other genotype-based resistance prediction tests, a drawback in characterizing *katG* codon 315 mutations and *inhA* mutations, as well as *emb* mutations for ethambutol resistance, is that no prediction can be made for samples containing the nonmutated genotypes (19). Therefore, the diagnostic categories cannot be "resistant" and "sensitive" but must be "resistant" and "no prediction possible." For these reasons, it is open to debate whether a rapid resistance prediction test with high specificity but low sensitivity is useful. The answer may depend of the current alternative, which means waiting for several weeks to detect isoniazid resistance by culture in all affected patients rather that detecting half of these cases within few hours. In addition, the mutations at codon 315 of the *katG* gene have been associated with a high level of isoniazid resistance (26), and thus the positive results obtained by this assay are more clinically useful. The mutation detection allows for a rapid result for resistance but not for susceptibility, and this rapid result may have value for the physician; however, for the testing of other antimicrobial agents and for all samples that are negative for mutations, backup culture for susceptibility testing would still be required.

This method could be used in combination with any of the previously described methods to identify *M. tuberculosis* (12, 13, 15), and the simultaneous identification and detection of

rifampin and isoniazid resistance in AR-positive sputa is possible.

Our results demonstrate that it is possible to implement real-time PCR in the diagnostic laboratory for the routine detection of *M. tuberculosis* from sputa and probably from liquid medium cultures. Slide microscopy for the detection of acid-fast bacilli is a convenient and economical test used for the determination of *M. tuberculosis* infection. However, recent studies have shown that up to half of the new cases of tuberculosis are smear negative (2). Although we have not determined the clinical specificity of the assay because we have included only auramine-rhodamine-positive sputa, this method could be applied directly to acid-fast bacilli smear-negative sputa from patients diagnosed with tuberculosis, especially in patients showing poor treatment compliance and with clinical relapses, among whom there is a high probability of resistant *M. tuberculosis* strain isolation.

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