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Current advances in the leprosy research activities

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[Received: 12 Nov. 2004]

Key words : Leprosy, *Mycobacterium leprae*, Topics

Due to the advent of multi-drug therapy (MDT) recommended by the WHO, for the treatment of leprosy, presently, leprosy is regarded as a "curable disease". The number of new cases in Japan is relatively very low, due to which the disease is likely to be neglected, but on scientific grounds, there is a necessity to perform in depth studies. Leprosy caused by *M. leprae* is still unclear on various aspects including transmission, immunology, nerve damage etc. Here we introduce the recent advances in the field of basic leprosy research.

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CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 2005, p. 44–51 Vol. 12, No. 1
1071-412X/05/$08.00+0 doi:10.1128/CDLI.12.1.44–51.2005
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Use of Glycopeptidolipid Core Antigen for Serodiagnosis of *Mycobacterium avium* Complex Pulmonary Disease in Immunocompetent Patients

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Received 6 February 2004/Returned for modification 20 May 2004/Accepted 6 October 2004

We report the development of a serodiagnostic method for *Mycobacterium avium* complex (MAC) disease with an enzyme immunoassay (EIA) with the MAC-specific glycopeptidolipid (GPL) core as the antigen. In this study, we confirmed by EIA that the GPL core antibody was in the sera of immunocompetent patients with MAC disease. The EIA for quantifying the GPL core antibody was evaluated as a clinical tool for serodiagnosis of pulmonary MAC disease. A significant increase in GPL core antibodies (immunoglobulins G, A, and M) was detected in sera of patients with MAC pulmonary diseases when they were compared to patients who were colonized with MAC, patients with *Mycobacterium kansasii* disease or tuberculosis, and healthy subjects. The sensitivities and specificities of the GPL core-based EIA for diagnosis of MAC pulmonary disease were 72.6% and 92.2%, respectively, for IgG, 92.5% and 95.1%, respectively, for IgA, and 78.3% and 91.0%, respectively, for IgM. The best sensitivity and specificity were obtained by measuring immunoglobulin A antibodies against GPL core antigen. The level of GPL core antibodies reflected disease activity, since it decreased in cured MAC patients who had responded to chemotherapy. Measurement of serum antibodies against GPL core is useful for both diagnosis and assessment of disease activity in MAC disease of the lung.

About 10 to 20% of mycobacterial diseases are caused by nontuberculous mycobacteria. Among nontuberculous mycobacteria, *Mycobacterium avium* and *M. intracellulare* are closely related and commonly grouped to form the *M. avium* complex (MAC). The diagnosis of pulmonary MAC disease is based on a combination of clinical, radiographic, and microbiologic criteria and the exclusion of other diseases that are similar clinically (1). MAC organisms are of low pathogenicity and single positive specimens with low numbers of organisms are frequently recovered from individuals with no apparent disease. The colonization of asymptomatic individuals, the possibility of environmental contamination of specimens, and the absence of standardized skin test antigens for confirming nontuberculous mycobacterial disease all combine to complicate interpretation by physicians of diagnostic tests for nontuberculous mycobacteria. The development of a serodiagnostic test to detect MAC infection is necessary to rapidly and accurately diagnose pulmonary MAC disease.

In a previous study, we reported the characteristics of an enzyme immunoassay (EIA) for MAC pulmonary diseases with a mixture of glycopeptidolipid (GPL) antigens from 11 reference strains of MAC and applied the assay to serodiagnosis of patients with MAC disease (7). However, there are problems with the transition of the assay from a research tool to widespread clinical use. Specifically, preparation of GPL antigen of consistent quality as well as quantity from 11 reference strains of MAC is both time- and cost-consuming. Identification of a simple and stable antigen for use in serodiagnostic tests for MAC disease is necessary. In addition, the natural history of MAC lung disease is unpredictable in immunocompetent patients. Some patients are resistant to multiple drug chemotherapy and show persistent excretion of MAC organisms and a steady worsening of chest radiographic findings until death. Other patients maintain a stable clinical and radiographic picture for years (1).

We have been investigating the relationship between the serotype of MAC isolates and the long-term survival of patients with pulmonary MAC disease. However, it was difficult to accurately identify the serotypes of clinical isolates with the seroagglutination test and thin-layer chromatography (TLC). The GPLs from different clinical isolates are serologically cross-reactive and have similar R_f values on TLC (17). When we used serodiagnosis to identify antibody serotypes against the different types of GPLs in some pulmonary MAC disease patients, we detected antibody against all 11 GPLs in each serum. We hypothesized that the antibody to the GPL core could be the reactive component in the sera of pulmonary MAC disease patients rather than all patients infected by every MAC serotype. Our hypothesis was supported by analysis of GPLs that shows that the fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-alaninol-*O*-(3,4-di-*O*-methyl-Rha), or GPL core, is common to all serotypes (3). Because the GPL core is generally considered

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TABLE 1. Characteristics of the subjects in a study of EIA for diagnosis of pulmonary MAC disease[a]

Characteristic	Group				
	MAC disease[b]	MAC colonization[c]	*M. kansasii* disease	Tuberculosis	Healthy subjects
No.	106	11	30	77	126
Mean age (yr) ± SD	65.4 ± 10.4	60.5 ± 15.8	52.1 ± 12.7[d]	50.9 ± 18.6[d]	47.8 ± 14.3[d]
Age range (yr)	37–86	27–80	29–80	14–83	26–88
No. male/no. female	40/66	3/8	28/2[d]	66/11[d]	70/56[d]
Mean duration of disease (yr) ± SD	3.8 ± 4.3	0	0.5 ± 0.8[d]	0.5 ± 1.1[d]	0

[a] All subjects were either seronegative for HIV or had no clinical symptoms consistent with AIDS.
[b] MAC disease included *M. avium* (n = 49), *M. intracellulare* (n = 22), and unclassified strains of MAC (n = 35).
[c] MAC colonization included *M. avium* (n = 4), *M. intracellulare* (n = 3), and unclassified strains of MAC (n = 4).
[d] Statistically significant difference compared to patients with MAC disease ($P < 0.05$).

nonantigenic, it has received less attention than the serologically active polar GPLs that can be identified by immunoassay.

In the present study, we show that the immunodominant epitope of GPLs is the GPL core antigen and we assessed whether an EIA with GPL core antigen is a useful clinical tool for diagnosis of pulmonary MAC diseases. Because whole GPL antigens are not cross-serotypic, it is necessary to prepare a mixture of GPL antigens from different serotypes of MAC (7). By contrast, GPL core antigen is the dominant epitope and cross-reacts with serum antibodies obtained from patients with MAC disease due to different serotypes of the organisms. We have asked whether EIA serodiagnosis with GPL core antigen could differentiate pulmonary MAC disease from MAC colonization and pulmonary tuberculosis and EIA serodiagnostic results could aid in evaluating the effectiveness of the chemotherapy as well as the timing of the future cessation of treatment.

MATERIALS AND METHODS

Study subjects. Sera were obtained from patients with pulmonary disease due to nontuberculous mycobacteria (MAC and *M. kansasii*), pulmonary tuberculosis, individuals with MAC colonization, and healthy subjects (Table 1). They were aliquoted into individual 1.0-ml doses in tubes, stored at −80°C until use, and thawed at room temperature just before the assay. Culture isolates of mycobacteria were identified by biochemical analyses and DNA probes. MAC (n = 106) included *M. avium* (n = 49), *M. intracellulare* (n = 22), and unclassified strains (n = 35). MAC disease was diagnosed according to the criteria of the American Thoracic Society (1). Subjects with a small amount of bacteria in a single positive sputum culture but no symptoms and normal findings on the chest computed tomograph were categorized as being colonized with MAC. Healthy subjects had no history of mycobacterial diseases. There were no subjects that were known to be positive for human immunodeficiency virus type 1 or type 2.

Twenty-seven patients had initially received combination chemotherapy for pulmonary MAC disease with clarithromycin, rifampin, and ethambutol for more than 1 year and/or streptomycin for 2 months according to the recommendation of the American Thoracic Society. Patients whose cultures converted to negative after treatment and whose sputum remained negative on culture for 6 months were categorized as cured. Patients whose cultures did not convert to negative despite treatment were classed as treatment failures. Serum specimens had been obtained sequentially before and after the chemotherapy. Informed consent was obtained from all subjects. This project was approved by the Toneyama National Hospital institutional review board for human subject experimentation and complies with international guidelines for studies involving human subjects.

Preparation of GPL core antigen. The 11 reference strains of MAC obtained from the American Type Culture Collection were serotypes 1 (ATCC 15769), 4 (ATCC 35767), 6 (ATCC 35773), 7 (ATCC 35847), 8 (ATCC 35771), 9 (ATCC 35774), 12 (ATCC 35762), 13 (ATCC 35768), and 14 (ATCC 35761), 16 (ATCC 13950), and 20 (ATCC 35764) (5). After culture in Middlebrook 7H9 (Difco, Detroit, Mich.) for 3 weeks, mycobacteria were autoclaved and lyophilized. Lyophilized bacteria were extracted with chloroform-methanol. Alkali-stable lipids were applied to a silica gel column (Analtech, Newark, Del.) and GPLs were eluted with methanol-chloroform. The eluted GPLs were purified repeatedly by one-dimensional thin-layer chromatography (TLC) that was developed with chloroform-methanol-distilled water until a single spot was obtained (2, 3, 7). Subsequently reductive β-elimination of GPL was used to prepare GPL core (11, 15). Briefly, purified GPL was dissolved in ethanol-sodium hydroxide-$NaBH_4$. The reaction mixture was heated, neutralized with acetic acid, and then evaporated. The organic phase was washed, and the resulting GPL core was collected. Both the purity and molecular weight of the GPL core were examined by two-dimensional TLC and by fast atom bombardment-mass spectrometry (FAB-MS) (Jeol, Tokyo, Japan).

EIA. EIA was done with slight modifications of the previously published method (9). Briefly, microtiter plates (Nunc Products, Roskilde, Denmark) were coated with 0.5 μg of GPLs and GPL core of *M. avium* serotype 4/well. Serum samples were diluted 40-fold with phosphate-buffered saline containing 1% bovine serum albumin. Diluted serum samples were added, followed by incubation for 1 h at 37°C. Plates were washed, then peroxidase-conjugated F(ab')2 of goat antibody against human immunoglobulin G (IgG), IgA, or IgM (Sigma, St. Louis, Mo.) was added, and plates were incubated for 2 h at 37°C. Unbound labeled antibody was removed by washing and the substrate, *o*-phenylenediamine dihydrochloride (Sigma), was added. Following color development, the optical densities of the wells on the plates were read for absorbance at 492 nm in a reader (model 550, Bio-Rad Laboratories, Tokyo, Japan). To determine the presence of an immunodominant epitope, inhibition of EIA was done by addition of either the mixture of GPL (7) or GPL core antigen at concentrations ranging from 1 to 5 μg/well. All assays were performed in triplicate and without prior knowledge of the clinical status of the patient.

Statistical analyses. All data were analyzed with the statistical analysis software package StatView 5.0 (SAS Institute, Cary, N.C.). Antibody EIA titers in individual patients or patient groups were expressed as the mean ± standard deviation. Further comparisons of data from patient groups were made with analysis of variance and nonparametric analysis. Spearman's correlation coefficient by rank was used to determine the relationship between EIA titers and clinical parameters. The chi-square test was used to determine the relationship between gender and patient group. $P < 0.05$ was considered significant.

RESULTS

Purification of GPL core antigens from MAC strains. One-dimensional TLC analysis of GPL antigens prepared from three serotypes (serotype 4 *M. avium*, and serotypes 16 and 20 *M. intracellulare*) of MAC reference strains showed a single spot, but each had distinct patterns (Fig. 1A), because the oligosaccharides were different in each strain. The two-dimensional TLC analysis of GPL core antigens prepared from serotype 4 (Fig. 1B), serotype 16 (Fig. 1C), and serotype 20 (Fig. 1D) exhibited a single spot that was identical among the strains. These results are consistent with the previous report that the common chemical structure of GPL is composed of C-mycoside, or GPL core (3, 7).

In a subsequent step, purified GPL cores of three MAC strains obtained by two-dimensional TLC were analyzed by

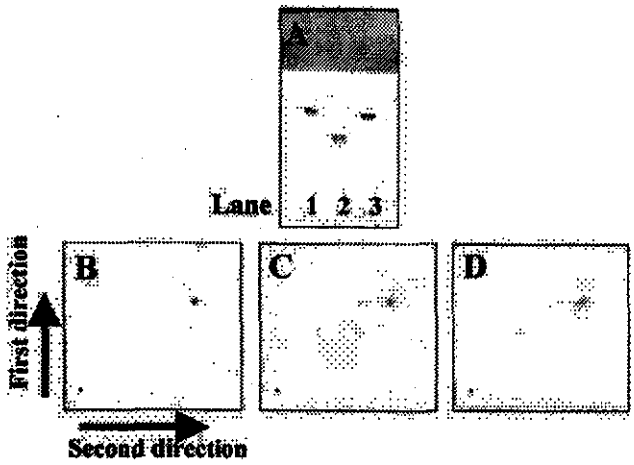

FIG. 1. One-dimensional (A) and two-dimensional TLC analysis (B to D) of GPLs and GPL core. Three reference strains of MAC were analyzed: serotypes 4 (ATCC 35767, lane 1), 16 (ATCC 13950, lane 2), and 20 (ATCC 35764, lane 3). GPLs were purified repeatedly by one-dimensional (1D) TLC developed with chloroform-methanol-distilled water until a single spot was obtained (A). Subsequently GPLs were β-eliminated to obtain GPL core, and then the purity of GPL core was examined by two-dimensional (2D) TLC (first in the vertical and second in the horizontal direction. B, serotype 4; C, serotype 16; D, serotype 20).

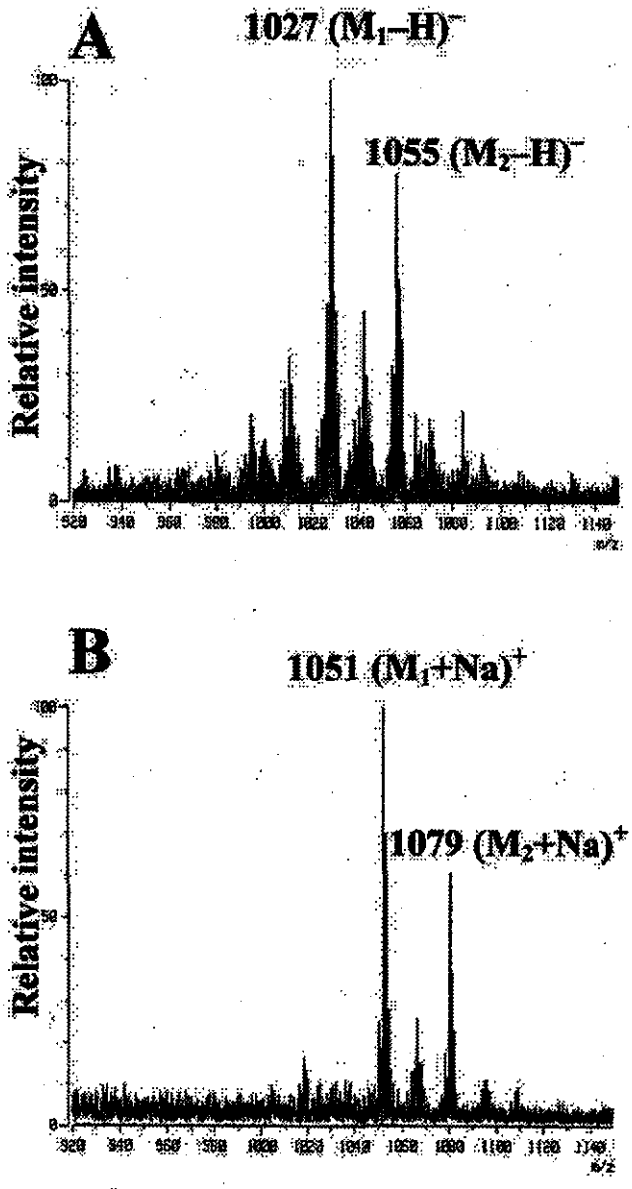

FIG. 2. FAB-MS of purified GPL core. GPL core of serotype 4 showed a main peak at an *m/z* of 1,027, corresponding to the (M-H)- ion in negative-ion mode (A) and an *m/z* of 1,051 corresponding to the (M+Na)+ in positive-ion mode (B). Based on these results, the molecular weight of GPL core of MAC serotype 4 was calculated to be 1,028.

FAB-MS to determine the molecular weight. The GPL core of serotype 4 showed a main peak at a *m/z* of 1,027 corresponding to the $(M-H)^-$ ion in negative-ion mode (Fig. 2A) and an *m/z* of 1051 corresponding to the $(M+Na)^+$ in positive-ion mode (Fig. 2B). Based on these results, the molecular weight of the GPL core of serotype 4 was calculated to be 1,028, which was consistent with the previous report (4). Similar results were obtained with serotypes 16 and 20 (data not shown).

Development of the EIA. We identified sera from pulmonary MAC disease patients with antibody against all 11 GPLs (Fig. 3). The titers of GPL antibody were reduced to background levels by adding GPL core antigens (5 μg/ml) to the assay for antibodies to every GPL, with the exceptions shown in Fig. 3. Antibodies to serotypes 1, 6, and 14 in patient A, serotype 1 in patient B, and serotypes 1, 14, and 20 in patient C were still detectable after adding core antigen. We considered the possibility that there was antibody against GPL core in the sera of pulmonary MAC disease patients and that the remaining antibodies after addition of the GPL core antigens might be to the serotype-specific oligosaccharide polar GPL antigens. We also examined the antigen specificity of the EIA by adding concentrations of GPL core antigens ranging from 1 to 5 μg to the assay for antibodies to either a mixture of GPLs prepared from 11 reference strains of MAC (five strains of *M. avium* and six strains of *M. intracellulare*) (7) or GPL core from MAC serotype 4 *M. avium*.

Sera were collected from five patients with pulmonary MAC disease (two patients with *M. avium* and three patients with *M. intracellulare*). Addition of GPL core inhibited the optical density levels in the EIA for IgG (Fig. 4A and 4D), IgA (Fig. 4B and 4E), and IgM (Fig. 4C and 4F) antibodies against both GPLs (Fig. 4A to 4C) and GPL core antigens (Fig. 4D to 4F) in a dose-dependent fashion (Fig. 4). Similar results were obtained by the addition of GPL core antigens purified from other serotypes of MAC, such as serotypes 16 and 20 (data not shown). These results point to the GPL core of MAC as having the immunodominant epitope of GPLs.

Levels of anti-GPL core IgG, IgA, and IgM antibodies in serum samples obtained from study subjects. The levels of IgG, IgA, and IgM antibodies against GPL core antigen was summarized in Table 2. IgG, IgA, and IgM antibody levels were significantly elevated in MAC disease patients but not in other patient groups ($P < 0.0001$). In the group of MAC disease patients, relationships between GPL core antibody levels of IgG and IgM and IgA and IgM were significant ($P <$

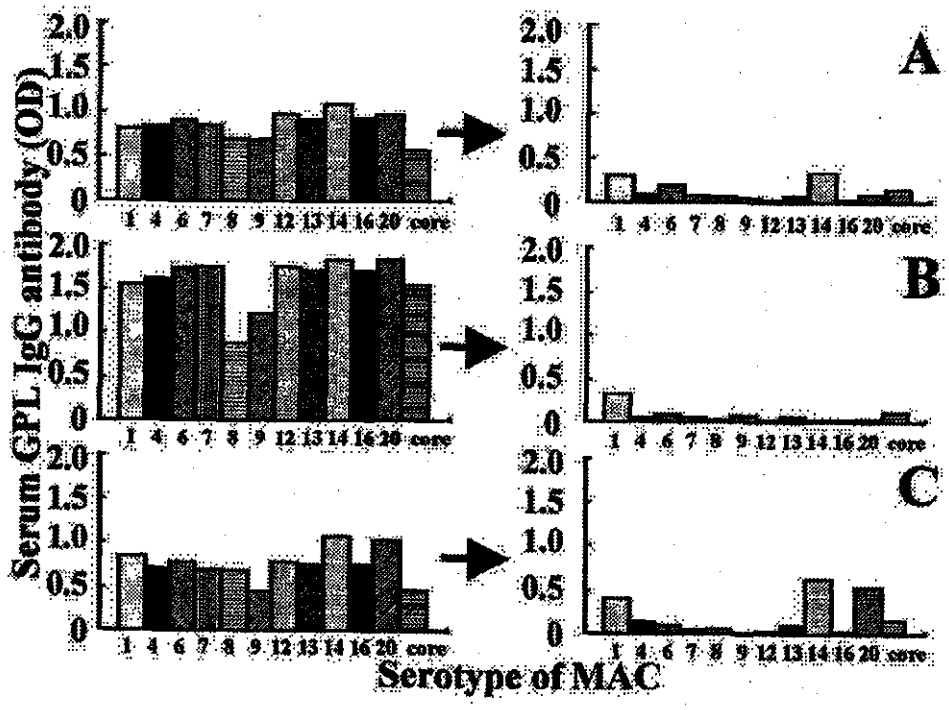

FIG. 3. Antibody titers to 11 serotypes of MAC and GPL core antigen in sera from three patients with MAC disease before and after adsorption with GPL core antigen. The serum titers before adsorption are shown in the left panel. After adsorption with 5 μg of GPL core antigen per ml, the titers of GPL antibody were reduced to background levels in most samples, with the exception of patient C, as shown in this figure. The titers of the three serum samples after adsorption are shown in the right panel.

0.05) but not IgG and IgA. No relationships were found between antibody levels and clinical parameters, including erythrocyte sedimentation rate, C-reactive protein, and the number of colonies after cultures, with the single exception of IgM antibody levels and erythrocyte sedimentation rate ($P < 0.05$).

Sensitivity and specificity of GPL core-based EIA for diagnosis of MAC disease. The cutoff levels, as defined with receiver operator characteristic (ROC) curves, were 0.064 for GPL core IgG, 0.072 for IgA, and 0.312 for IgM. The sensitivity and specificity of GPL core-based EIA for diagnosis of MAC pulmonary disease were 72.6% and 92.2%, respectively, for IgG, 92.5% and 95.1%, respectively, for IgA, and 78.3% and 91.0%, respectively, for IgM (Table 3). The best sensitivity and specificity for the diagnosis of MAC pulmonary disease was obtained by measuring IgA antibodies against GPL core. With national surveillance data for Japan, the prevalence of MAC pulmonary disease was estimated to be 2.45 cases per 100,000 population (13). The predictive values for positive results [true positive/(true positive + false positive)] and negative results [true negative/(true negative + false negative)] were 80.2% and 88.6%, respectively, (corrected values, 0.023% and 99.99%, respectively, by the prevalence rate) for IgG, 89.1% and 96.7%, respectively, (corrected values, 0.046% and 99.99%, respectively, by the prevalence rate) for IgA, and 79.0% and 90.6%, respectively, (corrected values, 0.021% and 99.99%, respectively, by the prevalence rate) for IgM.

Disease activity and level of GPL core antibodies. The levels of GPL core IgA antibody were quantified before implementation and after completion of antimicrobial chemotherapy for 27 patients with pulmonary MAC disease. The patients were divided into two groups according to the outcome of chemotherapy. There were 14 patients in the cured group and 13 patients in the treatment failure group. The GPL core IgA antibody levels before chemotherapy were the same in both groups. To determine whether the level of GPL core antibodies reflected MAC disease activity, we compared antibody levels sequentially in each patient before and after chemotherapy. In the cured patient group, the mean level of antibody before chemotherapy was 0.546 ± 0.369. After chemotherapy the mean level of antibody was 0.289 ± 0.293. In the treatment failure group, the mean level of antibody before chemotherapy was 0.704 ± 0.550 and after chemotherapy was 0.767 ± 0.429.

The mean IgA antibody titers in the cured group significantly decreased after chemotherapy ($P < 0.001$) but did not change in the treatment failure group (Fig. 5A). In the treatment failure group, a 61-year-old female patient had undergone lobectomy after chemotherapy. Her GPL core IgA antibody titer decreased rapidly after the surgery, and her sputum specimens converted to negative (Fig. 5B). No statistically significant differences were found between the cured and treatment failure groups for age, gender, duration of treatment, and timing of serum collection. Similar results were observed in changes of IgG and IgM antibodies to GPL core antigen before and after chemotherapy in both the cured ($P < 0.001$; IgG and IgM) and treatment failure groups (IgG, $P = 0.25$; IgM, $P = 0.55$).

DISCUSSION

In Japan, there are more than 30,000 new cases of mycobacterial diseases every year (incidence rate per 100,000, 25.8) and

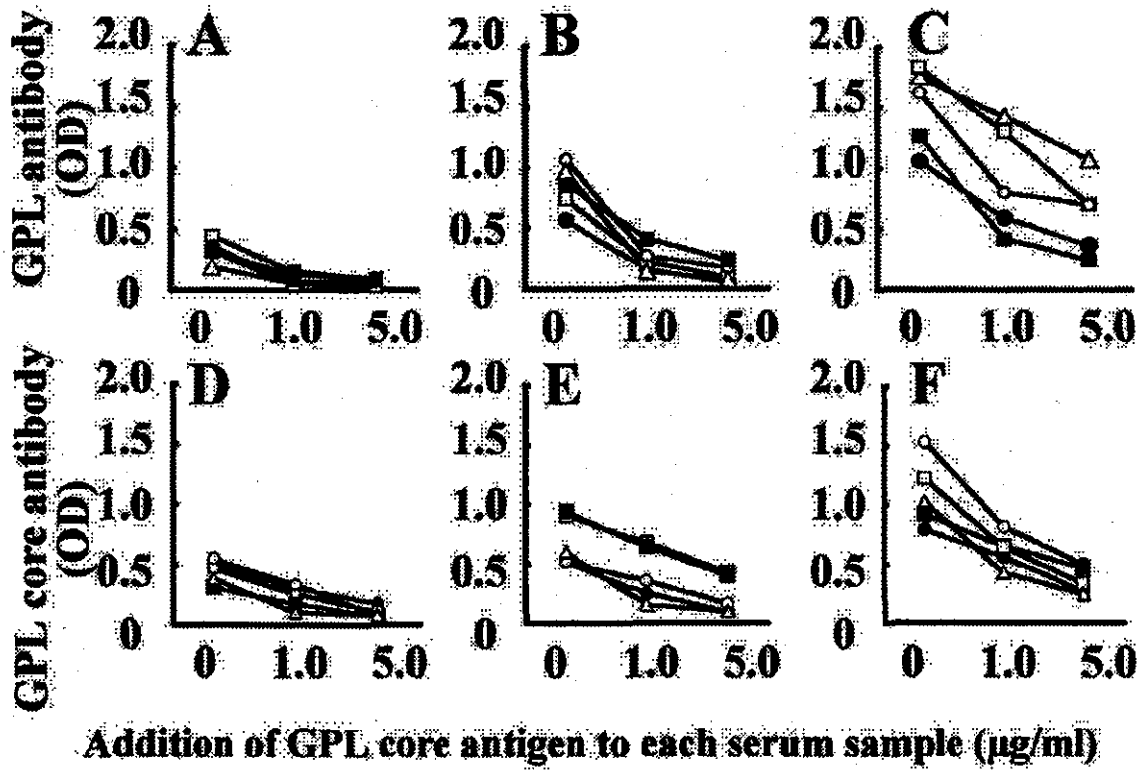

FIG. 4. Antigen specificity and recognition of an immunodominant epitope for GPLs. GPL core concentrations ranging from 1 to 5 μg/ml were added to the EIA with a mixture of GPLs prepared from 11 reference strains of MAC and GPL core of a serotype 4 strain. Sera were from five patients with MAC pulmonary disease, patient A (○), patient B (●), patient C (□), patient D (■), and patient E (△). Addition of GPL core antigen inhibited the level of optical density (OD) by IgG (panels A and D), IgA (panels B and E), and IgM (panels C and F) antibodies against GPLs (panels A, B, and C) and GPL core (panels D, E, and F) in a dose-dependent fashion.

about 20% of them in clinical practice are caused by nontuberculous mycobacteria such as MAC and *M. kansasii*. Of theses, 70% were MAC and 20% were *M. kansasii* (13). Because there are more than 500 patients with pulmonary disease due to nontuberculous mycobacteria in our hospital specializing in chest diseases and our previous study has demonstrated the availability of EIA with a mixture of GPL antigens for diagnosis of MAC pulmonary disease, we could conduct the present study.

The chemical structure of GPL is composed of a common GPL core, fatty acyl-D-Phe-D-allo-Thr-D-Ala-L-alaninol-*O*-(3,4-di-*O*-methyl-Rha), with the different oligosaccharide (polar GPL) moieties linked at the Thr substituent of the core. GPLs are the major cell surface antigens of slowly growing mycobacteria, such as MAC and *M. scrofulaceum*. There are 31 distinct GPL serotypes. In the present study, we report detection by EIA of an antibody against the purified MAC GPL core in sera of patients with pulmonary MAC disease. The GPL antibody levels were reduced in a dose-dependent fashion when different concentrations of GPL core were incorporated into the EIA. These results show that GPL core, the common component of all GPLs, has an immunodominant epitope in MAC strains. Although the present study has demonstrated that GPL core is the dominant epitope, there is a possibility that other components of GPLs, including oligosaccharide and oligosaccharide-GPL complex and whole GPLs, possess antigenicity. Indeed, serum antibodies to whole GPL antigen were incompletely adsorbed with GPL core with the serum of the

TABLE 2. Serum antibodies against GPL core antigen in patients with lung disease

Group	Titer[a]					
	IgG		IgA		IgM	
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI
MAC disease	0.281 ± 0.346	0.017–1.042	0.435 ± 0.385	0.051–1.599	0.714 ± 0.492	0.133–2.720
MAC colonization	0.026 ± 0.027*	0.005–0.104	0.021 ± 0.049*	0.006–0.143	0.164 ± 0.115*	0.038–0.472
M. kansasii disease	0.039 ± 0.015*	0.008–0.144	0.043 ± 0.054*	0.010–0.120	0.159 ± 0.131*	0.028–0.508
Tuberculosis	0.047 ± 0.031*	0.021–0.068	0.030 ± 0.018*	0.012–0.065	0.156 ± 0.113*	0.040–0.409
Healthy subjects	0.040 ± 0.028*	0.018–0.074	0.031 ± 0.042*	0.008–0.077	0.164 ± 0.092*	0.031–0.570

[a] CI, confidence interval. *, statistically significant difference compared to patients with MAC disease ($P < 0.0001$).

TABLE 3. Sensitivity and specificity of EIA for serodiagnosis of pulmonary MAC disease

Group	No.	IgG		IgA		IgM	
		Sensitivity (no. of seropositives)	Specificity (no. of seronegatives)	Sensitivity (no. of seropositives)	Specificity (no. of seronegatives)	Sensitivity (no. of seropositives)	Specificity (no. of seronegatives)
MAC disease	106	72.6 (77)	27.4 (29)	92.5 (98)	7.5 (8)	78.3 (83)	21.7 (23)
MAC colonization	11	9.1 (1)	90.9 (10)	9.1 (1)	90.9 (10)	18.2 (2)	81.8 (9)
M. kansasii disease	30	6.7 (2)	93.3 (28)	10.0 (3)	90.0 (27)	16.7 (5)	83.3 (25)
Tuberculosis	77	10.4 (8)	89.6 (69)	5.2 (4)	94.8 (73)	5.2 (4)	94.8 (73)
Healthy subjects	126	6.3 (8)	93.7 (118)	3.2 (4)	96.8 (122)	8.7 (11)	91.3 (115)

patient C (Fig. 3). Serotype-specific antisera were produced from rabbits by repeated immunizations with MAC (14). Thus, both the individual variability of humans and the species specificity in the immune response to antigens should be noted.

The recognition that there was an immunodominant antigenic epitope on the GPL core has minimized some of the problems of antigen preparation from an array of MAC strains. The GPL core based EIA described in this study had both high sensitivity and specificity when used to assay MAC-specific IgG, IgA, and IgM, and results were comparable to those previously obtained with mixtures of GPL antigens from 11 reference strains of MAC (9). It was likely that antibodies against both GPL core and polar GPL were being detected in the EIA when mixtures of GPL antigens were used since the addition of GPL core antigens reduced levels of GPL antibodies without reducing levels of GPL antibodies to serotype-specific oligosaccharide antigens. For the diagnosis of MAC pulmonary disease, the best sensitivity and specificity were obtained by measuring IgA antibodies against GPL core. The values for IgG antibodies against a mixture of GPL antigens were almost the same as IgA antibodies against GPL core. However both the ease and low cost of antigen preparation make the EIA for IgA with GPL core antigen a practical tool. Future studies would also benefit from the standardization of serum samples, as well as the methods for rapid and reliable serodiagnosis of MAC disease.

We previously reported the development of a rapid diagnostic EIA for tuberculosis, which is specific for antibodies to tuberculous glycolipid (9, 10). It was recently reported that the combination of lipoarabinomannan polysaccharide antigen, antigen-60, and tuberculous glycolipid appear to be the best choices as antigens for the serodiagnosis of tuberculosis (12). When we combined the results of three serodiagnostic tests which used lipoarabinomannan, antigen-60 and tuberculous glycolipid as antigens, the nontuberculous mycobacteria patients could not be differentiated serologically from tuberculosis patients because tuberculous glycolipid, lipoarabinomannan polysaccharide, and antigen-60 are common cell wall components of all acid-fast organisms, such as mycobacteria and nocardiae.

Nontuberculous mycobacteria cause a chronic, slowly progressive pulmonary infection resembling tuberculosis in immunocompetent hosts. MAC ranks first and *M. kansasii* ranks second among causes of human nontuberculous mycobacterial lung disease (6). We have used GPLs as an antigen for the differential serodiagnosis of pulmonary MAC disease and tuberculosis. This is feasible because GPLs are the major cell surface antigens of slowly growing mycobacteria, such as MAC and *M. scrofulaceum* (3). By contrast, *M. kansasii* and *M. tuberculosis* complex, including bacille Calmette-Guérin (BCG), do not have GPLs in their cell walls (3). In this study the low positive rate (Table 3) and low levels of serum GPL core antibodies in *M. kansasii* and tuberculosis patients confirmed that serodiagnosis by EIA with GPL core antigen could differentiate pulmonary MAC disease from both pulmonary tuberculosis and pulmonary nontuberculous mycobacterial disease caused by *M. kansasii*. Rapidly growing mycobacteria such as *M. chelonae* and *M. fortuitum* also have GPLs as major cell surface components but rarely cause pulmonary nontuberculous mycobacteria disease in humans (6).

MAC organisms are ubiquitous in nature. They have been isolated from water, soil, plants, house dust, and other environmental sources (6) and asymptomatic colonization of human hosts can occur. In this study, GPL core-based EIA could exclude MAC colonization as well as tuberculosis and *M. kansasii* disease because sera of individuals with MAC colonization have both a low positive rate and low antibody levels (Table 2, Table 3).

Despite the fact that most Japanese people (≥90%) have been given BCG (16), the rate of seropositivity for GPL core antibodies in healthy subjects is low (3.2 to 8.7%). One explanation is that GPL core-based EIA for MAC disease is not affected by prior vaccination with BCG, because GPLs are not present in *M. tuberculosis* complex (3). Although *M. tuberculosis* complex and *M. kansasii* do not have GPLs, a low positive rate (Table 3) and low levels of serum GPL antibodies in *M. kansasii* (6.7 to 16.7%), tuberculosis (5.2 to 10.4%), and healthy subjects (3.2 to 8.7%) could be detected. It is possible that latent subclinical infection with MAC leads to false-positive results since 7 to 12% of adults show evidence of subclinical MAC infection when tested for delayed-type skin reactivity to *M. avium* sensitin (18).

In our study, we were unable to determine the rate of subclinical infection with MAC because an appropriate test is not yet available in Japan. However, there are several possible explanations for the low levels of antibody against GPL core antigen. First, a low positive rate is directly related to the cutoff values defined by with the ROC curves. Second, subclinical infections with other nontuberculous mycobacteria, such as *M. chelonae* and/or *M. fortuitum*, must be excluded because such organisms possess GPL antigens in their cell surface (8), which may induce antibody production in the host. Third, a follow up of this antibody levels in these individuals needs to be done looking for individual variability according to the time. There are several possible explanations for the few false-negative patients (29 for IgG, 8 for IgA, and 23 for IgM); presence of

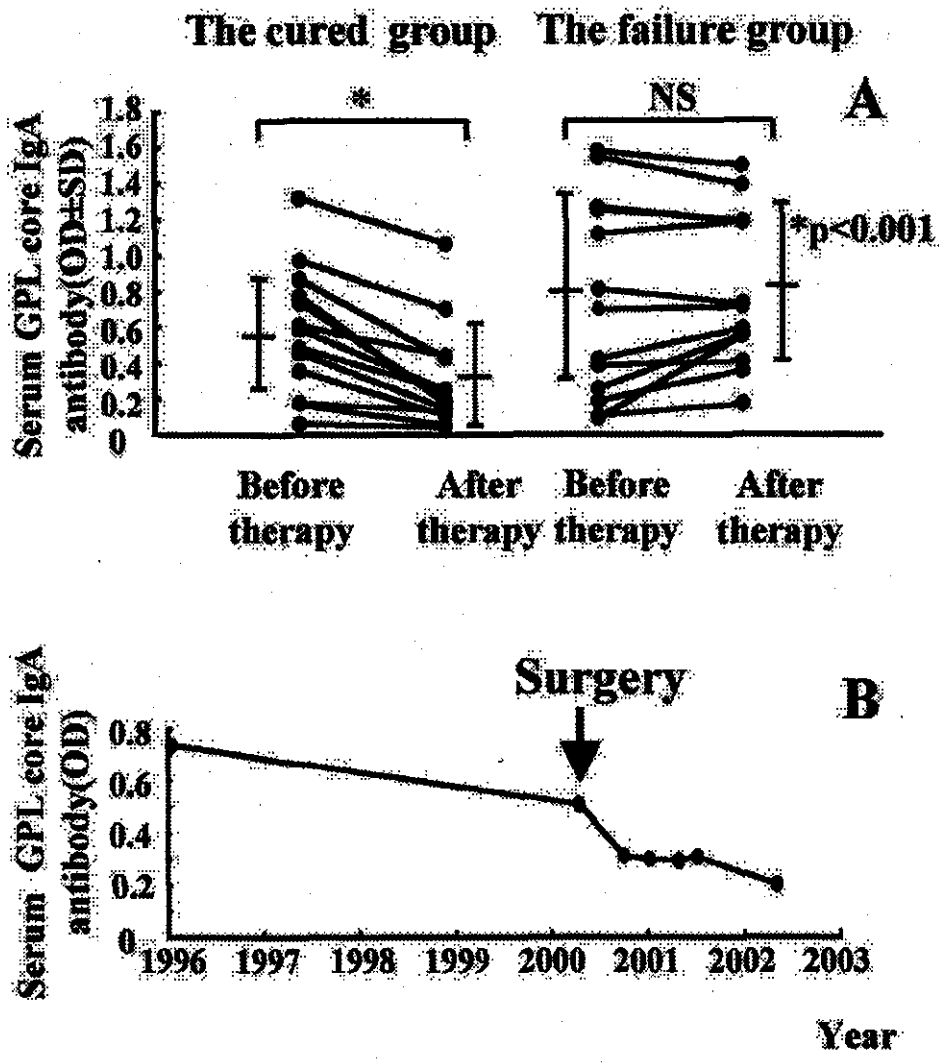

FIG. 5. Disease activity and level of antibodies to MAC GPL core. The levels of serum IgA antibodies to GPL core are shown before and after the completion of antimycobacterial chemotherapy for both the cured (14 MAC patients) and the failure groups (13 MAC patients). In the cured group, the culture results indicated conversion from positive to negative after successful chemotherapy, and in the failure group, the culture results indicated no conversion to negative despite treatment. All results are expressed as individual data (■), and the bars show the mean ± standard deviation for each group. The optical density levels decreased significantly (*, $P < 0.001$) in the cured group of patients but were not changed ($P = 0.381$) in the treatment failure group. The changes of serum IgA levels of to GPL core in a 61-year-old female MAC patient who had undergone lobectomy is shown in B. IgA levels decreased rapidly after the surgery, and sputum cultures converted from positive to negative.

circulating immune complexes, excess of GPL core antigens relative to antibodies, very low bacterial load, and recently diagnosed disease.

By obtaining EIA data before and after successful antimicrobial chemotherapy or surgery, we could demonstrate that the level of GPL core antibodies reflected disease activity of MAC infection. This result is consistent with our previous study on the level of GPL antibodies in MAC disease (7) and show that a merit of the assay is the ability to monitor disease activity. This finding is important because there is, as yet, no consensus as to when to discontinue chemotherapy for MAC disease (1). To validate the use of the EIA as an appropriate clinical tool for monitoring disease and scheduling treatment, data must be obtained in prospective, large-scale studies of active MAC disease.

The high sensitivity and specificity, combined with the simplicity, safety, and rapidity of obtaining results, point to the possibility that new avenues for serodiagnosis of MAC disease will become available with the introduction of the GPL core-based EIA. Results of serodiagnosis by EIA with MAC-specific GPL core antigen, when used in combination with acid-fast staining of sputum and culture confirmation and analyzed together with clinical, radiographic, and microbiologic criteria, may be a powerful tool for diagnosing MAC disease.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), Ministry of the Environment (Global Environment Research Fund), Ministry of Ed-

ucation, Culture, Sports, Science and Technology, Osaka City University (Urban Research Project), the United States-Japan Cooperative *Medical Science Program* against Tuberculosis and Leprosy, and the Grant-in-aid for Community Health and Medical Care from Ichou Association for Promotion of Medical Science.

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JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2004, p. 5277–5285 Vol. 42, No. 11
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.11.5277–5285.2004
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Dual-Probe Assay for Rapid Detection of Drug-Resistant *Mycobacterium tuberculosis* by Real-Time PCR

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Received 24 March 2004/Returned for modification 9 May 2004/Accepted 11 July 2004

Mutations in particular nucleotides of genes coding for drug targets or drug-converting enzymes lead to drug resistance in *Mycobacterium tuberculosis*. For rapid detection of drug-resistant *M. tuberculosis* in clinical specimens, a simple and applicable method is needed. Eight TaqMan minor groove binder (MGB) probes, which discriminate one-base mismatches, were designed (dual-probe assay with four reaction tubes). The target of six MGB probes was the *rpoB* gene, which is involved in rifampin resistance; five probes were designed to detect for mutation sites within an 81-bp hot spot of the *rpoB* gene, and one probe was designed as a tuberculosis (TB) control outside the *rpoB* gene hot-spot. We also designed probes to examine codon 315 of *katG* and codon 306 of *embB* for mutations associated with resistance to isoniazid and ethambutol, respectively. Our system was *M. tuberculosis* complex specific, because neither nontuberculous mycobacteria nor bacteria other than mycobacteria reacted with the system. Detection limits in direct and preamplified analyses were 250 and 10 fg of genomic DNA, respectively. The system could detect mutations of the *rpoB*, *katG*, and *embB* genes in DNAs extracted from 45 laboratory strains and from sputum samples of 27 patients with pulmonary TB. This system was much faster (3 h from DNA preparation) than conventional drug susceptibility testing (3 weeks). Results from the dual-MGB-probe assay were consistent with DNA sequencing. Because the dual-probe assay system is simple, rapid, and accurate, it can be applied to detect drug-resistant *M. tuberculosis* in clinical laboratories.

Tuberculosis (TB) presents a significant health threat to the world's population, with 8 million new cases of disease and 2 million deaths per year (36). To minimize the emergence and spread of drug-resistant TB, the basic principle of anti-TB treatment is to administer multiple drugs to which the organism is susceptible. Strains of *Mycobacterium tuberculosis* that are resistant to anti-TB drugs are being encountered with increased frequency (7). The major risk factors for drug resistance include inadequate prescription and delivery of chemotherapy, poor compliance, and an insufficient number of active drugs in the treatment regimen. The emergence of drug-resistant strains threatens our capability to control TB (2). Multidrug-resistant (MDR) *M. tuberculosis*, defined as simultaneous resistance to at least isoniazid (INH) and rifampin (RIF), is a serious problem. Strains of MDR *M. tuberculosis* appear to result from the stepwise acquisition of mutations in the genes encoding drug targets or drug-converting enzymes (10).

Rapid detection of susceptibility or resistance is crucial for TB treatment, because the initial choice of effective drugs is important. Major anti-TB drugs include INH, RIF, and ethambutol (EMB). Genetic studies have demonstrated that more than 95% of RIF resistance is associated with a mutation in the 81-bp core region of the *rpoB* gene (26, 31). RIF-susceptible mycobacteria do not possess the known mutations in core region of the *rpoB* gene. Therefore, mutations in the *rpoB* gene indicate that bacteria are RIF resistant. By contrast, INH-resistant strains exhibit mutations in several genes, such as *katG*, *inhA*, *oxyR*, and *ahpC* (22, 27, 30). The mutation of codon 315 (Ser) in the catalase-peroxidase (*katG*) gene is the most frequent site (30 to 65% of resistant strains) (4, 11). Furthermore, EMB-resistant strains have a point mutation at codon 306 (Met) in *embB* (3, 28), and the frequency is about 70% (3). Mutations of these sites are merely one mechanism leading to drug resistance. Many clinical isolates of drug-resistant *M. tuberculosis* do not bear mutations in these sites. Even if all of these mutations are examined, some resistant bacteria remain undetectable. However, the presence of mutations in the sites indicates that these bacilli are drug resistant (sensitivity, <100%; positive predictive value, 100%). In drug susceptibility testing, the culture method remains the "gold standard" (15). Since culture requires at least 2 weeks to obtain results (20), rapid diagnosis of drug resistance provides new opportunities for chemotherapeutic intervention in TB.

Because TaqMan minor groove binder (MGB) probes can distinguish one-base mismatches, the real-time PCR system in combination with MGB probes has been applied to analyze single-nucleotide polymorphisms (1, 12). It has been proven that the specificity of MGB probes is quite high (18). In the present study, we have developed a real-time PCR-based system with TaqMan MGB probes to detect the mutations asso-

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ciated with resistance of *M. tuberculosis* to INH, RIF, and EMB.

MATERIALS AND METHODS

Bacterial strains. To confirm the specificity of the real-time PCR system, we used DNAs extracted from *M. tuberculosis* H37Rv (ATCC 25618), *Mycobacterium bovis* (Ravenel), *M. bovis* BCG (Tokyo), *Mycobacterium africanum* (ATCC 25420), *Mycobacterium microti* (TC 77), *Mycobacterium avium* (ATCC 15769), *Mycobacterium intracellulare* (ATCC 13950), *Mycobacterium kansasii* (ATCC 12478), *Mycobacterium marinum* (ATCC 927), *Mycobacterium simiae* (ATCC 25275), *Mycobacterium asiaticum* (ATCC 25276), *Mycobacterium xenopi* (ATCC 19250), *Mycobacterium scrofulaceum* (ATCC 19981), *Mycobacterium gordonae* (ATCC 14470), *Mycobacterium malmoense* (ATCC 29571), *Mycobacterium shimoidei* (ATCC 27962), *Mycobacterium nonchromogenicum* (ATCC 19530), *Mycobacterium fortuitum* (ATCC 6841), *Mycobacterium abscessus* (ATCC 19977), *Mycobacterium tokaiense* (ATCC 27282), *Mycobacterium austroafricanum* (ATCC 33464), *Mycobacterium pulveris* (ATCC 35154), *Mycobacterium smegmatis* (ATCC 14468), and *Mycobacterium leprae* (Thai 53). *M. leprae* was a kind gift from M. Matsuoka, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan. In addition to mycobacteria, DNAs from *Klebsiella pneumoniae* (clinical isolate), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 29213) were used.

Drug-resistant clinical isolates and DNA isolation. Using the proportion method with Ogawa egg medium, drug resistance was defined as growth of at least 1% of the number of colonies that grew on drug-free medium at critical concentrations of the drugs (i.e., 40 mg of RIF per liter, 0.2 mg of INH per liter, and 2 mg of EMB per liter) (35). The present study examined 45 laboratory strains of RIF-resistant *M. tuberculosis* (resistant to RIF alone, 7 strains; resistant to RIF and INH, 12 strains; resistant to RIF and EMB, 9 strains; resistant to RIF, INH, and EMB, 17 strains). Genomic DNAs of mycobacteria were isolated from bacteria grown on Ogawa medium by combined chloroform extraction and mechanical disruption (16). DNAs from *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* were extracted with a QIAamp DNA Mini kit (Qiagen Inc., Valencia, Calif.). DNA concentration was estimated by UV absorbance at 260 nm.

Clinical samples and DNA preparation. We examined 27 clinical samples of sputum from patients with pulmonary TB. Drug susceptibility testing was performed as described in a previous study (35). A concentrated smear was prepared from specimens that were decontaminated by using the N-acetyl-L-cysteine–NaOH protocol (17). The basic fuchsin (Ziehl-Neelsen) staining procedure was employed. The DNAs were extracted by using the AMPLICOR respiratory specimen preparation kit (Roche Diagnostic Systems, Inc., Branchburg, N.J.) according to the manufacturer's instructions and were confirmed as *M. tuberculosis* complex with the COBAS AMPLICOR *M. tuberculosis* detection kit (Roche Diagnostic Systems, Inc.) (25).

PCR amplification and DNA sequencing. DNA sequences of *rpoB* (GenBank accession no. L27989), *embB* (U68480), and *katG* (X68081) were used for primer design. PCR was performed with each primer set (Table 1) as described previously (19). Both strands of PCR products of the *rpoB* and the *embB* genes were sequenced by using respective primers that were used in PCR. For DNA sequencing of the *katG* mutation sites, alternative sequencing primers were designed, because the amplicon (1,771 bp) was much longer than other genes (Table 1). Direct sequencing of PCR products was performed with a CEQ2000 automate sequencer (Beckman Coulter, Inc., Fullerton, Calif.) and a DTCS quick-start master mix kit (Beckman Coulter, Inc.).

Preparation of TaqMan MGB probes. The TaqMan MGB probes were designed to hybridize with wild-type DNA by using the Primer Express program (Applied Biosystems, Foster City, Calif.). The MGB probes were synthesized by Applied Biosystems. The primers and probes used in the present study are shown in Table 1. Four of eight probes were labeled with 6-carboxyfluorescein (FAM) (emission wavelength, 518 nm) and the remaining four probes were labeled with VIC (emission wavelength, 552 nm), because these two dyes emit luminescence of different wavelengths and can be distinguished individually in one tube.

Real-time PCR and nested PCR. The real-time PCR mixture was prepared in a final volume of 25 μl with 12.5 μl of Universal PCR Master Mix (Applied Biosystems), 25 pmol of primer, the optimal concentration of FAM- and VIC-labeled TaqMan MGB probes (Table 1), and 5 ng of purified DNA or 1 μl of extracted DNA. Real-time PCR analysis was performed with an ABI PRISM 7700 instrument (Applied Biosystems). The conditions of PCR amplification were 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed with Sequence Detector software (Applied Biosystems). Fluorescence of hybridized probes was expressed as ΔRn (normalized reporter signal). The number of amplification cycles required for emission of a certain luminescence intensity by each probe (ΔRn = 0.2) reflected the amount of DNA in the sample. This cycle number was called the threshold cycle (Ct). Therefore, the presence of a mutation would result in an increase in Ct.

Nested PCR was performed when signals were undetected in real-time PCR. The PCR was carried out in a 25-μl reaction volume. The reaction mixture contained 1× PCR buffer, 1 U of GC-rich enzyme mix (Roche Diagnostics Corp., Indianapolis, Ind.), a 200 μM concentration of each of the four deoxynucleoside triphosphates, a 1 μM concentration of three kinds of primer sets (Table 1), 1 M GC-rich solution, 1.5 mM $MgCl_2$, and 10 μl of template DNA. The primer pairs for the *rpoB*, *katG*, and *embB* genes amplified 534-, 464-, and 408-bp fragments, respectively. The PCR conditions were as follows: initial denaturation at 94°C for 5 min and then 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. After the first PCR, the amplified product was diluted 100-fold with sterilized water. One microliter of this solution was used for the real-time PCR analysis as described above.

IPC for detecting PCR inhibitors. For analysis of sputum samples from TB patients, an internal process control (IPC) was performed to detect PCR inhibitors PCR simultaneously. As IPC primers, each rpoB primer was added to the 5′ end of the corresponding lambda phage primer (Table 1). The identical sequence of the lambda phage IPC-R probe, which was 5′ labeled with FAM and quenched with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end, was used for analysis as described previously (13).

Electrophoresis. Amplification products were separated on 3% agarose gels with 1× Tris-acetate-EDTA buffer for 45 min at 100 V.

RESULTS

Real-time PCR. For rapid detection of mutations in the *rpoB*, *katG*, and *embB* genes involving *M. tuberculosis* resistance to RIF, INH, and EMB, respectively, three primer pairs and eight MGB probes were designed (Fig. 1 and Table 1). Five probes (rpo510/514, rpo514/520, rpo520/524, rpo524/529, and rpo529/533) were used for detection of mutations in the hot spot of *rpoB* (81 bp between codons 507 and 533 [equivalent to *Escherichia coli* numbering system {31}]). One probe (TB control probe) was designed outside the hot spot in *rpoB* as a control for determining the amount of DNA and for identifying *M. tuberculosis*. Polymorphisms in the 81-bp region of *rpoB* could be analyzed by using three tubes. One probe each for *embB* (codon 306) and *katG* (codon 315) was labeled with FAM and VIC, respectively. These probes were mixed with their four corresponding primers in one tube. Four tubes (three tubes for control and RIF resistance and one tube for INH and EMB resistance) were employed in the assay. Luminescence of all eight probes was detectable by real-time PCR with genomic DNA extracted from *M. tuberculosis* H37Rv as the template (Fig. 2A). When the DNA had mutations in *rpoB* at position 516 and in *katG* at position 315, the corresponding probes showed no luminescence signal (Fig. 2B). Typical results are shown in Fig. 2C and D. These results indicate that the probes used in this study can identify mutations of the target genes. Similar results were obtained by using autoclaved supernatants of *M. tuberculosis* suspensions instead of purified DNAs (data not shown).

Specificity and sensitivity. No luminescence was found when *M. avium* DNA (up to 50 ng) was analyzed in this system (Fig. 2E). In addition, luminescence was not detected even when more than 50 ng of DNA from other mycobacteria, such as *M. intracellulare*, *M. kansasii*, *M. marinum*, *M. simiae*, *M. asiaticum*, *M. xenopi*, *M. scrofulaceum*, *M. gordonae*, *M. malmoense*, *M. shimoidei*, *M. nonchromogenicum*, *M. fortuitum*, *M. abscessus*, *M. tokaiense*, *M. austroafricanum*, *M. pulveris*, *M. smegmatis*, and *M. leprae*, was used as the template. The presence of sufficient amounts of DNA in the system was confirmed by

TABLE 1. Designed primers and probes used in this study

Primer or probe	Target	Conc (μM)	Sequence[a]	Product size (bp)
Real-time PCR				
PCR primer	*rpoB*	1.0	5′-ACACCGCAGACGTTGATCA-3′	363
		1.0	5′-CTAGTGATGGCGGTCAGGTAC-3′	
	embB	1.0	5′-CGTGGTGATATTCGGCTTCCT-3′	130
		1.0	5′-GCCGAACCAGCGGAAATAG-3′	
	katG	1.0	5′-TGGGCTGGAAGAGCTCGTAT-3′	105
		1.0	5′-GGAAACTGTTGTCCCATTTCG-3′	
MGB probe	TB control	0.1	5′-FAM-TCTTCGGCACCAGC-MGB-3′	
	rpo520/524	0.1	5′-VIC-TCAACCCCGACAGC-MGB-3′	
	rpo510/514	0.08	5′-FAM-CCATGAATTGGCTCAGC-MGB-3′	
	rpo514/520	0.06	5′-VIC-TTCATGGACCAGAACAA-MGB-3′	
	rpo529/533	0.12	5′-FAM-CAGCGCCGACAGT-MGB-3′	
	rpo524/529	0.04	5′-VIC-TGACCCACAAGCGC-MGB-3′	
	emb306	0.08	5′-FAM-CTCGGGCCATGCC-MGB-3′	
	kat315	0.04	5′-VIC-CACCAGCGGCATC-MGB-3′	
Nested PCR				
	rpoB	1.0	5′-GGGAGCGGATGACCACCCA-3′	534
		1.0	5′-TGTAGTCCACCTCAGACGAG-3′	
	embB	1.0	5′-CTGAAACTGCTGGCGATCAT-3′	408
		1.0	5′-CAGGCGCATCCACAGACT-3′	
	katG	1.0	5′-GAGCCCGATGAGGTCTATTG-3′	464
		1.0	5′-CTCTTCGTCAGCTCCCACTC-3′	
Internal process control				
IPC primer	*rpoB*/lambda	1.0	5′-ACACCGCAGACGTTGATCATTTCCGGGACGTATCATGCT-3′	187
		1.0	5′-CTAGTGATGGCGGTCAGGTACACCGCTCAGGCATTTGCT-3′	
IPC probe	Lambda	0.2	5′-FAM-TCCTTCGTGATATCGGACGTTGGCTG-TAMRA-3′	
DNA sequencing				
PCR and sequence	*rpoB*	1.0, 0.2	5′-ACCGACGACATCGACCACTT-3′	528
		1.0, 0.2	5′-GGCGGTCAGGTACACGATCT-3′	
	embB	1.0, 0.2	5′-CGACCACGCTGAAACTGCT-3′	561
		1.0, 0.2	5′-CGTTGTTGAACGGCATCCAC-3′	
PCR	*katG*	1.0	5′-CAACCGGCTCAATCTGAAGG-3′	1,771
		1.0	5′-CCACCTACCAGCACCGTCAT-3′	
Sequence		0.2	5′-GCAGATGGGGCTGATCTACG-3′	
		0.2	5′-AACTCGTCGGCCAATTCCTC-3′	

[a] Each TaqMan MGB probe was labeled with either FAM or VIC at the 5′ end and with both a nonfluorescent quencher and an MGB at the 3′ end. The IPC probe was labeled with FAM at the 5′ end and TAMRA at the 3′ end. The sequences of IPC primers in boldface correspond to the lambda phage DNA sequences (13).

alternative PCR with mycobacterial 16S rRNA gene primers (6) or *dnaJ* gene primers (29) (data not shown). As expected, we detected no luminescence with DNAs prepared from *K. pneumoniae*, *P. aeruginosa*, and *S. aureus*. By contrast, the other members of *M. tuberculosis* complex, such as *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti*, exhibited similar luminescence in the real-time PCR system (data not shown). Therefore, the specificity of this system for the *M. tuberculosis* complex was sufficiently high.

By using purified genomic DNA from *M. tuberculosis* H37Rv, the sensitivity of this system was determined. In direct real-time PCR with the *rpoB* primer set and TB control probe, 100 fg of genomic DNA could be detected. Therefore, the mutation was detectable efficiently in the presence of 250 fg (Ct = 37) of TB genomic DNA. The detection limit of the real-time PCR was 10 fg of DNA when combined with nested PCR (data not shown).

Assessment of real-time PCR. We extracted and sequenced genomic DNAs from 45 laboratory strains of RIF-resistant *M. tuberculosis*. They were classified into 20 groups based on their genotypes by nucleotide sequencing of *M. tuberculosis* DNAs (Table 2). The luminescence intensity of TaqMan MGB probes in the real-time PCR amplification system was expressed as Ct. The Ct was higher when mutations were present in the genes. Using 45 RIF-resistant strains, we measured the Ct derived from an internal TB control probe bound outside the hot spots of the *rpoB* gene and ΔCt, which expressed the difference between the control and each MGB probe (Table 2). It was found that the ΔCts of probes hybridizing with the region without mutations were low (from −2.44 to 1.61) and that the

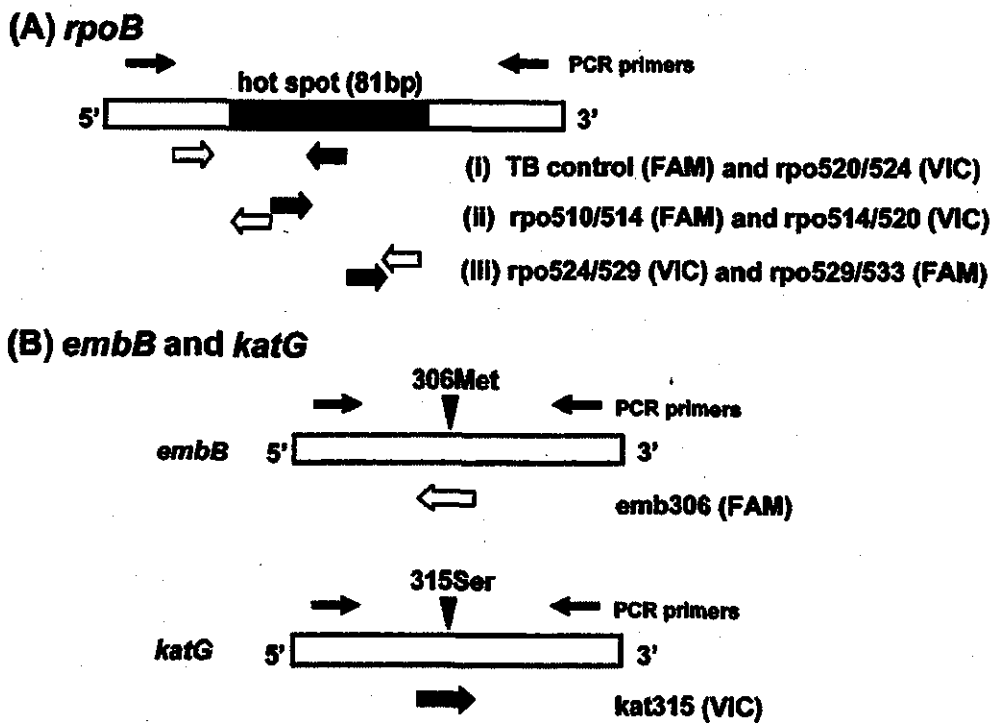

FIG. 1. Design of TaqMan MGB probes for detection of mutations in the *rpoB*, *embB*, and *katG* genes. MGB probes were labeled with two different dyes (FAM or VIC). The DNA sample and PCR primers for *rpoB* amplification were mixed with each set of probes in three different tubes: (i) TB control and rpo520/524, (ii) rpo510/514 and rpo514/520, and (iii) rpo524/529 and rpo529/533 (A). The emb306 and kat315 probes and *embB* and *katG* PCR primers were mixed and reacted in another tube (B). The sequences of primers and probes are listed in Table 1.

ΔCt was higher (≥7) when mutations existed in the target DNA that should hybridize with the MGB probe. These results suggest that a ΔCt of more than 7 was associated with a mutation in the nucleotide sequence. No strains that had mutations within the codons 510 to 514 of the *rpoB* gene were found. To check to the specificity of the rpo510/514 probe, the *rpoB* gene was cloned and mutations were constructed at codons 511 (CTG→CCG) and 513 (CAA→CTA). Real-time

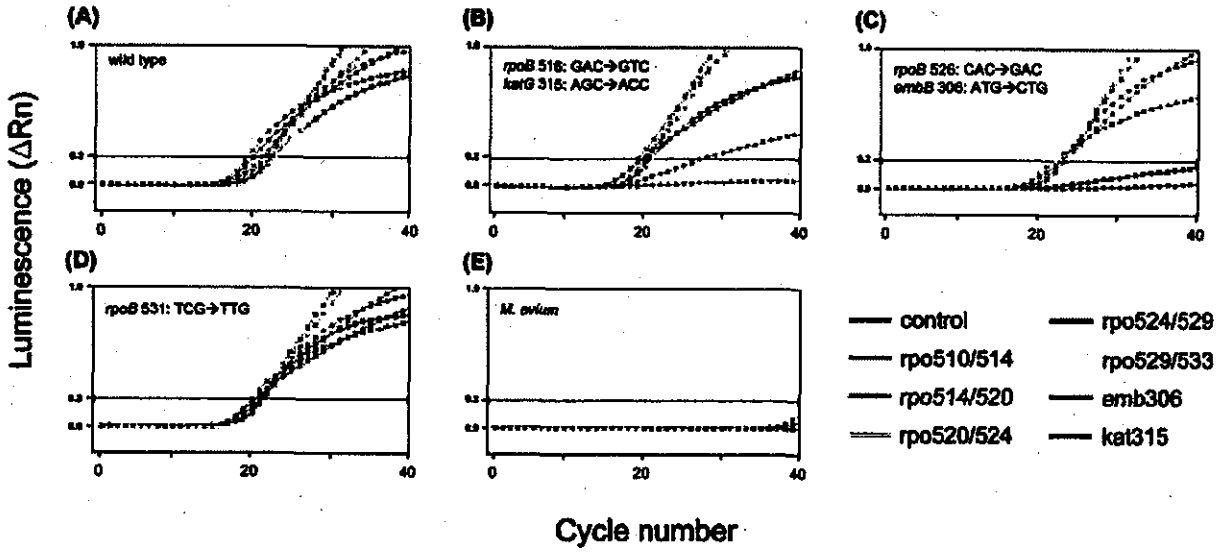

FIG. 2. Analysis of DNAs from mycobacteria with eight TaqMan MGB probes by real-time PCR. The templates were genomic DNAs extracted from *M. tuberculosis* H37Rv (A) as a control, certain mutants of *M. tuberculosis* (B, *rpoB* and *katG*; C, *rpoB* and *embB*; and D, *rpoB*), and *M. avium* (E). The *x* axis shows cycle numbers of PCR, and the *y* axis represents the normalized reporter signal (ΔRn). A horizontal line indicates the threshold (ΔRn = 0.2). The Ct is expressed as the number of cycles to reach the threshold.

TABLE 2. Luminescent patterns of real-time PCR with DNAs from 45 laboratory strains of RIF-resistant *M. tuberculosis*[a]

Mutant position[b]	No. of strains	Control Ct[c] (mean ± SD)	ΔCt[d] (mean ± SD)						
			rpo510/514	rpo514/519	rpo520/524	rpo524/529	rpo529/533	emb306	kat315
wt[e]	7	21.33 ± 0.89	0.19 ± 1.22	−0.48 ± 0.82	−0.42 ± 0.15	0.65 ± 0.68	1.11 ± 0.85	−0.82 ± 1.22	−1.69 ± 0.98
rpoB 516, GAC→GTC	1	20.67	−0.25	**8.11**	0.02	1.14	1.61	0.31	−0.45
rpoB 516, GAC→GTC *katG* 315, AGC→ACC	7	21.43 ± 1.38	0.89 ± 0.49	**7.19 ± 0.33**	−0.27 ± 0.27	0.84 ± 0.38	1.09 ± 0.55	−1.05 ± 0.40	**>17.19**
rpoB 516, GAC→GTC *rpoB* 522, TCG→TTG *katG* 315, AGC→ACC	1	22.18	−0.57	**>17.82**	**12.96**	0.79	0.94	−1.08	**>17.82**
rpoB 520, CCG→TCG *rpoB* 522, TCG→TTG	1	20.53	0.64	−0.35	**14.47**	0.64	0.85	−0.89	−1.74
rpoB 520, CCG→TCG *rpoB* 522, TCG→TTG *katG* 315, AGC→ACC	1	22.29	0.25	−0.34	**16.06**	0.46	0.76	−1.62	**>17.71**
rpoB 522, TCG→TTG	1	22.36	−0.37	−0.43	**15.58**	0.37	0.54	−1.78	−2.44
rpoB 526, CAC→TAC *katG* 315, AGC→ACC	1	23.20	−0.55	−0.70	−0.43	**>16.80**	−0.21	−1.25	**>16.80**
rpoB 526, CAC→CGC *katG* 315, AGC→ACC	1	19.33	0.02	−1.04	−0.24	**>20.67**	−0.36	−1.16	**>20.67**
rpoB 526, CAC→CGC *embB* 306, ATG→ATA	1	22.87	−1.11	−0.59	−0.29	**>17.13**	−0.51	**>17.13**	−2.12
rpoB 526, CAC→GAC *embB* 306, ATG→CTG	1	23.09	−0.49	−0.37	−0.60	**>16.91**	−1.24	**>16.91**	−1.25
rpoB 526, CAC→AGC *embB* 306, ATG→GTG *katG* 315, AGC→ACC	1	23.44	−1.14	−0.54	−0.40	**>16.56**	−0.40	**>16.56**	**>16.56**
rpoB 531, TCG→TTG	11	21.73 ± 1.77	0.22 ± 1.18	−0.28 ± 0.33	−0.31 ± 1.88	0.05 ± 0.32	**>16.50**	−0.84 ± 1.12	−1.55 ± 1.34
rpoB 531, TCG→TGG	1	19.10	1.00	0.47	−0.28	0.78	**20.90**	0.23	−0.53
rpoB 531, TCG→TTG *katG* 315, AGC→ACC	2	20.89 ± 0.76	0.20 ± 0.59	−0.33 ± 0.33	0.07 ± 0.10	0.11 ± 0.32	**>18.36**	−0.96 ± 0.42	**>18.36**
rpoB 531, TCG→TTG *embB* 306, ATG→ATC	2	21.77 ± 1.16	0.86 ± 0.24	−0.04 ± 0.11	−0.33 ± 0.14	0.37 ± 0.20	**>17.07**	**>17.07**	−0.78 ± 0.16
rpoB 531, TCG→TTG *embB* 306, ATG→GTG	3	20.90 ± 0.47	0.63 ± 0.65	−0.02 ± 0.23	−0.24 ± 0.17	0.35 ± 0.30	**>18.63**	**>18.63**	−0.78 ± 0.70
rpoB 531, TCG→TTG *embB* 306, ATG→CTG	1	20.78	−0.28	−0.07	−0.22	0.48	**>18.22**	**>18.22**	−1.87
rpoB 531, TCG→TTG *embB* 306, ATG→ATC *katG* 315, AGC→ACC	5	22.04 ± 1.52	−0.01 ± 0.35	−0.30 ± 0.28	−0.21 ± 0.27	0.00 ± 0.12	**>16.44**	**>16.44**	**>16.44**
rpoB 531, TCG→TGG *embB* 306, ATG→GTG *katG* 315, AGC→ACC	1	22.24	−1.03	−0.52	−0.08	−0.04	**>17.76**	**>17.76**	**>17.76**
rpoB 531, TCG→TGG *embB* 306, ATG→ATC *katG* 315, AGC→ACC	2	21.20 ± 0.16	0.40 ± 0.11	−0.45 ± 0.03	0.05 ± 0.06	−0.17 ± 0.15	**>18.64**	**>18.64**	**>18.64**

[a] Forty-five laboratory strains of RIF-resistant *M. tuberculosis*, including MDR *M. tuberculosis*.
[b] Gene, codon number, and allele of the mutation(s) in the strain.
[c] Cycle number required when luminescence of control probe reaches the threshold (ΔRn = 0.2).
[d] Difference in Ct between control probe and corresponding probe. The mutations resulted in an increased ΔCt, which is indicated by boldface.
[e] Wild-type, pansusceptible strains, including *M. tuberculosis* H37Rv.

TABLE 3. Luminescence patterns of real-time PCR with DNA extracted from sputua of TB patients[a]

Sample	Smear	Culture	Resistance phenotype	Mutant position[b]	Control Ct[c]	ΔCt[d]						
						rpo510/514	rpo514/519	rpo520/524	rpo524/529	rpo529/533	emb306	kat315
C1	−	+	−	−	>40.00	ND[e]	ND	ND	ND	ND	ND	ND
Nested PCR					18.91	0.38	0.77	−0.11	1.57	1.34	−1.44	−0.36
C2	+	+	−	−	34.07	−0.12	−1.10	0.06	2.46	2.05	−3.30	−2.47
C3	−	+	−	−	27.95	−1.99	−2.85	−0.22	−0.63	−1.47	−1.86	−0.77
C4	−	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					17.00	1.38	0.55	1.55	1.40	0.03	0.55	0.62
C5	−	+	INH	*katG* 315, AGC→ACC	33.11	3.15	2.49	0.20	0.85	0.24	−3.28	**>6.79**
C6	−	+	−	−	37.31	0.65	0.29	0.02	1.65	1.77	−3.35	−3.07
C7	−	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					20.06	0.38	0.90	−0.24	1.31	1.20	−0.55	−0.87
C8	+	+	−	−	31.70	0.92	0.82	0.82	2.79	2.40	−0.79	−0.37
C9	+	+	−	−	39.39	ND	ND	ND	ND	ND	ND	ND
Nested PCR					15.23	2.01	2.13	−0.04	2.01	1.40	−0.69	−1.39
C10	+	+	−	−	36.35	−2.12	−1.36	−0.08	−0.26	0.06	−2.52	−3.40
C11	−	+	RIF, INH, EMB	*rpoB* 531, TCG→TTG; *katG* 315, AGC→ACC; *embB* 306, ATG→GTG	31.08	0.50	0.04	0.01	0.14	**>8.92**	**>8.92**	**>8.92**
C12	−	+	−	−	34.70	−0.15	0.18	0.59	1.51	1.81	−3.36	−2.60
C13	−	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					19.11	0.64	−0.37	−0.12	0.87	1.35	1.36	−1.06
C14	−	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					21.80	0.45	−0.45	−0.11	0.94	1.17	−1.52	0.67
C15	−	+	−	−	13.94	1.35	0.43	−0.26	0.93	1.42	0.75	0.05
C16	−	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					19.96	0.69	−0.10	−0.09	1.04	1.03	−1.65	−1.39
C17	−	+	−	−	31.73	1.12	1.12	−0.06	2.34	2.16	−2.37	−0.77
C18	−	+	−	−	33.19	1.04	0.26	0.42	1.81	1.36	−2.72	−1.94
C19	−	+	−	−	36.23	−2.22	0.42	−0.04	1.90	2.28	−2.69	−2.07
C20	+	−	ND	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					21.28	0.62	−0.27	0.00	1.24	1.70	−0.72	−2.33
C21	−	+	−	−	35.52	0.60	−0.47	0.15	1.19	1.37	−3.24	−2.49
C22	+	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					19.93	0.23	−0.58	−0.13	0.66	0.93	−0.93	−2.15
C23	−	−	ND	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					15.71	−0.08	−0.92	−0.19	0.13	−0.15	−0.72	−2.33
C24	−	+	−	−	36.45	0.92	0.05	0.81	1.97	1.72	−3.04	−2.23
C25	−	+	−	−	32.70	2.16	2.57	0.21	1.96	1.36	−3.04	−1.95
C26	+	+	−	−	36.40	0.10	0.20	0.21	2.00	2.02	−3.33	−2.61
C27	+	+	−	−	>40.00	ND	ND	ND	ND	ND	ND	ND
Nested PCR					18.68	1.68	0.64	0.12	1.30	2.37	1.29	1.11

[a] DNAs derived from *M. tuberculosis* were detected with the COBAS AMPLICOR PCR system.
[b] Gene, codon number, and allele of mutations in *M. tuberculosis* DNA extracted from sputa of TB patients.
[c] Cycle number required when luminescence of control probe reaches the threshold (ΔRn = 0.2).
[d] Difference in Ct between control probe and corresponding probe. The mutations resulted in an increased ΔCt, which is indicated by boldface.
[e] ND, not detected.

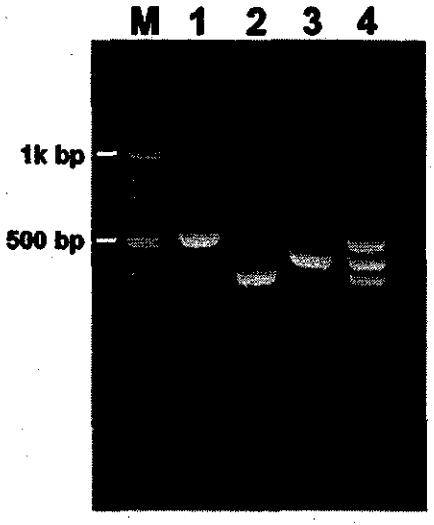

FIG. 3. DNA analysis of amplicons by simplex and multiplex PCRs. DNA from sputum sample C1 was amplified by using primers for the *rpoB*, *embB*, and *katG* genes. The product sizes of their amplicons were 534, 408, and 464 bp, respectively (lanes 1 to 3, respectively). Three kinds of primer sets for the *rpoB*, *embB*, and *katG* genes were mixed, and multiplex PCR was performed (lane 4). The M lane was a DNA molecular size marker which contained the 100-bp ladder.

PCR analysis with each construct as the template showed that the luminescence that was derived from probe rpo510/514 disappeared completely (data not shown).

Clinical application of real-time PCR. DNAs extracted from sputa of patients with pulmonary TB were examined to assess to possibility of rapid detection of drug-resistant *M. tuberculosis* in clinical specimens by real-time PCR. Twenty-five clinical samples were culture positive, and two samples were culture negative. Nine samples were smear positive, and 18 samples were negative (Table 3). Real-time PCR was used to analyze sputum samples for detection of mutations. Sample C5 had a mutation at codon 315 in the *katG* gene, and sample C11 possessed mutations at codon 531 in the *rpoB* gene, codon 315 in the *katG* gene, and codon 306 in the *embB* gene. These findings were in agreement with the results obtained by DNA sequencing. The drug susceptibility phenotypes of clinical isolates assessed by conventional culture methods were consistent with the genotypes (Table 3). Thus, our real-time PCR system can detect mutations even when clinical samples, such as sputa, are used.

A total of 16 of 27 sputum samples (59.3%) showed strong luminescence in real-time PCR. By contrast, 11 samples showed no luminescence even after 40 cycles of PCR amplification. This was probably due to low concentrations, because IPC was positive for all 11 samples. Consequently, we performed site-specific nested PCR when the amount of DNA was small. The *rpoB*, *katG*, and *embB* genes were amplified by PCR with their corresponding primer sets (Fig. 3). By optimizing the PCR conditions with six primers, three fragments that contained the target sites at a similar concentration were amplified (Fig. 3, lane 4). The targets (*rpoB*, *katG*, and *embB*) were amplified by nested PCR with one tube. The targets were then analyzed by using real-time PCR. Nested PCR products could be analyzed for the presence of mutations associated with drug resistance, although DNA was undetectable in a single-step real-time PCR in these 11 samples. When nested PCR was used before real-time PCR, analysis of all 27 samples could be performed appropriately (Table 4). However, in analysis of clinical samples, the Ct was more variable, resulting in a larger ΔCt (from −3.40 to 3.15) than for purified DNAs extracted from laboratory strains (from −2.44 to 1.61).

DISCUSSION

The TaqMan MGB probes are currently used for detection of single-nucleotide polymorphisms, because they can distinguish one-base mismatches (1, 12, 18). In the present study, we have attempted to apply these probes to detect drug resistance on a genetic basis. The implication of all studies on the genetic basis of antimicrobial resistance in *M. tuberculosis* is that the MDR phenotype (defined as simultaneous resistance to at least INH and RIF) is the result of accumulative mutations (10, 23). The major anti-TB drugs are INH, RIF, and EMB. Among them, INH and RIF are the most potent agents. More than 90% of RIF-resistant *M. tuberculosis* isolates possess a point mutation at the hot spot in the 81-bp region of *rpoB* (3, 26). For that reason, detection of mutations in the *rpoB* gene is a quite useful strategy for diagnosis.

The present study also analyzed extracted DNAs from *M. tuberculosis* by real-time PCR for the presence of mutations in the *katG* (codon 315) and *embB* (codon 306) genes. Codons 315 in *katG* and 306 in *embB* were selected because mutations at these sites have been observed frequently in INH- and EMB-resistant *M. tuberculosis* (3, 4, 11, 28). However, the role of codon 306 in the *embB* gene for EMB resistance remains controversial, because the mutation has also been found in EMB-susceptible *M. tuberculosis*. It has been reported that *embB*306 mutations were detected in 48% of EMB-resistant strains and in 31% of EMB-susceptible strains (21). In particular, 60% of EMB-susceptible strains were resistant to rifampin and isoniazid (i.e., MDR), but none of pansusceptible strains harbored an *embB*306 mutation. A discrepancy between the results of phenotypic and genotypic analyses of EMB resistance tests was restricted to the strains that were already resistant to other anti-TB drugs, such as MDR *M. tuberculosis*. Our results have shown that 42% of EMB-susceptible strains of MDR *M. tuberculosis* had the mutation, in contrast to 0% of pansusceptible strains. When a mutation exists at *embB*306 in MDR *M. tuberculosis*, it is possible that the strain is susceptible to EMB. Nevertheless, 70% of EMB-resistant strains have a point mutation at codon 306 in the *embB* gene (3, 28). The mutation of codon 315 (Ser) in the *katG* gene is the most frequently encountered mutation that is associated with INH resistance (30 to 65%) (4, 11). In the present study, we found that 70% of INH-resistant strains and 6% of INH-susceptible strains had the mutation at this site. These results are consistent with previous reports (4, 11, 21).

The results obtained from the real-time PCR system in this study were consistent with DNA sequencing analyses of 45 laboratory strains and 27 clinical samples. The system did not react with DNAs prepared from *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, and nontuberculous mycobacteria but reacted solely with DNAs from isolates belonging to the *M. tuberculosis* com-

TABLE 4. Detection of genotypes by real-time PCR

Sample type and analysis	No. of samples	No. (%) detected	No. (%) undetected
DNAs from cultured bacilli	45	45 (100)	0 (0)
DNAs from sputa of TB patients[a]			
Direct analysis	27	16 (59.3)	11 (40.7)
Preamplified DNA (after nested PCR)[b]	11	11 (100)	0 (0)

[a] DNAs were extracted from sputa of TB patients by use of the AMPLICOR respiratory specimen preparation kit.
[b] DNAs from sputa were analyzed after preamplification by nested PCR.

plex. These results imply that the primers and probes used here are specific for *M. tuberculosis* complex.

Although understanding the mechanisms of drug resistance has practical implications for rapid detection of drug-resistant TB by molecular methods, there are a number of limitations to widespread use of PCR-based techniques. For one, resistance to anti-TB agents involves changes in multiple genes and at multiple possible locations within a gene. This fact complicates testing for the various genes. In addition, not all possible genes or mechanisms of resistance have been identified, which represents a significant drawback for diagnostics.

It has been reported previously that real-time PCR with MGB probes was applied for detection of INH-resistant *M. tuberculosis* (34). In that study, DNAs prepared from smear-positive sputa were used. However, the results for certain samples were inconsistent with those of DNA sequencing. One possible explanation for this is that the amount and/or quality of DNA was not standardized in the study (34). Our results suggest that the amount of DNA in the sample is critical for analysis with real-time PCR using TaqMan MGB probes. The quality of DNA (e.g., whether it is inhibitor free) is also important. In the present study, two control probes were designed for the real-time PCR. One was a TB control probe for identification of *M. tuberculosis* and confirmation of DNA amount; the other was the IPC for detection of PCR inhibitors. If luminescence is not detected, the reason mentioned above can be suspected.

Several molecular methods to detect drug-resistant *M. tuberculosis* have been reported (8). These methods are fundamentally based on the detection of point mutations. PCR-DNA sequencing is a straightforward technology to detect mutation (14), although it takes 1 to 2 days until the result is obtained. Methods based on real-time PCR have utilized fluorescence resonance energy transfer (FRET) probes (9, 32), molecular beacons (6, 23, 24), and TaqMan MGB probes (34). By using the real-time PCR as described here, the time to obtain results for drug susceptibility or resistance can be shortened to as little as 3 h from the preparation of DNAs from isolates of *M. tuberculosis* and sputum samples. Rapid detection of drug resistance or susceptibility may open new therapeutic avenues for intervention in diseases in which drug resistance is a major impediment to treatment. The method based on FRET probes requires a longer probe, because it utilizes both sensor and anchor probes (33). The shift of melting temperature was unclear in some cases when long probes were used in FRET analysis to detect mutations. By contrast, molecular beacons and TaqMan MGB probes, which can be designed to be shorter than FRET probe, detect the mutations on the basis of either emission or lack of emission of luminescence. The luminescence can be measured with a fluorescence plate reader after conventional PCR, even without a real-time PCR analyzer (5).

Molecular beacons are an alternative approach to detect drug-resistant organisms in a real-time format. It has been reported that RIF-resistant *M. tuberculosis* can be detected in a one-tube reaction by using the beacons (6). This is a convenient and simple method to detect mutations. However, it is necessary to use a real-time PCR analyzer that can detect five kinds of different luminescence simultaneously. Although the assay described here requires the use of four tubes per sample, only two wavelengths are employed, enabling the use of less advanced equipment.

Our present study suggests that drug-resistant *M. tuberculosis* can be detected by ΔCt with TaqMan MGB probes in real-time PCR. Based on the result for 45 laboratory strains and 27 clinical samples of *M. tuberculosis*, ΔCt was less than 3.5 when organisms had no mutation (Tables 2 and 3). When ΔCt was more than 6, the DNA samples contained mutations within the target region. These isolates or samples are strongly inferred to be drug-resistant *M. tuberculosis* (Tables 2 and 3). When wild-type and mutated DNAs were mixed at ratios ranging from 8:2 to 2:8, ΔCt was distributed between 3.5 and 6.0 (data not shown). Indeed, ΔCt of a mixture of drug-susceptible and -resistant bacilli ranged from 3.5 to 6.0. When ΔCt is distributed within the range of 3.5 to 6.0, DNA sequencing analysis should be performed to confirm the mixture. In analysis of clinical samples without mutations, the range of ΔCt became larger (−3.40 to 3.15) than that of purified DNAs extracted from laboratory strains (−2.44 to 1.61). It is thought that ΔCt obtained from the real-time PCR is a useful marker for discriminating between drug-susceptible and drug-resistant *M. tuberculosis* strains. These criteria are applicable to detect drug-resistant *M. tuberculosis* in clinical laboratories.

Real-time-based PCR shows sufficient specificity and sensitivity to detect drug-resistant *M. tuberculosis* even with sputum samples from TB patients without culture. The real-time PCR described here can detect drug-resistant *M. tuberculosis* within 3 h from DNA preparation. For those reasons, it may be a powerful tool for control of drug-resistant *M. tuberculosis*.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health, Labour and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants); the Ministry of Education, Culture, Sports, Science and Technology; the Ministry of the Environment (Global Environment Research Fund); Osaka City University (Urban Research Project); and the United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy.

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