Rodriguez, G. M., Gold, B., Gomez, M., Dussurget, O. & Smith, I. (1999). Identification and characterization of two divergently transcribed iron regulated genes in *Mycobacterium tuberculosis*. *Tuber Lung Dis* **79**, 287–298.

Rodriguez, G. M., Voskuil, M. I., Gold, B., Schoolnik, G. K. & Smith, I. (2002). *ideR*, an essential gene in *Mycobacterium tuberculosis*: role of IdeR in iron-dependent gene expression, iron metabolism, and oxidative stress response. *Infect Immun* **70**, 3371–3381.

Romano, M., Rindi, L., Korf, H., Bonanni, D., Adnet, P. Y., Jurion, F., Garzelli, C. & Huygen, K. (2008). Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). *Vaccine* **26**, 6053–6063.

Rosenkrands, I. & Andersen, P. (2001). Preparation of culture filtrate proteins from *Mycobacterium tuberculosis*. In *Mycobacterium tuberculosis Protocols*, pp. 205–215. Edited by T. Parish & N. G. Stoker. Totowa, NJ: Humana Press. doi: 10.1385/1-59259-147-7: 205.

Schnappinger, D., Ehrt, S., Voskuil, M. I., Liu, Y., Mangan, J. A., Monahan, I. M., Dolganov, G., Efron, B., Butcher, P. D. & other authors (2003). Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. *J Exp Med* **198**, 693–704.

Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H. & other authors (1991). New use of BCG for recombinant vaccines. *Nature* **351**, 456–460.

Surewicz, K., Aung, H., Kanost, R. A., Jones, L., Hejal, R. & Toossi, Z. (2004). The differential interaction of p38 MAP kinase and tumor necrosis factor-α in human alveolar macrophages and monocytes induced by *Mycobacterium tuberculosis*. *Cell Immunol* **228**, 34–41.

Tundup, S., Pathak, N., Ramanadham, M., Mukhopadhyay, S., Murthy, K. J. R., Ehtesham, N. Z. & Hasnain, S. E. (2008). The co-operonic PE25/PPE41 protein complex of *Mycobacterium tuberculosis* elicits increased humoral and cell mediated immune response. *PLoS ONE* **3**, e3586.

Voskuil, M. I., Schnappinger, D., Rutherford, R., Liu, Y. & Schoolnik, G. K. (2004). Regulation of the *Mycobacterium tuberculosis* PE/PPE genes. *Tuberculosis (Edinb)* **84**, 256–262.

Wang, J., Qie, Y., Zhang, H., Zhu, B., Xu, Y., Liu, W., Chen, J. & Wang, H. (2008). PPE protein (Rv3425) from DNA segment RD11 of *Mycobacterium tuberculosis*: a novel immunodominant antigen of *Mycobacterium tuberculosis* induces humoral and cellular immune responses in mice. *Microbiol Immunol* **52**, 224–230.

Wolfe, F., Michaud, K., Anderson, J. & Urbansky, K. (2004). Tuberculosis infection in patients with rheumatoid arthritis and the effect of infliximab therapy. *Arthritis Rheum* **50**, 372–379.

Zhang, Y., Broser, M. & Rom, W. N. (1994). Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-κB. *Proc Natl Acad Sci U S A* **91**, 2225–2229.

Hindawi Publishing Corporation
Tuberculosis Research and Treatment
Volume 2011, Article ID 940642, 9 pages
doi:10.1155/2011/940642

Clinical Study

Stratified Threshold Values of QuantiFERON Assay for Diagnosing Tuberculosis Infection in Immunocompromised Populations

Haruyuki Ariga,[1] Hideaki Nagai,[1] Atsuyuki Kurashima,[1] Yoshihiko Hoshino,[2] Syunsuke Shoji,[1] and Yutsuki Nakajima[1]

[1] *Center for Respiratory Medicine, National Hospital Organization, Tokyo National Hospital, 3-1-1 Takeoka, Kiyose, Tokyo 204-8585, Japan*
[2] *Department of Mycobacteriology, National Institute of Infectious Diseases, 4-2-1 Aoba, Higashi-Murayama, Tokyo 189-0002, Japan*

Correspondence should be addressed to Yoshihiko Hoshino, yhoshino@nih.go.jp

Received 7 February 2011; Accepted 13 April 2011

Academic Editor: A. S. Apt

Background. The detection of latent tuberculosis (TB) is essential for TB control, but T-cell assay might be influenced by degree of immunosuppression. The relationship between immunocompetence and interferon (IFN)-γ response in QuantiFERON-TB Gold (QFT) is uncertain, especially in HIV-negative populations. *Methods and Results.* QFT has been performed for healthy subjects and TB suspected patients. Of 3017 patients, 727 were diagnosed as pulmonary TB by culture. The absolute number of blood lymphocyte in TB patients was significantly associated with QFT. Definitive TB patients were divided into eight groups according to lymphocyte counts. For each subgroup, receiver operating characteristic curve analysis was conducted from 357 healthy control subjects. The optimal cut-off for the patient group with adequate lymphocyte counts was found, but this was reduced for lymphocytopenia. *Conclusions.* The lymphocyte count was positively associated with QFT. Positive criteria should be calibrated in consideration of cell-mediated immunocompetence and risk of progression to active TB.

1. Introduction

Treatment of persons with latent tuberculosis infection (LTBI) is essential to prevent the development of active tuberculosis (TB). Although the greatest risk of TB disease is traditionally associated with recent exposures, immunocompromised hosts represent a setting in which reactivation of TB from more remote exposures is a concern. The US Centers for Disease Control and Prevention (CDC) recommends targeted testing for LTBI in specific at-risk groups, including immunocompromised individuals with HIV infection, receiving immunosuppressive therapy, chronic renal failure, solid organ transplant, silicosis, cancer of the head or neck and lung, malnutrition, and diabetes [1]. The targeted treatment of LTBI in these populations is also central to TB control at the public health level [1, 2].

Until recently, tuberculin skin testing (TST) was the only test for the detection of LTBI, but now in vitro T-cell-based IFN-γ assays that are highly specific for mycobacterium tuberculosis (Mtb) have been developed as alternative diagnostic test [3]. In contrast to TST, T-cell assays are unaffected by previous BCG vaccination and infection with most environmental nontuberculous mycobacteria [4]. QuantiFERON-TB Gold in tube (QFT-IT) (Cellestis, Carnegie, Australia) and T-SPOT. TB (Oxford Immunotec, Oxford, UK) are two types of commercially available T-cell assay. QuantiFERON-TB Gold (QFT; second generation) and QFT-IT (third generation) are whole blood assays based on enzyme-linked immunosorbent assay, whereas the T-SPOT. TB uses peripheral blood mononuclear cells for antigen stimulation in an enzyme-linked immunospot assay. Although there is no gold standard for LTBI diagnosis, when surrogate markers of recent exposure to Mtb are used, T-cell assays have been shown in many studies to be as good if not better than the TST in a contact investigation [5, 6]. CDC

guidelines recommend the single use of QFT in place of the TST for all circumstances in which the TST is used [7].

The TST is well known to have diminished sensitivity in the setting of immunocompromised hosts. False-negative results in TST were reported, for example, in elderly people and in malnutrition [8, 9]. A recent study suggested that T-cell assays identified more immunocompromised patients with LTBI than did the TST [10]. Because these new T-cell assays rely on measuring specific acquired cell-mediated immune responses, any immunomodulating factors affecting cellular immunity in vivo may impact on the test performance in vitro [11]. However, the correlation between the degree of immunocompetence and specific IFN-γ responses is not clear. Furthermore, most studies have analysed data from T-cell assays as dichotomous results using a single cutoff value, and little work has been done on validation of thresholds in consideration of immunological diversity. There is epidemiological evidence to support the use of risk-stratified cutoff values, as in the interpretation of TST [1]. However, no such data exist presently for T-cell assays. We hypothesized that the appropriate positive cutoff value in QFT for diagnosing LTBI would be different in immunocompetent populations and immunosuppressed hosts with impaired cell-mediated immunity. The objective of this study is therefore to identify immune-related clinical indicators associated with the degree of antigen-specific IFN-γ production using a large immunologically unselected population with obvious TB infection. Secondly, based on these findings, we revised the threshold values of QFT for populations with different immune status in clinical practice.

2. Methods

2.1. Participants. From January 2006 to October 2008, new patients and healthy subjects in our hospital verbally consenting to the study were consecutively enrolled after the research protocol was approved by the institutional review board of National Tokyo Hospital (IRB). QFT assay is now approved by national medical insurance in Japan and should be indicated to diagnose TB infection in clinical practice. IRB approves a verbal informed consent for QFT assay. QFT was prescribed by hospital physicians for inpatients and outpatients in any ward, without any influence of the investigators. For all patients recruited into the study, information on their previous medical history, clinical symptoms and signs, and radiological and microbiological data were collected at the time of enrollment. Bronchial lavage fluid samples obtained by bronchofiberscopy were collected and cultured for mycobacteria when it was judged necessary. Clinicians did not take QFT results into account for their final diagnosis. Several routine laboratory tests for clinical diagnosis were simultaneously performed. We also routinely performed QFT on healthy employees at the start of their employment. We collected information on history of prior TB, previous working in any healthcare settings or recent exposure to a patient with active TB and other TB risk factors such as taking immunosuppressive drugs.

2.2. Whole Blood QuantiFERON-TB Gold Assay. The test was performed according to the manufacturer's instructions. The interpretation of the results was performed according to the guidelines proposed by CDC [7]. Previously we evaluated that this test would have 74% sensitivity in all patients including HIV-negative immunosuppressive patients [12]. However, it was increased to 88% in patients with peripheral lymphocyte counts $\geq$1000 cells/mm^3. Two specialized technicians running the test in our hospital were completely blinded to individual clinical information and final diagnosis.

2.3. Statistical Analysis. Associations between antigen-specific IFN-γ production in the QFT assay and several clinical characteristics were examined by fitting a linear model. Regression diagnostic exercises included inspection of residual distributions (quantile-quantile and normal-probability plots, residuals-versus-fitted plots), nonlinearity in the functional relationship with predictors (augmented component-versus-residual plots), and examination for collinearity (variance inflation factor). Predictions made from the fitted model were expressed in the original metric with the aid of Duan's smearing estimation. Assay results were subjected to maximum-likelihood logistic regression against the categorized subpopulations. Pairwise differences in logistic regression coefficients were compared at a Type I error rate of 0.05, adjusted for multiple comparisons by the Bonferroni method. Pairwise comparisons were made between areas under the receiver operating characteristic (ROC) curve (AUROC) for the categorized groups. Adjustment for multiple comparisons was made using the Dunn-Sida'k method. P values $< .05$ were considered significant. Statistical analysis was conducted with Stata, Release 10.1 and 11 (StataCorp LLP, College Station, Texas, U.S.A.), and GraphPad Prism 5 (GraphPad software, San Diego, California).

3. Results

3.1. Characteristics of the Patients. A total of 3762 subjects underwent initial QFT testing. As shown in Figure 1, of the 3017 TB suspects, 727 patients were finally diagnosed as having definite active pulmonary TB based on the microbiological detection of Mtb from sputum or bronchial lavage fluid obtained using bronchofiberscopy. We excluded patients with probable TB in which no bacteriological evidence was obtained, persons with recent exposure to smear-positive TB patients, and those who had been receiving of antituberculous treatment for more than 14 days at the time of testing. Characteristics of patients with active TB are shown in Table 1. More males than females participated in the study, and most of the study participants were Japanese. The majority (98.9%) was HIV seronegative. Positive sputum smears were found in 552 of 727 patients (75.9%). Clinical immunomodulatory factors pertaining to the TB patients are also shown in Table 1. Patients could have multiple factors at the same time.

3.2. Clinical Predictors Related to the Specific IFN-γ Response in TB Patients. Associations between specific IFN-γ

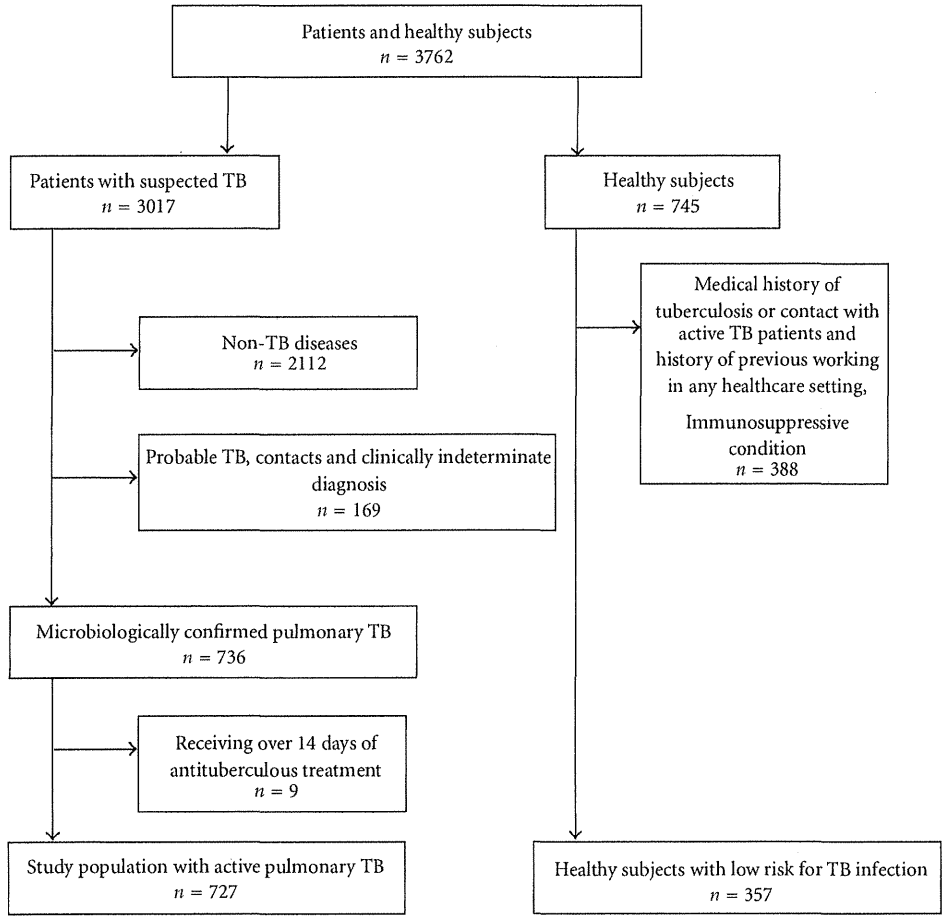

FIGURE 1: Study participants recruitment profile. TB: tuberculosis.

production in unselected TB patients and several patient characteristics were examined by fitting to a linear model. For this, the response variable was the maximum IFN-γ after stimulating with ESAT-6 and CFP-10, logarithmically transformed in order to make the distribution of the model residuals approximate more closely to normal and homoscedastic. The following patient characteristics were used as predictors of antigen-stimulated IFN-γ production: age, absolute counts of lymphocytes, monocytes, neutrophils and eosinophils, mitogen-stimulated IFN-γ production (positive control) and nil (background) negative control IFN-γ, and various clinical laboratory test results that might relate to immunocompetence and general condition (serum albumin and blood hemoglobin concentrations), inflammatory status (C-reactive protein level; CRP), serum creatinine concentration, and serum liver enzyme activity levels. None of the predictors were transformed. The values for certain clinical laboratory test results were scaled as needed in order to bring them into similar numerical domains; in practice, this meant that leukogram-related regression coefficients are given in units of thousands of formed elements cells/mm^3. Three values were missing for albumin, and three for CRP. The total observations available for regression were therefore 721. Regression coefficients, their standard errors, P values, and 95% confidence intervals are presented in Table 2. Both background and mitogen-background control conditions are positively associated with antigen-specific IFN-γ production ($P < .0005$ and $P = .006$, resp.). Lymphocyte count is likewise positively associated with the test kit response ($P < .0005$). Neutrophil count is negatively associated with specific response ($P < .0005$).

3.3. QFT Results in Lymphocyte-Stratified TB Populations. Next, the TB patient population was divided into eight subgroups based on the absolute number of peripheral blood lymphocyte. Ranges for categories were chosen as 0–299, 300–499, 500–699, 700–999, 1000–1199, 1200–1499, 1500–1799, and >1800 cells/mm^3 (Figure 2(a)). Figure 2(b) displays the QFT results for patients according to these eight categories of lymphocyte counts. The proportion of positive assay results (at a cutoff of 0.35 IU/mL) and mitogen-stimulated IFN-γ responses were found to be positively associated with lymphocyte count (Figures 2(b) and 2(c)). Conversely, indeterminate assay results showed a negative relationship with lymphocyte count (Figure 2(b)). Indeterminate result rates significantly increased in the categories with less than

Table 1: Demographic and baseline clinical characteristics of pulmonary tuberculosis patients.

Total, *n*		727	
Age, median, range		63	15–97
Female, *n*, %		208	28.8
Nationality (other than Japanese), *n*, %		9	1.2
	0	167	23.0
	1+	187	25.7
Sputum smear status[a], *n*, %	2+	113	15.5
	3+	252	34.7
	unknown	7	1.0
Lung cavitary lesion, *n* (%)		276	38.0
Underlying preexisting conditions[b], *n*, %			
Administration of immunosuppressant before TB onset[c]		39	5.4
Malignant diseases		44	6.1
Serum albumin less than 3.0 g/dL		248	34.1
Bedridden		78	10.7
Diabetes mellitus		69	9.5
HIV positive		8	1.1
Liver cirrhosis		5	0.7
Renal failure		5	0.7
History of cerebral infarction		17	2.3
Silicosis		3	0.4
Dementia		45	6.2
Alcoholism		15	2.1
Homeless		34	4.7
Drug abuser		0	0.0

Note. [a]Smear by sputum microscopy: 0 (no acid fast bacilli (AFB) on smear), 1+ (1–99 AFB per 100 field), 2+ (1–10 AFB per field), and 3+ (more than 10 AFB per field).
[b]More than one condition can coexist in the same patient.
[c]Including chronic systemic steroids, antitumor necrosis alpha agents and immunosuppressive agents.

700 cells/mm^3. Most markedly, in severe lymphocytopenia with less than 300 cells/mm^3, the fraction of test with indeterminate result was 37.8%.

3.4. Optimum Thresholds in Lymphocyte-Stratified TB Populations. Of the 745 employees in our hospital, 357 immunocompetent individuals had no history of TB disease or contact with active TB patients, and no prior history of working in any health care setting (Figure 1). Median age was 25 years old (range of 21–28). More females (79.3%) than males participated. None of these subjects had any immunosuppressive conditions. All healthy subjects had received BCG vaccination at least once. A positive QFT result was observed in only 3 participants (0.8%), and there were no indeterminate results. Median antigen-specific IFN-γ response was 0.01 IU/mL (interquartile range of 0–0.02). Using these QFT data from 357 healthy subjects, for the 8 groups of TB patients stratified by lymphocyte counts as described above, ROC curve analyses were performed to determine the optimum cutoff value (Table 3). In the subgroup with lymphocyte counts of 1800 cells/mm^3 and over, the AUROC curve was 0.996 and the appropriate cutoff was determined to be 0.19 IU/mL (sensitivity 96% and specificity 98%) (Table 3 and Figure 3). A negative trend of AUROC curve with decreasing lymphocyte counts was observed. Notably, a sudden decrease in AUROC curve was found in patients with lymphocytopenia (less than 700 cells/mm^3). An appropriate cut-off for moderate lymphocytopenia (700–1500 cells/mm^3) was determined to be 0.1 IU/mL. Although the test performance was considerably decreased in severe lymphocytopenia group (<700 cells/mm^3), optimal cutoff value was found to be 0.040 IU/mL.

4. Discussion

Different interrelated immunomodulatory factors may generally affect lymphocyte condition in vivo. Malnutrition predisposes to a greater incidence of clinically apparent infection and increased morbidity and mortality due to infection. Depression of circulating lymphocytes and interleukin-2 production following mitogen stimulation has been observed after a fast of only 7 days [13]. Administration of systemic corticosteroid may suppress lymphocyte proliferation and function [14]. T cells from aged animals and humans consistently show depressed responsiveness to mitogens [15]. The presence of a variety of solid tumors is associated with impaired recall delayed-type hypersensitivity and decreased in vitro T-cell proliferation to mitogens [16]. Patients receiving hemodialysis display reduced T-cell function in vitro, diminished antibody production, and compromised neutrophil and dendritic cell function [17]. A recent study has indicated that indeterminate QFT results were increased in HIV-infected individuals with lower CD4$^+$ T cell counts [18, 19]. Persons under these clinical immunosuppressive conditions may have a high likelihood of developing active TB disease if they are infected with Mtb. Thus, lymphocytopenia or lymphocyte dysfunction is closely associated with the risk of active TB disease in compromised hosts. The present study revealed that, in an immunologically unselected population with TB infection, absolute lymphocyte counts and mitogen-stimulated IFN-γ response are positively associated with the level of antigen-specific IFN-γ response in the whole blood T-cell assay. Because CD4$^+$ T cells are the major source of IFN-γ in T-cell assay, this finding is not unexpected (although our unpublished data using intracellular cytokine-staining method suggested that CD8$^+$ T cells also produce IFN-γ in some patients). On the other hand, because some patients with normal lymphocyte counts nonetheless had decreased specific IFN-γ production, other so far-unidentified lymphocyte count-independent factors such as regulatory T-cell effects [20] could explain decreased IFN-γ responses. Although a poor mitogen response was rarely observed in patients with normal lymphocyte counts, good mitogen response could still be mediated in some patients with severe lymphocytopenia. This may suggest that

Table 2: Regression coefficients and associated statistics.

Predictors	Coefficient	SE	t	P	95% CI	
					Lower	Upper
Age (y)	−0.0030	0.0027	−1.12	.26	−0.0083	0.0023
background (IU/mL)	0.57	0.089	6.46	<.0005	0.40	0.75
Mitogen background (IU/mL)	0.016	0.0056	2.78	.006	0.0046	0.027
Albumin (g/dL)	0.0055	0.11	0.05	.96	−0.21	0.22
Hemoglobin (g/dL)	−0.023	0.029	−0.79	.43	−0.080	0.034
Neutrophil (1000/mm^3)	−0.14	0.021	−6.51	<.0005	−0.18	−0.095
Monocyte (1000/mm^3)	0.26	0.23	1.13	.26	−0.19	0.72
Lymphocyte (1000/mm^3)	0.61	0.13	4.65	<.0005	0.35	0.87
Eosinophil (1000/mm^3)	−0.63	0.44	−1.43	.15	−1.5	0.23
CRP (mg/dL)	−0.015	0.012	−1.27	.21	−0.038	0.0081
AST (IU/L)	−0.0023	0.0030	−0.76	.45	−0.0082	0.0036
ALT (IU/L)	0.0047	0.0032	1.48	.14	−0.0015	0.011
Creatinine (mg/dL)	0.18	0.12	1.48	.14	−0.058	0.41
Mitogen × Lymphocyte	−0.0073	0.0034	−2.16	.031	−0.014	−0.00067
Intercept (ln IU/mL)	0.22	0.47				

Note. Regression coefficients for linear regression of log-transformed antigen-stimulated IFN-γ production by the QuantiFERON-TB Gold. Wald (t) test statistics are based upon 698 degrees of freedom. P values are not adjusted for multiplicity. Diagnostic plots do not indicate significant departures from normality for the distribution of residuals or nonlinearity in the predictions. The maximum variance inflation factor (<4) is within an acceptable range. CRP: C-reactive protein; AST: aspartate amino transferase; ALT: alanine aminotransferase; SE: standard error; CI: confidence inter.

Table 3: Analysis of area under the receiver operating characteristic curves for lymphocyte-stratified patients groups.

Lymphocyte count/mm^3	n	AUROC curve	95% CI	SE	cutoff value IU/mL	sensitivity %	95% CI	specificity %	95% CI
<300	45	0.86	0.777–0.943	0.04	0.04	80	65.4–90.4	86	81.7–89.2
300–500	57	0.82	0.806–0.938	0.03	0.04	79	66.1–88.6	86	81.7–89.2
500–700	79	0.93[a]	0.883–0.973	0.02	0.05	89	81.0–95.5	89	85.7–92.4
700–1000	145	0.97[b]	0.962–0.987	0.01	0.1	87	80.3–91.9	94	91.2–96.3
1000–1200	98	0.98[b]	0.957–0.994	0.01	0.1	91	83.3–95.7	94	91.2–96.3
1200–1500	121	0.97[b]	0.954–0.993	0.01	0.1	93	87.4–97.1	95	91.8–96.8
1500–1800	78	0.97[b]	0.949–0.989	0.01	0.15	91	82.4–96.3	96	93.2–97.6
>1800	104	0.99[b]	0.992–0.9997	0.002	0.19	96	90.4–98.9	98	96.0–99.2

Note. AUROC: area under the receiver operating characteristic; SE: standard error; CI: Confidence Interval Categories for lymphocyte counts exclude the value shown for the upper end of the range.

[a]: $P < .05$ versus <300/mm^3 category and $P < .05$ versus 300–500/mm^3 category.

[b]: $P < .05$ versus <300/mm^3 category, $P < .05$ versus 300–500/mm^3 category, and $P < .05$ versus 500–700/mm^3 category.

certain leukocytes other than T cells may produce IFN-γ in response to whole blood stimulation by mitogen. The present study also suggested that neutrophil count was negatively associated with specific IFN-γ response. One possibility could be due to neutrophil-dominant high inflammatory status in advanced tuberculosis with reduced lymphocyte count [21].

Because the risk of LTBI is closely associated with duration of exposure to and proximity to sputum-smear-positive pulmonary TB patient, TB exposure is used as a surrogate for LTBI. However, as there is no proper gold standard for the diagnosis of LTBI, one cannot prove the presence or absence of LTBI [22]. Therefore, for estimating sensitivity and specificity of T-cell assay, untreated patients with culture-positive TB and healthy and low risk individuals without known exposure to TB have generally been used [23, 24].

However, host immunity would be expected to be different between active TB disease and asymptomatic LTBI. Recently, Harari et al. showed that dominant TNF-α TB-specific CD4$^+$ T-cell response would discriminate between latent infection and active disease [25]. However, they excluded patients with immunosuppression (such as HIV$^+$), malnutrition (body weight < 50 kg), and lymphocytepenia (<3000 cells/mm^3). TB disease severity is associated with greater depression of the total lymphocyte and CD4$^+$ T cell counts. The CD4$^+$ T-cell counts return to normal levels in most patients after one month of antituberculous chemotherapy [26]. Active TB attenuates antigen-stimulated IFN-γ production due to immunosuppression by the disease process itself and migration of specific T cells out of the peripheral blood [27]. This immune suppression in active

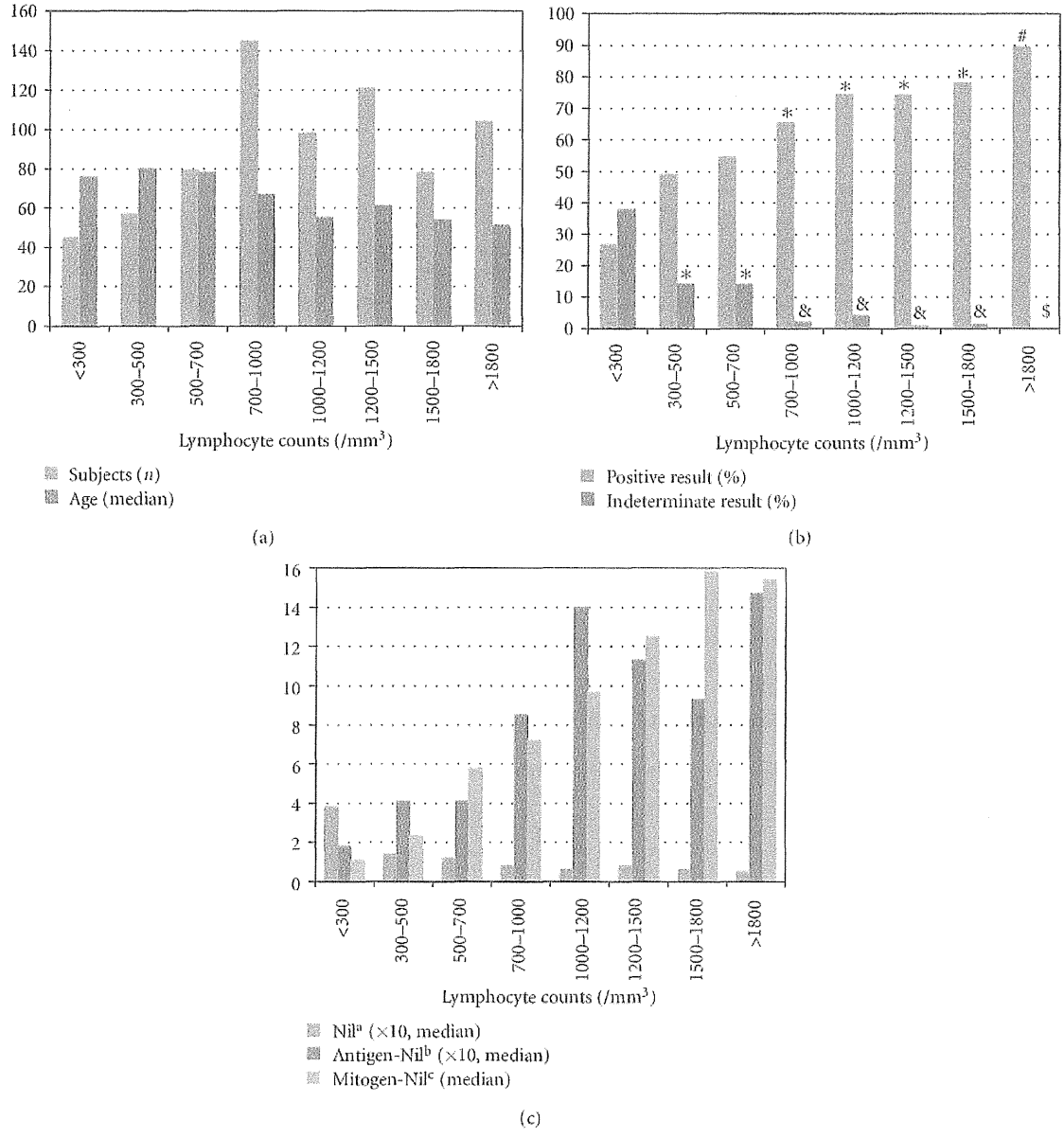

FIGURE 2: QuantiFERON-TB GOLD results in culture-proven pulmonary tuberculosis subgroups stratified by absolute number of peripheral blood lymphocyte. (a) Numbers and ages of patients divided into eight subgroups based on peripheral lymphocytes. (b) Proportion of QFT results with eight subgroups. *: $P < .05$ versus <300/mm³ category. &: $P < .05$ versus <300/mm³ category, $P < .05$ versus 300–500/mm³ category, and $P < .05$ versus 500–700/mm³ category. #: $P < .05$ versus <300/mm³ category, $P < .05$ versus 300–500/mm³ category, $P < .05$ versus 500–700/mm³ category, and $P < .05$ versus 700–1000/mm³ category. $: $P < .05$ versus <300/mm³ category, $P < .05$ versus 300–500/mm³ category, $P < .05$ versus 500–700/mm³ category, $P < .05$ versus 700–1000/mm³ category, and $P < .05$ versus 1000–1200/mm³ category. (c) Antigen-stimulated and mitogen-stimulated IFN-γ responses with lymphocyte count (IU/mL). [a]: Nil (background) IFN-γ concentration. [b]: Difference between the higher IFN-γ concentration after stimulation with either antigenic peptides ESAT-6 or CFP-10 and nil (background) IFN-γ concentration. [c]: Difference between the determined IFN-γ concentration after stimulation with mitogen and the nil (background) IFN-γ concentration.

TB may confound the use of an active TB population as a surrogate for LTBI. We need to select immunocompetent healthy persons known to have TB infection as a surrogate, as far as possible. From the opposite point of view, a population with active TB disease and associated immunosuppression could be used as a surrogate for immunosuppressed hosts with LTBI.

In general, the single cutoff value of 0.35 IU/mL as given in the manufacturer's instruction is uniformly applied to the interpretation of the positive result of QFT at present.

TABLE 4: Proposed Immunocompetence-stratified positive criteria and borderline zone for whole blood T-cell assay (QuantiFERON-TB Gold).

Compromised host[a]		Immunocompetent host (adult)		
≥0.1[b]	<0.1	≥0.3	0.1–0.3	<0.1
positive	Negative	postive	borderline	negative

Note: [a]: compromised hosts with impaired cell-mediated immunity and lymphocytopenia, such as patients receiving chronic immunosuppressive therapy, advanced malignant diseases, malnutrition, chronic renal failure on hemodialysis, HIV infection, and hospitalised elderly. [b]: IU/ml.

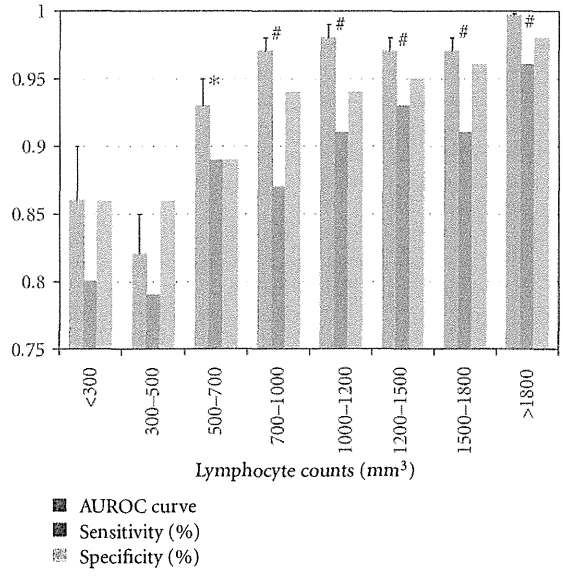

FIGURE 3: Analysis of area under the receiver operating characteristic curves for lymphocyte-stratified patients groups. AUROC: area under the receiver operating characteristic. Error bar; standard error. *: $P < .05$ versus <300/mm³ category and $P < .05$ versus 300–500/mm³ category. #: $P < .05$ versus <300/mm³ category, $P < .05$ versus 300–500/mm³ category, and $P < .05$ versus 500–700/mm³ category.

Because QFT is used for evaluating individuals suspected of having been infected with TB, it is necessary to decide whether the test result is "positive" or "negative" by using a single cutoff for clinical decision making. However, because the test measures antigen-stimulated IFN-γ production from cells in whole blood, the results are inherently continuous variables. Interpretation of the test results including conversions and reversions may require comprehension of nonspecific variability and reproducibility of the continuous data [28]. Moreover, specific IFN-γ production might be affected by impaired cell-mediated immunity such as in lymphocytopenia as described above. Here, using the current cutoff value of 0.35 IU/mL, sensitivity was 89% in population with adequate lymphocyte count, whereas in severe (<700 cells/mm³) and moderate (700–1500 cells/mm³) lymphocytopenia population, sensitivity ranged from 33% to 75% (Table 3). Antigen-specific responses and the test performance were markedly attenuated especially in severe lymphocytopenia. Thus, immunosuppression with severe lymphocytopenia may compromise the test performance. A recent report indicated that QFT sensitivity using the 0.35 IU/mL cutoff in high-incidence settings (69%) might be lower compared with low-burden countries (83%) probably due to several factors including HIV coinfection, advanced disease, and malnutrition [29]. The manufacturer defined assay cut-off 0.35 IU/mL allows for high specificity but likely at some cost in sensitivity, especially under immunosuppressive conditions with lymphocytopenia. Our data suggest that a cut-off of 0.2 IU/mL might be more appropriate for TB-infected immunocompetent subjects with normal lymphocyte count and low inflammatory reaction level which would be consistent with LTBI. Similarly, a cut-off of 0.1 IU/mL maximized the sensitivity without loss of specificity in moderate lymphocytopenic population. In the low burden setting, we propose that the test result should be considered positive if the specific response is greater than 0.1 IU/mL, especially under immunosuppressive conditions associated with lymphocytopenia (Table 4).

The cutoff value and definition of conversion in the T-cell assays for serial testing is a matter of debate and research both in high- and low-burden settings [30]. Since there is likely to be both individual immunological diversity as well as inherent variability in the determination of IFN-γ value, it could be relevant to establish a borderline grey zone [31]. We propose that in immunocompetent healthy subjects, borderline zone on both sides of the optimal cut-off 0.2 IU/mL from our analysis would be relevant (i.e., 0.1–0.3 IU/mL) in low endemic country. Any value less than 0.1 IU/mL is considered "definitely negative", and any value equal to or greater than 0.3 IU/mL is considered "definitely positive" (Table 4). In Japan, negative criteria for QFT are taken as less than 0.1 IU/mL at present, and the result of the test is considered "equivocal" borderline if the response to the specific antigens is equal or greater than 0.1 and less than 0.35 IU/mL. In the present long-term study, we used the QFT assay with two specific antigens: ESAT-6 and CFP-10. In the QFT-IT assay, new TB-Antigen 7.7 in addition to the two conventional antigens has been introduced and might incrementally improve sensitivity [32, 33]; however, the cut-off 0.35 IU/mL remains unchanged.

In conclusion, our large longitudinal study revealed that the absolute number of peripheral blood lymphocyte was closely associated with antigen-stimulated IFN-γ production in whole blood assay in unselected TB patients. Criteria for assigning a positive result should be stratified to allow for greater performance depending on the immune status of the subjects, especially in immunocompromised patients with impaired cell-mediated immunity who are at increased risk of progression to active TB disease.

Acknowledgments

The authors thank Ayako Watanabe and Yasuko Inoue, for their technical assistance with the laboratory assays, and Drs. Toshiki Tamura, Hajime Goto, and all physicians in their hospital for their support. This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan for Y. Hoshino. and by a Grant-in-Aid for Scientific Research (C) from The Ministry of Education, Culture, Sport, Science and Technology of Japan for Y. Hoshino.

References

[1] "Targeted tuberculin testing and treatment of latent tuberculosis infection. This official statement of the American Thoracic Society was adopted by the ATS Board of Directors, July 1999. This is a Joint Statement of the American Thoracic Society (ATS) and the Centers for Disease Control and Prevention (CDC). This statement was endorsed by the Council of the Infectious Diseases Society of America. (IDSA), September 1999, and the sections of this statement," *American Journal of Respiratory and Critical Care Medicine*, vol. 161, no. 4, part 2, pp. S221–S247, 2000.

[2] C. R. Horsburgh Jr., "Priorities for the treatment of latent tuberculosis infection in the United States," *New England Journal of Medicine*, vol. 350, no. 20, pp. 2060–2067, 2004.

[3] P. Andersen, M. E. Munk, J. M. Pollock, and T. M. Doherty, "Specific immune-based diagnosis of tuberculosis," *Lancet*, vol. 356, no. 9235, pp. 1099–1104, 2000.

[4] A. D. Lein, C. F. von Reyn, P. Ravn, C. R. Horsburgh Jr., L. N. Alexander, and P. Andersen, "Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to Mycobacterium avium complex and those with pulmonary disease due to Mycobacterium tuberculosis," *Clinical and Diagnostic Laboratory Immunology*, vol. 6, no. 4, pp. 606–609, 1999.

[5] S. M. Arend, S. F. Thijsen, E. M. Leyten et al., "Comparison of two interferon-gamma assays and tuberculin skin test for tracing tuberculosis contacts," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 6, pp. 618–627, 2007.

[6] K. Ewer, J. Deeks, L. Alvarez et al., "Comparison of T-cell-based assay with tuberculin skin test for diagnosis of Mycobacterium tuberculosis infection in a school tuberculosis outbreak," *Lancet*, vol. 361, no. 9364, pp. 1168–1173, 2003.

[7] G. H. Mazurek, J. Jereb, P. Lobue, M. F. Iademarco, B. Metchock, and A. Vernon, "Guidelines for using the QuantiFERON-TB Gold test for detecting Mycobacterium tuberculosis infection, United States," *Morbidity and Mortality Weekly Report*, vol. 54, no. 15, pp. 49–55, 2005.

[8] W. W. Stead and T. To, "The significance of the tuberculin skin test in elderly persons," *Annals of Internal Medicine*, vol. 107, no. 6, pp. 837–842, 1987.

[9] T. Kardjito, M. Donosepoetro, and J. M. Grange, "The Mantoux test in tuberculosis: correlations between the diameters of the dermal responses and the serum protein levels," *Tubercle*, vol. 62, no. 1, pp. 31–35, 1981.

[10] L. Richeldi, M. Losi, R. D'Amico et al., "Performance of tests for latent tuberculosis in different groups of immunocompromised patients," *Chest*, vol. 136, no. 1, pp. 198–204, 2009.

[11] G. Ferrara, M. Losi, M. Meacci et al., "Routine hospital use of a new commercial whole blood interferon-gamma assay for the diagnosis of tuberculosis infection," *American Journal of Respiratory and Critical Care Medicine*, vol. 172, no. 5, pp. 631–635, 2005.

[12] K. Komiya, H. Ariga, H. Nagai et al., "Impact of peripheral lymphocyte count on the sensitivity of 2 IFN-gamma release assays, QFT-G and ELISPOT, in patients with pulmonary tuberculosis," *Internal Medicine*, vol. 49, no. 17, pp. 1849–1855, 2010.

[13] L. Sävendahl and L. E. Underwood, "Decreased interleukin-2 production from cultured peripheral blood mononuclear cells in human acute starvation," *Journal of Clinical Endocrinology and Metabolism*, vol. 82, no. 4, pp. 1177–1180, 1997.

[14] J. L. Chiang, R. Patterson, J. J. McGillen et al., "Long-term corticosteroid effect on lymphocyte and polymorphonuclear cell function in asthmatics," *Journal of Allergy and Clinical Immunology*, vol. 65, no. 4, pp. 263–268, 1980.

[15] R. Schwab, C. A. Walters, and M. E. Weksler, "Host defense mechanisms and aging," *Seminars in Oncology*, vol. 16, no. 1, pp. 20–27, 1989.

[16] D. Y. Kavanaugh and D. P. Carbone, "Immunologic dysfunction in cancer," *Hematology/Oncology Clinics of North America*, vol. 10, no. 4, pp. 927–951, 1996.

[17] S. de Marie, "Diseases and drug-related interventions affecting host defence," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 12, supplement 1, pp. S36–S41, 1993.

[18] E. Raby, M. Moyo, A. Devendra et al., "The effects of HIV on the sensitivity of a whole blood IFN-gamma release assay in Zambian adults with active tuberculosis," *PLoS ONE*, vol. 3, no. 6, article e2489, 2008.

[19] M. G. Aabye, P. Ravn, G. PrayGod et al., "The impact of HIV infection and CD4 cell count on the performance of an interferon gamma release assay in patients with pulmonary tuberculosis," *PLoS ONE*, vol. 4, no. 1, article e4220, 2009.

[20] V. Guyot-Revol, J. A. Innes, S. Hackforth, T. Hinks, and A. Lalvani, "Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis," *American Journal of Respiratory and Critical Care Medicine*, vol. 173, no. 7, pp. 803–810, 2006.

[21] M. P. Berry, C. M. Graham, F. W. McNab et al., "An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis," *Nature*, vol. 466, no. 7309, pp. 973–977, 2010.

[22] D. Menzies, M. Pai, and G. Comstock, "Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research," *Annals of Internal Medicine*, vol. 146, no. 5, pp. 340–354, 2007.

[23] D. J. Jeffries, P. C. Hill, A. Fox et al., "Identifying ELISPOT and skin test cut-offs for diagnosis of Mycobacterium tuberculosis infection in the Gambia," *International Journal of Tuberculosis and Lung Disease*, vol. 10, no. 2, pp. 192–198, 2006.

[24] T. Mori, M. Sakatani, F. Yamagishi et al., "Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens," *American Journal of Respiratory and Critical Care Medicine*, vol. 170, no. 1, pp. 59–64, 2004.

[25] A. Harari, V. Rozot, F. B. Enders et al., "Dominant TNF-α+ Mycobacterium tuberculosis-specific CD4+ T cell responses discriminate between latent infection and active disease," *Nature Medicine*, vol. 17, no. 3, pp. 372–377, 2011.

[26] B. E. Jones, M. M. Oo, E. K. Taikwel et al., "CD4 cell counts in human immunodeficiency virus-negative patients with tuberculosis," *Clinical Infectious Diseases*, vol. 24, no. 5, pp. 988–991, 1997.

[27] J. Vekemans, C. Lienhardt, J. S. Sillah et al., "Tuberculosis contacts but not patients have higher gamma interferon

responses to ESAT-6 than do community controls in The Gambia," *Infection and Immunity*, vol. 69, no. 10, pp. 6554–6557, 2001.

[28] A. Veerapathran, R. Joshi, K. Goswami et al., "T-cell assays for tuberculosis infection: deriving cut-offs for conversions using reproducibility data," *PLoS ONE*, vol. 3, no. 3, article e1850, 2008.

[29] K. Dheda, R. Z. Smit, M. Badri, and M. Pai, "T-cell interferon-gamma release assays for the rapid immunodiagnosis of tuberculosis: clinical utility in high-burden vs. low-burden settings," *Current Opinion in Pulmonary Medicine*, vol. 15, no. 3, pp. 188–200, 2009.

[30] M. Pai, R. Joshi, S. Dogra et al., "Serial testing of health care workers for tuberculosis using interferon-gamma assay," *American Journal of Respiratory and Critical Care Medicine*, vol. 174, no. 3, pp. 349–355, 2006.

[31] M. Pai, R. Joshi, S. Dogra et al., "T-cell assay conversions and reversions among household contacts of tuberculosis patients in rural India," *International Journal of Tuberculosis and Lung Disease*, vol. 13, no. 1, pp. 84–92, 2009.

[32] N. Harada, K. Higuchi, T. Yoshiyama et al., "Comparison of the sensitivity and specificity of two whole blood interferon-gamma assays for M. tuberculosis infection," *Journal of Infection*, vol. 56, no. 5, pp. 348–353, 2008.

[33] H. Mahomed, E. J. Hughes, T. Hawkridge et al., "Comparison of Mantoux skin test with three generations of a whole blood IFN-gamma assay for tuberculosis infection," *International Journal of Tuberculosis and Lung Disease*, vol. 10, no. 3, pp. 310–316, 2006.

NOTE *Bacteriology*

Mycobacterium pseudoshottsii Isolated from 24 Farmed Fishes in Western Japan

Kazue NAKANAGA[1)]*, Yoshihiko HOSHINO[1)], Yoko HATTORI[2)], Atsushi YAMAMOTO[2)], Shinpei WADA[3)], Kishio HATAI[3)], Masahiko MAKINO[1)] and Norihisa ISHII[1)]

[1)]*Leprosy Research Center, National Institute of Infectious Diseases, 4–2–1 Aoba, Higashimurayama, Tokyo 189–0002, Japan*
[2)]*Faculty of Fisheries, Kagoshima University, 4–50–20 Shimoarata, Kagoshima 890–0056, Japan*
[3)]*School of Veterinary Medicine, Nippon Veterinary and Life Science University, 1–7–1 Kyonan, Musasino, Tokyo 180–8602, Japan*

(Received 11 May 2011/Accepted 27 September 2011/Published online in J-STAGE 11 October 2011)

ABSTRACT. Mycobacteria isolated from epizootics of farmed fishes in western Japan were examined for the first time using multigenotypic analysis. By analysis of the sequences of the internal transcribed spacer between the 16S and 23S rRNA genes (ITS) region and the partial 16S rRNA, *hsp65* and *rpoB* genes, *M. pseudoshottsii* was identified as the causative agent in these infections. Prior to this study, only *M. marinum* has been known as the causative agent of lethal mycobacterial disease in marine fishes in Japan.
KEY WORDS: lethal fish infection, *Mycobacterium pseudoshottsii*, mycolactone.

doi: 10.1292/jvms.11-0226; *J. Vet. Med. Sci.*74(2): 275–278, 2012

Mycobacterium (M.) marinum, M. salmoniphilum, M. fortuitum, M. chelonae and *M. abscessus* are the most commonly identified mycobacterial fish pathogens [1, 4]. In particular, *M. marinum* is found in a wide range of saltwater species [2]. Molecular and phylogenetic analyses have facilitated the worldwide recovery of novel mycobacterial species, strains and isolates, such as *M. shottsii* [9] and *M. pseudoshottsii,* from wild marine fishes [3, 10].

M. pseudoshottsii, a slow-growing, photochromogenic mycobacterium, was initially isolated in 2005 from striped bass [10]. Its biochemical reactions, growth characteristics and mycolic acid profiles resemble those of *M. shottsii*, a nonpigmented mycobacterium that was isolated during the same epizootic outbreak [10]. However, the sequences of the 16S rRNA gene and the gene encoding the 65 kDa heat shock protein (hsp65) revealed that the isolate was unique [9, 10]. Initially, *M. pseudoshottsii* was found only in wild Chesapeake Bay striped bass; however, both the range of host species and the area of disease distribution have expanded to a variety of fishes and locations [13, 15]. In Japan, molecular and genotypic examinations of piscine-related nontuberculous mycobacteria (NTM) are rare. Here, we report on the genotypic analysis of mycobacteria isolated from infected fishes raised on farms in western Japan.

Twenty-four isolates were recovered from moribund yellowtails (*Seriola quinqueradiata*) , greater amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*), sevenband grouper (*Epinephelus septemfasciatus*), and yellowtail amberjack (*Seriola lalandi*) at fish farms in the western part of Japan from 1999 to 2008 (Table 1). The diseased fish generally showed lethargy, anorexia, emaciation and abdominal distension with ascites. Sometimes, mass culling of the same fish group at a farm was needed because of mass mortality. In some cases, skin ulceration and eye corneal ulceration were observed. White nodules were often found in several internal organs especially in enlarged spleens and kidneys. Isolation was attempted with the affected organ, kidneys, spleen, liver and gills of each fish. These tissues were aseptically dissected, homogenized in phosphate buffered saline, inoculated on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) or homogenized with 4% NaOH for 10 min and inoculated on 1% Ogawa egg slant (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Incubation was performed at 23 to 25°C for 2 to 3 months. Subculture were performed for colony purification with 2% Ogawa egg slant and/or Middlebrook 7H11 agar supplemented with 10% OADC enrichment (Becton, Dickinson and Company, Fukushima, Japan).

Multigenotypic analysis was used to identify the resulting isolates. One loopful of mycobacterial colonies on Ogawa egg slant or 7H11 agar was suspended in 400 μl sterilized phosphate-buffered saline supplemented with 0.05% Tween 80 and was stored at −80°C until DNA was extracted. A frozen bacterial suspension was crushed in a bead-beating instrument (Magnalizer; Roche Diagnostics Japan, Tokyo, Japan) at 3,000 rpm for 90 sec with zirconia beads (diameter, 2 mm). Total genomic DNA was purified from the crashed suspension using a High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics Japan, Tokyo, Japan) and was stored at −20°C.

An approximately 1,500-bp fragment of the 16S rRNA gene, the partial sequences of the *hsp65* and *rpoB* genes and the internal transcribed spacer between the 16S and 23S rRNA genes (ITS region) were amplified by PCR using AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, U.S.A.) with the primers listed in Table 2. The amplicons of the isolates were sequenced using an ABI

* CORRESPONDENCE TO: NAKANAGA, K., Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4–2–1 Aoba, Higashimurayama, Tokyo 189–0002, Japan.
e-mail: nakanaga@nih.go.jp

Table 1. Origin of the mycobacterial strains used in this study

Strain	Host fish	Isolation date	Site isolated	Location of fish farm (Prefecture)
MF01	Yellow tail (*Seriola quinqueradiata*)	Oct/19/2004	Kidney	Kagoshima
MF06	Yellow tail (*Seriola quinqueradiata*)	Sep/08/2008	Kidney	Kagoshima
MF09	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	Kidney	Oita
MF10	Yellow tail (*Seriola quinqueradiata*)	Oct/31/2001	Kidney	Oita
MF12	Yellow tail (*Seriola quinqueradiata*)	Aug/19/2008	Kidney	Ehime
MF14	Yellow tail (*Seriola quinqueradiata*)	Aug/29/2008	Kidney	Ehime
MF31	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Spleen	Kagoshima
MF32	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF33	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF34	Yellow tail (*Seriola quinqueradiata*)	Oct/18/2004	Kidney	Kagoshima
MF35	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF36	Yellow tail (*Seriola quinqueradiata*)	Feb/02/2005	Kidney	Kagoshima
MF44	Yellow tail (*Seriola quinqueradiata*)	Jul /19/2001	NC[a)]	Oita
MF45	Yellow tail (*Seriola quinqueradiata*)	Oct/01/2001	NC	Oita
MF46	Yellow tail (*Seriola quinqueradiata*)	NC/– /2004	NC	Ehime
MF02	Greater amberjack (*Seriola dumerili*)	Jan/06/2005	Kidney	Kagoshima
MF05	Greater amberjack (*Seriola dumerili*)	Nov/15/2005	Kidney	Kagoshima
MF07	Greater amberjack (*Seriola dumerili*)	NC/– /2006	Miliary nodule	Miyazaki
MF40	Greater amberjack (*Seriola dumerili*)	Jan/05/2004	NC	Kagoshima
MF13	Sevenband grouper (*Epinephelus septemfasciatus*)	Aug/18/2008	Kidney	Ehime
MF15	Sevenband grouper (*Epinephelus septemfasciatus*)	Oct/– /2008	Kidney	Ehime
MF04	Striped jack (*Pseudocaranx dentex*)	Nov/15/2005	Kidney	Kagoshima
MF08	Striped jack (*Pseudocaranx dentex*)	Sep/06/1999	Kidney	Oita
MF11	Yellowtail amberjack (*Seriola lalandi*)	Aug/09/2007	Spleen	Oita

a) Not clear.

Table 2. Primers used in this study

Primer	Sequence (positions)	PCR target (fragment size)	Reference
8F16S	5'-AGAGTTTGATCCTGGCTCAG- 3' (8–27)	16S rRNA gene (app. 1,500 bp)	12
1047R16S	5'-TGCACACAGGCCACAAGGGA- 3' (1,047–1,028)		
830F16S	5'-GTGTGGGTTTCCTTCCTTGG- 3' (830–849)		
1542R16S	5'-AAGGAGGTGATCCAGCCGCA- 3' (1,542–1,523)		
ITSF	5'-TTGTACACACCGCCCGTC- 3' (16S, 1,390–)	16S-23S ITS region (app. 340 bp)	11
ITSR	5'-TCTCGATGCCAAGGCATCCACC- 3' (23S, 44–)		
TB11	5'-ACCAACGATGGTGTGTCCAT- 3' (398–417)	*hsp65* (439bp)	16
TB12	5'-CTTGTCGAACCGCATACCCT- 3' (836–817)		
MF	5'-CGACCACTTCGGCAACCG- 3'	*rpoB* (342 bp)	5
MR	5'-TCGATCGGGCACATCCGG- 3'		

Prism 310 PCR Genetic Analyzer (Applied Biosystems) [6] and compared to the sequences of six strains of mycobacteria: "*M. ulcerans* subsp. *shinshuense*" ATCC33728 [6], *M. ulcerans* ATCC19423 (type strain), *M. ulcerans* Agy99 [14], *M. marinum* ATCC 927 (type strain), *M. marinum* clinical isolate strain 112509 (the preceding 5 strains originated in humans) and *M. pseudoshottsii* JCM15466 (type strain). The JCM strain was distributed by the Microbe Division of the Riken BioResource Center (BRC; Saitama, Japan). Isolate and reference sequences were deposited into the DNA Data Bank of Japan (DDBJ) under accession numbers AB548704 to AB548734 and AB642161 to AB642165.

The sequences of the 1,475-bp fragment of 16S rRNA gene from the piscine isolates showed almost complete identity with the *M. pseudoshottsii* reference strain (99.93–100% identity). Only a single mismatch was found at nucleotide position 487 or 488 in 9 of 24 piscine isolates compared with the DNA sequence of *M. pseudoshottsii* JCM15466. However, conserved mismatches with the 5 strains that originated in humans were found at nucleotide positions 95, 969, 1,007 and 1,215 (Table 3). *M. ulcerans* Agy99 had sum upped 3-base pair insertion (TTT) at nucleotide position 1,449–1,451. Similarly, the ITS regions of the piscine isolates and the *M. pseudoshottsii* reference strain were either identical or differed at position 57, while conserved mismatches with the strains originating in humans were at nucleotide positions 30 and 62. All of the sequences of *hsp65* and *rpoB* gene fragments from the iso-

Table 3. Alignment of the 16S rRNA, ITS, *hsp65* and *rpoB* gene sequences from 24 piscine isolates and 6 reference strains[a)]

Strain	Prefecture/ Country	Nucleotide sequence positions																			
		16S rRNA[b)]								ITS region				*hsp65*[c)]						*rpoB*[d)]	
		95	487–8	492	969	1007	1215	1247	1288	30	57	62	83	455	571	637	639	647	797	92	143
M. shinshuense ATCC 33728	Nagano/Japan	T	GG	G	A	G	T	G	G	G	G	T	A	C	T	C	C	A	T	C	C
M. ulcerans ATCC 19423^T	NC[e)]/ Australia	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. ulcerans Agy99	NC / Ghana	T	GG	A	A	G	T	G	C	G	G	T	A	T	C	C	C	A	C	T	C
M. marinum ATCC 927^T	NC / USA	T	GG	A	A	G	T	A	A	G	G	T	A	C	C	C	T	G	C	C	G
M. marinum 112509	Tokyo/Japan	T	GG	A	A	G	T	A	A	G	G	T	G	C	C	C	T	G	C	C	G
M. pseudoshottsii JCM 15466^T	NC / USA	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF01 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF06 (yellow tail)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF09 (yellow tail)	Oita/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF10 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF12 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF14 (yellow tail)	Ehime/Japan	C	AA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF31 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF32 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF33 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF34 (yellow tail)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF35 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF36 (yellow tail)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF44 (yellow tail)	Oita/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF45 (yellow tail)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF46 (yellow tail)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF02 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF05 (greater amberjack)	Kagoshima/Japan	C	GG	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF07 (greater amberjack)	Miyazaki /Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF40 (greater amberjack)	Kagoshima/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF13 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF15 (sevenband grouper)	Ehime/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF04 (striped jack)	Kagoshima/Japan	C	AA	A	G	T	C	A	A	A	T	C	A	C	C	T	C	G	C	C	C
MF08 (striped jack)	Oita/Japan	C	GG	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C
MF11 (yellowtail amberjack)	Oita/Japan	C	GA	A	G	T	C	A	A	A	G	C	A	C	C	T	C	G	C	C	C

a) Only nucleotide differences are noted. Nucleotide positions were based on the b) *E. coli* 16S rRNA gene (accession No. J01859). c) *M. tuberculosis hsp65* gene. (accession No. M15467) and d) *rpoB* gene (accession No. AF057454). e) Not clear.

lates showed complete identity with those of the *M. pseudoshottsii* sequences (Table 3). A conserved mismatch between piscine and human isolates in *hsp65* gene fragments was only found at nucleotide position 637. The results showed that the 24 piscine isolates were all identified as *M. pseudoshottsii* rather than *M. marinum*.

A lethal case of *M. marinum* in cultured yellowtails, which was identified using biological, biochemical and 16S rRNA sequence analyses, has been reported in Japan [17]. In our study, *M. pseudoshottsii* was identified as an additional source of atypical piscine mycobacteriosis and (the bacteria) had been distributed in farmed fisheries in the west part of Japan since 1999. Further studies are needed to develop an easier method to distinguish *M. pseudoshottsii* from *M. marinum* because both strains might have not been differentiated before in Japan. Their differences in susceptibility to antimicrobial agents and in capacity for human pathogenesis should be elucidated. In addition, *M. pseudoshottsii* produces a unique plasmid-encoded toxic macrolide, mycolactone F [7], suggesting that *M. pseudoshottsii* provides a reservoir in aquatic environments for the horizontal transfer of the plasmid-borne genes that encode mycolactone F. Interestingly the potency of mycolactone F with regard to apoptosis in a mammalian cell line was significantly less than that of mycolactone A/B, which is produced by *M. ulcerans*, a causative agent of Buruli ulcer [17]. Further molecular, biochemical and drug susceptibility studies are needed to understand the possible role of mycolactone F in mycobacteriosis and to fully characterize piscine mycobacterial infections in Japan.

ACKNOWLEDGMENTS. We thank Prof. Yoshida (University of Miyazaki, Japan), Dr. Fukuda (Oita Prefectural Agriculture, Forestry and Fisheries Research Center) and Dr. Yamashita (Ehime Prefectural Agriculture, Forestry and Fisheries Research Center) for providing mycobacterial isolates obtained from affected fishes. This work was supported in part by a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labour, and Welfare of Japan to Y. H., M. M. and N. I., by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Tech-

nology of Japan to Y. H. and by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science to K. N.

REFERENCES

1. Chemlal, K. and Portaels, F. 2003. Molecular diagnosis of non-tuberculous mycobacteria. *Curr. Opin. Infect. Dis.* **16**: 77–83.
2. Decostere, A., Hermans, K. and Haesebrouck, F. 2004. Piscine mycobacteriosis: a literature review covering the agent and the disease it causes in fish and humans. *Vet. Microbiol.* **99**: 159–166.
3. Devulder, G., Pérouse de Montclos, M. and Flandrois, J. P. 2005. A multigene approach to phylogenetic analysis using the genus Mycobacterium as a model. *Int. J. Syst. Evol. Microbiol.* **55**: 293–302.
4. Frerichs, G. N. 1993. Mycobacteriosis: nocardiosis. pp. 219–234. *In*: Bacterial Diseases of Fish (Inglis, V., Roberts , R. J. and Bromage, N. R. eds.), Halsted Press, New York.
5. Kim, B. J., Lee, S. H., Lyu, M. A., Kim, S. J., Bai, G. H., Kim, S. J., Chae, G. T., Kim, E. C., Cha, C. Y. and Kook, Y. H. 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (*rpoB*). *J. Clin. Microbiol.* **37**: 1714–1720.
6. Nakanaga, K., Ishii, N., Suzuki, K., Tanigawa, K., Goto, M., Okabe, T., Imada, H., Kodama, A., Iwamoto, T., Takahashi, H. and Saito, H. 2007. "*Mycobacterium ulcerans* subsp. *shinshuense*" isolated from a skin ulcer lesion: identification based on 16S rRNA gene sequencing. *J. Clin. Microbiol.* **45**: 3840–3843.
7. Pidot, S. J., Hong, H., Seemann, T., Porter, J. L., Yip, M. J., Men, A., Johnson, M., Wilson, P., Davies, J. K., Leadlay, P. F. and Stinear, T. P. 2008. Deciphering the genetic basis for polyketide variation among mycobacteria producing mycolactones. *BMC Genomics* **9**: 462.
8. Ranger, B. S., Mahrous, E. A., Mosi, L., Adusumilli, S., Lee, R. E., Colorni, A., Rhodes, M. and Small, P. L. C. 2006. Globally distributed Mycobacterial fish pathogens produce a novel plasmid-encoded toxic macrolide, mycolactone F. *Infect. Immun.* **74**: 6037–6045.
9. Rhodes, M. W., Kator, H., Kotob, S., van Berkum, P., Kaattari, I., Vogelbein, W., Floyd, M. M., Butler, W. R., Quinn, F. D., Ottinger, C. and Shotts, E. 2001. A unique Mycobacterium species isolated from an epizootic of striped bass (Morone saxatilis). *Emerg. Infect. Dis.* **7**: 896–899.
10. Rhodes, M. W., Kator, H., McNabb, A., Deshayes, C., Reyrat, J., Brown-Elliott, B. A., Wallace, R., Trott, K., Parker, J. M., Lifland, B., Osterhout, G., Kaattari, I., Reece, K., Vogelbein, W. and Ottinger, C. A. 2005. *Mycobacterium pseudoshottsii* sp. nov., a slowly growing chromogenic species isolated from Chesapeake Bay striped bass (Morone saxatilis). *Int. J. Syst. Evol. Microbiol.* **55**: 1139–1147.
11. Roth, A., Fischer, M., Hamid, M. E., Michalke, S., Ludwig, W. and Mauch, H. 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *J. Clin. Microbiol.* **36**: 139–147.
12. Springer, B., Wu, W. K., Bodmer, T., Haase, G., Pfyffer, G. E., Kroppenstedt, R. M., Schroder, K. H., Emler, S., Kilburn, J. O., Kirschner, P., Telenti, A., Coyle, M. B. and Böttger, E. C. 1996. Isolation and characterization of a unique group of slowly growing mycobacteria: description of *Mycobacterium lentiflavum* sp. nov. *J. Clin. Microbiol.* **34**: 1100–1107.
13. Stine, C. B., Jakobs, J. M., Rhodes, M. R., Overton, A., Fast, M. and Baya, A. M. 2009. Expanded range and new host species of *Mycobacterium shottsii* and *M. pseudoshottsii*. *J. Aquat. Anim. Health* **21**: 179–183.
14. Stinear, T. P., Mve-Obiang, A., Small, P. L., Frigui, W., Pryor, M. J., Brosch, R., Jenkin, G. A. Johnson, P. D., Davies, J. K., Lee, R. E., Adusumilli, S., Garnier, T., Haydock, S. F., Leadlay, P. F. and Cole, S. T. 2004. Giant plasmid-encoded polyketide synthases produce the macrolide toxin of *Mycobacterium ulcerans*. *Proc. Natl. Acad. Sci. U.S.A.* **101**: 1345–1349.
15. Stragier, P., Hermans, K., Stinear, T. and Portaels, F. 2008. First report of a mycolactone-producing Mycobacterium infection in agriculture in Belgium. *FEMS Microbiol. Lett.* **286**: 93–95.
16. Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E. C. and Bodmer, T. 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J. Clin. Microbiol.* **31**: 175–178.
17. Weerakhun, S., Aoki, N., Kurata, O., Hatai, K., Nibe, H. and Hirae, T. 2007. *Mycobacterium marinum* infection in cultured yellowtail Seriiola quinqueradiata in Japan. *Fish Pathol.* **42**: 79–84.

AAC
Journals.ASM.org

Mutation Analysis of Mycobacterial *rpoB* Genes and Rifampin Resistance Using Recombinant *Mycobacterium smegmatis*

Noboru Nakata, Masanori Kai, and Masahiko Makino

Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Rifampin is a major drug used to treat leprosy and tuberculosis. The rifampin resistance of *Mycobacterium leprae* and *Mycobacterium tuberculosis* results from a mutation in the *rpoB* gene, encoding the β subunit of RNA polymerase. A method for the molecular determination of rifampin resistance in these two mycobacteria would be clinically valuable, but the relationship between the mutations and susceptibility to rifampin must be clarified before its use. Analyses of mutations responsible for rifampin resistance using clinical isolates present some limitations. Each clinical isolate has its own genetic variations in some loci other than *rpoB*, which might affect rifampin susceptibility. For this study, we constructed recombinant strains of *Mycobacterium smegmatis* carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without mutation and disrupted their own *rpoB* genes on the chromosome. The rifampin and rifabutin susceptibilities of the recombinant bacteria were measured to examine the influence of the mutations. The results confirmed that several mutations detected in clinical isolates of these two pathogenic mycobacteria can confer rifampin resistance, but they also suggested that some mutations detected in *M. leprae* isolates or rifampin-resistant *M. tuberculosis* isolates are not involved in rifampin resistance.

Leprosy and tuberculosis persist as important global public health concerns. Rifampin, a major drug used to treat these two infectious diseases, has a molecular mechanism of activity involving the inhibition of DNA-dependent RNA polymerase (15). In *Escherichia coli*, this enzyme is a complex oligomer comprised of four subunits, α, β, β′, and σ, encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD*, respectively. Rifampin binds to the β subunit of RNA polymerase and results in transcription inhibition (15). Mutations in the *rpoB* gene, encoding the β subunit of RNA polymerase, reportedly result in resistance to rifampin in several mycobacterial species, including *Mycobacterium leprae* and *Mycobacterium tuberculosis* (9, 21). The former has not yet been cultured on artificial media; it requires 11 to 14 days to double in experimentally infected mice. Therefore, it is difficult to determine the rifampin susceptibilities of *M. leprae* isolates. The standardized method using a mouse footpad takes more than half a year to determine the rifampin susceptibility of *M. leprae* isolates and requires 5×10^3 *M. leprae* bacilli (3), which require almost a year to prepare. *In vitro* drug susceptibility testing for *M. leprae* using a radioactive reagent requires more (10^7) *M. leprae* cells (7). In contrast, mutations in the *rpoB* gene of *M. leprae* can be detected in a few days or less. It would be very helpful if mutations responsible for rifampin resistance could be determined without performing mouse footpad testing. The main mutations that confer rifampin resistance to *M. tuberculosis* are located in the 81-bp core region of the *rpoB* gene, encompassing codons 507 to 533, known as the rifampin resistance-determining region (RRDR) (17, 18). About 95% of rifampin-resistant *M. tuberculosis* strains have a mutation in this region (18, 20). Four mutations, D516V, H526Y, H526D, and S531L, are most commonly associated with the high-level rifampin resistance of *M. tuberculosis* strains (4, 10, 19), but some other mutations in the 81-bp region have not yet been confirmed completely as being responsible for rifampin resistance.

We have established a method to determine the mutations responsible for the dapsone resistance of *M. leprae* using recombinant *Mycobacterium smegmatis* strains (16). In the present study, we assessed the applicability of the determination of rifampin resistance for analysis. We then analyzed *rpoB* mutations conferring rifampin resistance to *M. leprae* and *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* DH5α was used for DNA cloning. *M. smegmatis* mc^{2}155 was used as a mycobacterial host to produce strains for drug susceptibility testing. Plasmids pYUB854 and phAE87 were kindly provided by W. R. Jacobs, Jr. (Department of Microbiology and Immunology, Albert Einstein College of Medicine, New York, NY). *M. smegmatis* mc^{2}155 and its transformants were grown in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% bovine serum albumin (fraction V), 0.2% glucose, 0.085% NaCl, 0.2% glycerol, and 0.1% Tween 80.

Site-directed mutagenesis. The wild-type *rpoB* genes of *M. leprae* and *M. tuberculosis* were amplified from *M. leprae* Thai-53 and *M. tuberculosis* H37Rv by PCR and cloned into pMV261. Site-directed mutagenesis was performed by using PCR with DNA polymerase (Takara PrimeStar HS; Takara Bio Inc., Kyoto, Japan) and the primers presented in Table 1. PCR products were purified and phosphorylated with T4 kinase and ATP and were then ligated to make them circular. The ligation mixture was used to transform *E. coli* DH5α cells, and kanamycin-resistant colonies were isolated. Plasmids were extracted from the transformants. The mutated sequences were then confirmed by sequencing. The inserts of the plasmids were also cloned into pNN301 (16). Mutations introduced into the *M. leprae rpoB* or *M. tuberculosis rpoB* gene are listed in Table 2.

Disruption of the *rpoB* gene on the *M. smegmatis* chromosome. *M. smegmatis* mc^{2}155 cells were transformed with plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation. Recombinants were selected on LB medium containing kanamycin. Allel-

Received 30 September 2011 Returned for modification 31 October 2011
Accepted 4 January 2012
Published ahead of print 17 January 2012
Address correspondence to Noboru Nakata, n-nakata@nih.go.jp.
Supplemental material for this article may be found at http://aac.asm.org/.

doi:10.1128/AAC.05831-11

TABLE 1 Primers used for this study

Primer	Sequence[a]	Application
M. smegmatis		
MSRBUF	GCCTTAAGGAGGAGAAGGACGAGGCCAC	*rpoB* disruption, upstream forward
MSRBUR	GCTCTAGACAAGATGCATCCTTCCAGCA	*rpoB* disruption, upstream reverse
MSRBDF	GCAAGCTTTCGCGCAACGAATCCGCGTC	*rpoB* disruption, downstream forward
MSRBDR	GCACTAGTAGCGCACGCAGCTTCTTCTG	*rpoB* disruption, downstream reverse
MSRBF	TGGTCAAGCAGTTCCTCAAC	Detection of *rpoB* disruption, forward
MSRBR	CGTTGTTGACGATGATCTCG	Detection of *rpoB* disruption, reverse
M. leprae		
MLRBWTF	GCGGATCCGTGCTGGAAGGATGCATCTT	Cloning of *M. leprae rpoB*, forward
MLRBWTR	GCGTTAACCTAAGCCAGATCTTCTATGG	Cloning of *M. leprae rpoB*, reverse
MLRBWTF1	CAGTTCATGGATCAGAACAACCCTC	Introduction of point mutation at codons 507 and 508
MLRBWTF2	TGTCGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation at codon 526
MLRBWTF3	TTCGCACTACGGCCGGATGTGCCCG	Introduction of point mutation at codon 547
MLRBWTR1	CGACAGCTGGCTGGTGCCGAAGAAT	Introduction of point mutation at codons 513, 516, and 517
MLRBWTR2	GCCGGCGCTTGTGGGTCAGGCCCGA	Introduction of point mutation at codons 531, 532, and 533
MLRB507GGG	CGACAGCTGGCTGGTCCCGAAGAAT	Introduction of point mutation GGC507→GGG
MLRB507AGC	CGACAGCTGGCTGGTGCTGAAGAAT	Introduction of point mutation GGC507→AGC
MLRB508ACA	CGACAGCTGGCTTGTGCCGAAGAAT	Introduction of point mutation ACC508→ACA
MLRB513GTG	GTGTTCATGGATCAGAACAACCCTC	Introduction of point mutation CAG513→GTG
MLRB516AAT	CAGTTCATGAATCAGAACAACCCTC	Introduction of point mutation GAT516→AAT
MLRB517CAT	CAGTTCATGGATCATAACAACCCTC	Introduction of point mutation CAG517→CAT
MLRB526TAC	GCCGGCGCTTGTAGGTCAGGCCCGA	Introduction of point mutation CAC526→TAC
MLRB531TTG	TGTTGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TTG
MLRB531TGG	TGTGGGCGCTGGGCCCGGGTGGTTT	Introduction of point mutation TCG531→TGG
MLRB532TCG	TGTCGTCGCTGGGCCCGGGTGGTTT	Introduction of point mutation GCG532→TCG
MLRB533CCG	TGTCGGCGCCGGGCCCGGGTGGTTT	Introduction of point mutation CTG533→CCG
MLRB547ATC	GGGTGCACGTCACGGATCTCTAGCC	Introduction of point mutation GTC547→ATC
M. tuberculosis		
MTRBWTF	GCGAATTCTTGGCAGATTCCCGCCAGAG	Cloning of *M. tuberculosis rpoB*, forward
MTRBWTR	GCAAGCTTTTACGCAAGATCCTCGACAC	Cloning of *M. tuberculosis rpoB*, reverse
MTRBWTF1	AATTCATGGACCAGAACAACCCGCT	Introduction of point mutation at codons 507, 508, 510, 511, 512, and 513 and deletion of codons 506-508
MTRBWTF2	CTGTCGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation at codons 522, 523, 526, and 531
MTRBWTR1	GGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of mutation at codons 514, 516, 518, 519, and 521; deletion of codon 518; and insertion of TTC between codons 514 and 515
MTRBWTR2	TCGGCGCTTGTGGGTCAACCCCGAC	Introduction of point mutations TCG531→TTC and TCG531→TTG
MTRB507AGC	GGCTCAGCTGGCTGGTGCTGAAGAA	Introduction of point mutation GGC507→AGC
MTRB507GAT	GGCTCAGCTGGCTGGTATCGAAGAA	Introduction of point mutation GGC507→GAT
MTRB508CAC	GGCTCAGCTGGCTGTGGCCGAAGAA	Introduction of point mutation ACC508→CAC
MTRB508GCC	GGCTCAGCTGGCTGGCGCCGAAGAA	Introduction of point mutation ACC508→GCC
MTRB510CAT	GGCTCAGATGGCTGGTGCCGAAGAA	Introduction of point mutation CAG510→CAT
MTRB511CCG	GGCTCGGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CTG511→CCG
MTRB513AAT1	TGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→AAT
MTRB513AAT2	ATTTCATGGACCAGAACAACCCGCT	Introduction of point mutation CAA513→AAT
MTRB513GAA	CGCTCAGCTGGCTGGTGCCGAAGAA	Introduction of point mutation CAA513→GAA
MTRB516GAG	AATTCATGGAGCAGAACAACCCGCT	Introduction of point mutation GAC516→GAG
MTRB516CAC	AATTCATGCACCAGAACAACCCGCT	Introduction of point mutation GAC516→CAC
MTRB516GTC	AATTCATGGTCCAGAACAACCCGCT	Introduction of point mutation GAC516→GTC
MTRB521ATG	AATTCATGGACCAGAACAACCCGAT	Introduction of point mutation CTG521→ATG
MTRB522TTG	TCGGCGCTTGTGGGTCAACCCCAAC	Introduction of point mutation TCG522→TTG
MTRB523GCG	TCGGCGCTTGTGGGTCAACGCCGAC	Introduction of point mutation GGG523→GCG
MTRB523GGC	TCGGCGCTTGTGGGTCAAGCCCGAC	Introduction of point mutation GGG523→GGC
MTRB526CTC	TCGGCGCTTGAGGGTCAACCCCGAC	Introduction of point mutation CAC526→CTC
MTRB526TAC	TCGGCGCTTGTAGGTCAACCCCGAC	Introduction of point mutation CAC526→TAC
MTRB526GAC	TCGGCGCTTGTCGGTCAACCCCGAC	Introduction of point mutation CAC526→GAC
MTRB526TTC	TCGGCGCTTGAAGGTCAACCCCGAC	Introduction of point mutation CAC526→TTC
MTRB526AAC	TCGGCGCTTGTTGGTCAACCCCGAC	Introduction of point mutation CAC526→AAC
MTRB526CGC	TCGGCGCTTGCGGGTCAACCCCGAC	Introduction of point mutation CAC526→CGC
MTRB526CAA	TCGGCGCTTTTGGGTCAACCCCGAC	Introduction of point mutation CAC526→CAA
MTRB529AAA	TTTGCGCTTGTGGGTCAACC	Introduction of point mutation CGA529→AAA
MTRB531TTC	CTGTTCGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTC
MTRB531TTG	CTGTTGGCGCTGGGGCCCGGCGGTC	Introduction of point mutation TCG531→TTG
MTRB506d	GGCTCAGCTGGCTGAACTCCTTGAT	Introduction of mutation 506-508del
MTRBin514TTC	AATTCTTCATGGACCAGAACAACCC	Introduction of mutation 514insTTC
MTRBd518	AATTCATGGACCAGAACCCGCTGTC	Introduction of mutation 518del

[a] Restriction sites are underlined.

TABLE 2 Rifampin and rifabutin susceptibilities of the recombinant *M. smegmatis* strains

Mutation	Rifampin		Rifabutin		Reference(s)
	MIC (μg/ml)	Fold increase[a]	MIC (μg/ml)	Fold increase	
M. leprae					
Wild type	1		0.25		
GGC507→GGG (silent)	1	1	0.25	1	This study
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	3
ACC508→ACA (silent)	1	1	0.25	1	This study
CAG513→GTG (Q513V)	32	32	8	32	3
GAT516→AAT (D516N)	32	32	2	8	14
CAG517→CAT (Q517H)	1	1	0.25	1	11
CAC526→TAC (H526Y)	32	32	8	32	14
TCG531→TTG (S531L)	32	32	4	16	3, 14
TCG531→TGG (S531W)	32	32	8	32	14
GCG532→TCG (A532S)	1	1	0.25	1	11
CTG533→CCG (L533P)	32	32	4	16	14
GTC547→ATC (V547I)	1	1	0.25	1	This study
M. tuberculosis					
Wild type	1		0.25		
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	1
GGC507→GAT (G507D)	0.5	0.5	0.125	0.5	1
ACC508→CAC (T508H)	0.5	0.5	0.125	0.5	1
ACC508→GCC (T508A)	1	1	0.25	1	1
CAG510→CAT (Q510H)	1	1	0.25	1	22
CTG511→CCG (L511P)	16	16	1	4	1, 12
CAA513→AAT (Q513N)	8	8	0.5	2	1
CAA513→GAA (Q513E)	32	32	2	8	1
GAC516→GAG (D516E)	8	8	0.5	2	12
GAC516→CAC (D516H)	1	1	0.25	1	1
GAC516→GTC (D516V)	32	32	2	8	12, 21, 22
CTG521→ATG (L521M)	1	1	0.125	0.5	21
TCG522→TTG (S522L)	>32	>32	8	32	21
GGG523→GCG (G523A)	1	1	0.125	0.5	1
GGG523→GGC (silent)	1	1	0.25	1	1
CAC526→CTC (H526L)	32	32	4	16	12, 22
CAC526→TAC (H526Y)	>32	>32	8	32	12, 22
CAC526→GAC (H526D)	>32	>32	8	32	12, 22
CAC526→TTC (H526F)	>32	>32	4	16	1
CAC526→AAC (H526N)	32	32	2	8	8
CAC526→CGC (H526R)	32	32	8	32	12, 22
CAC526→CAA (H526Q)	8	8	0.5	2	1
CGA529→AAA (R529K)	32	32	4	16	22
TCG531→TTC (S531F)	32	32	4	16	1
TCG531→TTG (S531L)	32	32	8	32	21, 22
506-508del[b]	16	16	0.5	2	5
514insTTC[c]	>32	>32	8	32	12 22
518del[d]	32	32	2	8	22

[a] Fold increase in MIC compared to the wild-type sequence.
[b] Deletion of codons 506 to 508.
[c] Insertion of TTC between codons 514 and 515.
[d] Deletion of codon 518.

ic-exchange mutants were constructed by using a temperature-sensitive mycobacteriophage method described in a previous report (2). Using the *M. smegmatis* mc^{2}155 genome sequence (GenBank accession number CP000480), the upstream and downstream flanking DNA sequences were used to generate a deletion mutation in the *rpoB* gene (MSMEG_1367). To disrupt the *rpoB* gene, DNA segments from 1,119 bp upstream through 21 bp downstream of the initiation codon of *M. smegmatis rpoB* and from 39 bp upstream through 941 bp downstream of the termination codon were cloned directionally into the cosmid vector pYUB854, which contains a *res-hyg-res* cassette and a *cos* sequence for lambda phage assembly. The plasmids thus produced were digested with PacI and ligated into PH101 genomic DNA excised from the phage-plasmid hybrid (phasmid) phAE87 by PacI digestion. The ligated DNA was packaged (GigaPackIII Gold packaging extract; Stratagene, La Jolla, CA). The resultant mixture was used for the transduction of *E. coli* STBL2 cells (Life Technologies Inc., Carlsbad, CA) to yield cosmid DNA. After *E. coli* was transduced and the transductants were plated onto hygromycin-containing medium, phasmid DNA was prepared from the pooled antibiotic-resistant transductants and electroporated into *M. smegmatis* mc^{2}155. Bacterial cells were incubated at 30°C to produce the recombinant phage. The *M. smeg-*

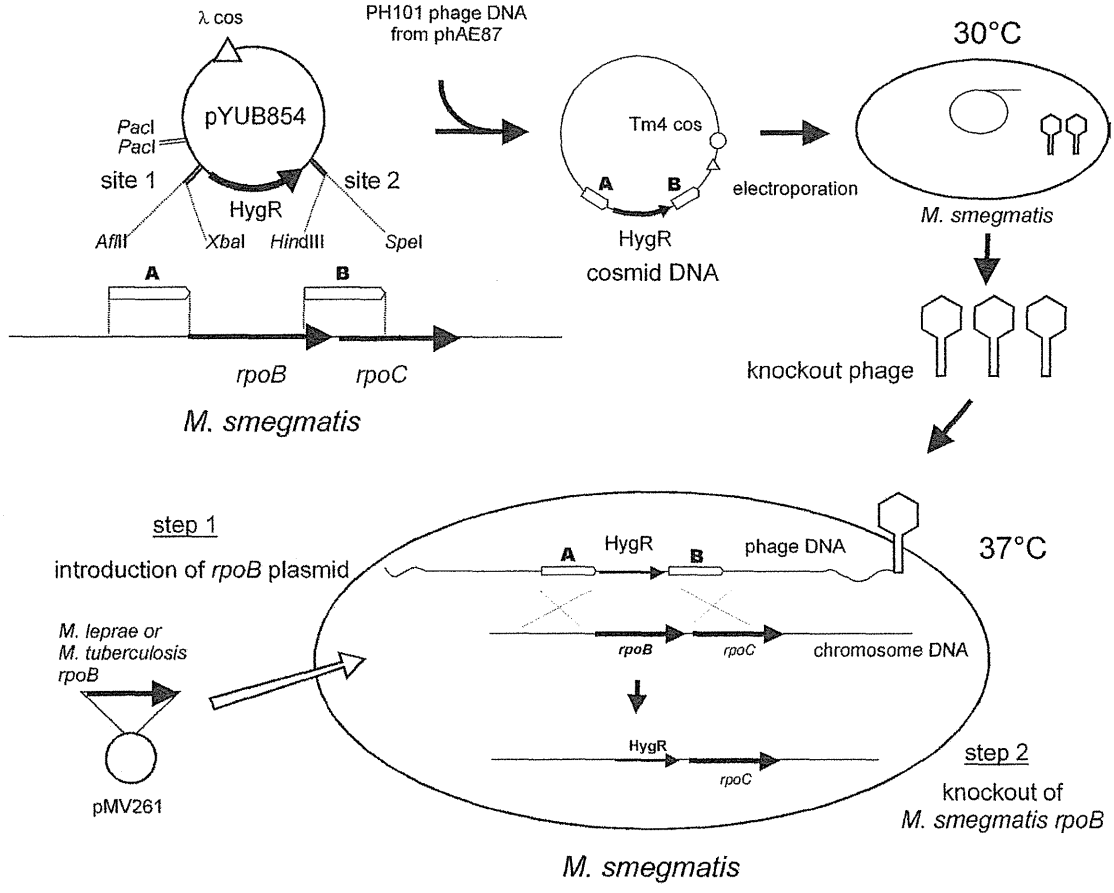

FIG 1 Construction of recombinant *M. smegmatis* strains for rifampin susceptibility testing.

matis transformant carrying the *M. leprae* or *M. tuberculosis rpoB* gene was infected with the produced temperature-sensitive phage at 37°C for allelic exchange, and kanamycin-resistant and hygromycin-resistant colonies were isolated. Two colonies for each point mutation were subjected to subsequent tests.

Drug susceptibility testing. The MIC values for *M. smegmatis* recombinant clones were determined by culture on Middlebrook 7H10 agar plates containing 2-fold serial dilutions of rifampin (0.25 to 32 μg/ml) or rifabutin (0.0625 to 8 μg/ml). The MIC value for each strain was defined as the lowest concentration of the drug necessary to inhibit bacterial growth.

RESULTS

Construction of recombinant *M. smegmatis* strains. In our previous study, we sequenced the *rpoB* regions of *M. leprae* clinical samples isolated in Vietnam and detected several mutations (11). In addition to these mutations, we detected some mutations (GGC→GGG at codon 507, ACC→ACA at codon 508, and GTC→ATC at codon 547) in clinical specimens from Vietnam and other countries (our unpublished data). We prepared plasmids with mutations in the *M. leprae* and *M. tuberculosis rpoB* genes. Each plasmid has one of 40 mutations (12 for *M. leprae rpoB* and 28 for *M. tuberculosis rpoB*) presented in Table 2. The mutated sequences were confirmed by sequencing. Plasmids carrying the *M. leprae* or *M. tuberculosis rpoB* gene with or without a point mutation were introduced individually into *M. smegmatis*. The *M. smegmatis* transformants were subjected to allelic exchange to disrupt the *rpoB* gene on their own chromosome (Fig. 1). The isolation of *rpoB*-disrupted mutants carrying the pNN301-*rpoB* constructs was unsuccessful. Consequently, the recombinant strains with pMV261-*rpoB* constructs were used for subsequent tests. PCR analysis confirmed that the *M. smegmatis rpoB* sequences in the recombinant strains with pMV261-*rpoB* constructs were replaced by hygromycin resistance gene sequences (see Fig. S1 in the supplemental material). All strains showed growth rates comparable to that of wild-type *M. smegmatis*.

Drug susceptibility. The rifampin susceptibilities and rifabutin susceptibilities of the recombinant *M. smegmatis* strains were tested (see Fig. S2 in the supplemental material). The MIC values of rifampin and rifabutin for the recombinant *M. smegmatis* strains and the fold increases in MIC compared to the wild-type sequences are presented in Table 2. It should be noted that the MIC values for the *M. smegmatis* strains might be shifted from those for *M. leprae* or *M. tuberculosis* because of their differences in cell wall permeability and other factors. The MIC value of rifampin for the recombinant *M. smegmatis* strain with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene was 1 μg/ml. Most strains that had a mutation at codon 511, 513, 516, 522, 526, 531, or 533 showed rifampin resistance. In contrast, strains that had a mutation at codon 507, 508, 517, 521, 523, or 532 showed MIC values of rifampin comparable to those for the wild-type sequence. The MIC values of rifabutin for the recombinant

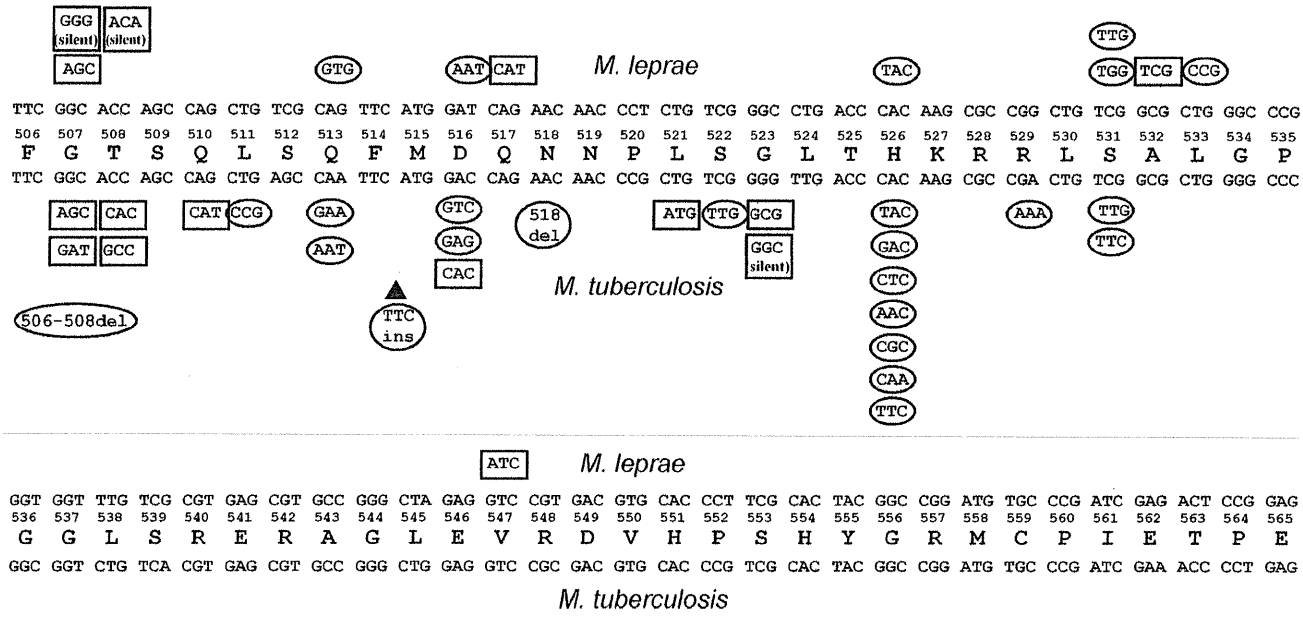

FIG 2 Mutations introduced into the *M. leprae rpoB* gene or *M. tuberculosis rpoB* gene and rifampin susceptibility. The consensus amino acid sequence of *M. leprae* RpoB and *M. tuberculosis* RpoB between codons 506 and 565 is shown. The *M. leprae rpoB* sequence and codons are shown above the consensus amino acid sequence. The *M. tuberculosis rpoB* sequence and codons are shown below the consensus sequence. Mutated codons that gave rise to rifampin resistance are surrounded by ovals. Mutated codons that showed levels of rifampin susceptibility comparable to those of the wild-type sequences are surrounded by rectangles.

M. smegmatis strains with the wild-type sequence of the *M. leprae rpoB* or *M. tuberculosis rpoB* gene were 0.25 μg/ml. Generally, rifabutin was more efficacious than rifampin in terms of concentration.

DISCUSSION

To functionally replace the *rpoB* gene of *M. smegmatis* with the *M. leprae* or *M tuberculosis* counterpart, we used a method established in our previous study (16). Because *rpoB* is a necessary gene for bacterial growth, this genetic locus cannot be disrupted without compensating for its activity. Therefore, we first introduced the *rpoB* gene of *M. leprae* or *M. tuberculosis* into *M. smegmatis* using vector plasmids of two types before disrupting the *rpoB* gene on the *M. smegmatis* chromosome. One vector was pMV261, a multicopy shuttle plasmid. The other was a single-copy integrative shuttle plasmid, pNN301. However, the isolation of *rpoB*-disrupted mutants carrying pNN301-*rpoB* constructs was unsuccessful, probably because of insufficient RpoB expression.

We tested 2 silent mutations and 10 mutations that change amino acid residues for *M. leprae* (Fig. 2). Codons 516, 526, 531, and 533 in the *M. leprae rpoB* gene are known to be codons responsible for rifampin resistance. However, it remains unclear whether or not mutations that have not been reported previously can confer rifampin resistance. Our results show that not all mutations in the *rpoB* gene detected in *M. leprae* clinical samples confer rifampin resistance. *M. leprae* is not cultivable. Therefore, it has been very difficult to analyze the mutation-susceptibility relationship. Using recombinant *M. smegmatis*, however, we can analyze it in a few weeks. We also tested 1 silent mutation, 24 mutations that change amino acids, 2 deletions, and 1 insertion for *M. tuberculosis*. Some mutations did not confer rifampin resistance, which is inconsistent with the susceptibility of the *M. tuberculosis* clinical isolates reported previously. Most mutations at codon 516, 526, or 531 showed rifampin resistance. It is interesting that the strains with the mutation GAC516→CAC for D516H were not rifampin resistant. All other mutations at codon 516 showed rifampin resistance. The mutation GAC516→CAC in *M. tuberculosis* was reported for a strain with multiple mutations and should not be involved in rifampin resistance.

Rifabutin, a spiropiperidyl rifampin, is a rifamycin derivative that is more active than rifampin against slow-growing mycobacteria, including *M. tuberculosis* and *M. avium-M. intracellulare* complex strains, *in vitro* and *in vivo*. It is also active against some rifampin-resistant strains of *M. tuberculosis* (6, 13). Our results indicate that some mutations (e.g., GAT516→AAT of *M. leprae* and GAC516→GAG of *M. tuberculosis*) show weak resistance to rifabutin.

Molecular methods designed to detect drug resistance have some limitations. In some cases, the identified mutations are not related to the acquisition of resistance. Caution is necessary when considering mutations, especially if the mutation detected in clinical isolates is not reported very often. For example, Q510H and L521M mutations were detected in rifampin-resistant *M. tuberculosis* isolates (21, 22), but our results suggest that these mutations are not responsible for rifampin resistance (Table 2). The method used for this study can directly assess the influence of designated mutations in *rpoB*. If the mutations can confer rifampin resistance, we can eliminate the possibility that genetic variation in some region other than *rpoB* on the chromosome of the clinical isolates is responsible for the resistance. Bahrmand et al. previously reported the high-level rifampin resistance of *M. tuberculosis* isolates with multiple mutations within the *rpoB* gene (1). Our method might also be useful for analyzing multiple mutations

detected in the *rpoB* gene of clinical isolates to determine the contribution of each single mutation to rifampin resistance.

ACKNOWLEDGMENTS

This work was supported by grants from the Ministry of Health, Labor, and Welfare (Emerging and Re-Emerging Infectious Diseases).

REFERENCES

1. **Bahrmand AR, Titov LP, Tasbiti AH, Yari S, Graviss EA.** 2009. High-level rifampin resistance correlates with multiple mutations in the rpoB gene of pulmonary tuberculosis isolates from the Afghanistan border of Iran. J. Clin. Microbiol. **47**:2744–2750.
2. **Bardarov S, et al.** 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in *Mycobacterium tuberculosis, M. bovis* BCG and *M. smegmatis.* Microbiology **148**: 3007–3017.
3. **Cambau E, et al.** 2002. Molecular detection of rifampin and ofloxacin resistance for patients who experience relapse of multibacillary leprosy. Clin. Infect. Dis. **34**:39–45.
4. **Cavusoglu C, Turhan A, Akinci P, Soyler I.** 2006. Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. J. Clin. Microbiol. **44**: 2338–2342.
5. **Chikamatsu K, Mizuno K, Yamada H, Mitarai S.** 2009. Cross-resistance between rifampicin and rifabutin among multi-drug resistant *Mycobacterium tuberculosis* strains. Kekkaku **84**:631–633. (In Japanese.)
6. **Dickinson JM, Mitchison DA.** 1987. In vitro activity of new rifamycins against rifampicin-resistant M. tuberculosis and MAIS-complex mycobacteria. Tubercle **68**:177–182.
7. **Franzblau SG, Hastings RC.** 1988. In vitro and in vivo activities of macrolides against Mycobacterium leprae. Antimicrob. Agents Chemother. **32**:1758–1762.
8. **Hauck Y, Fabre M, Vergnaud G, Soler C, Pourcel C.** 2009. Comparison of two commercial assays for the characterization of rpoB mutations in *Mycobacterium tuberculosis* and description of new mutations conferring weak resistance to rifampicin. J. Antimicrob. Chemother. **64**:259–262.
9. **Honore N, Cole ST.** 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae.* Antimicrob. Agents Chemother. **37**:414–418.
10. **Huitric E, Werngren J, Jureen P, Hoffner S.** 2006. Resistance levels and rpoB gene mutations among in vitro-selected rifampin-resistant *Mycobacterium tuberculosis* mutants. Antimicrob. Agents Chemother. **50**:2860–2862.
11. **Kai M, et al.** 2011. Analysis of drug-resistant strains of *Mycobacterium leprae* in an endemic area of Vietnam. Clin. Infect. Dis. **52**:e127–e132.
12. **Kapur V, et al.** 1994. Characterization by automated DNA sequencing of mutations in the gene (rpoB) encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. J. Clin. Microbiol. **32**:1095–1098.
13. **Luna-Herrera J, Reddy MV, Gangadharam PR.** 1995. In-vitro and intracellular activity of rifabutin on drug-susceptible and multiple drug-resistant (MDR) tubercle bacilli. J. Antimicrob. Chemother. **36**:355–363.
14. **Maeda S, et al.** 2001. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob. Agents Chemother. **45**:3635–3639.
15. **McClure WR, Cech CL.** 1978. On the mechanism of rifampicin inhibition of RNA synthesis. J. Biol. Chem. **253**:8949–8956.
16. **Nakata N, Kai M, Makino M.** 2011. Mutation analysis of the *Mycobacterium leprae* folP1 gene and dapsone resistance. Antimicrob. Agents Chemother. **55**:762–766.
17. **Ramaswamy S, Musser JM.** 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber. Lung Dis. **79**:3–29.
18. **Rattan A, Kalia A, Ahmad N.** 1998. Multidrug-resistant *Mycobacterium tuberculosis*: molecular perspectives. Emerg. Infect. Dis. **4**:195–209.
19. **Rigouts L, et al.** 2007. Newly developed primers for comprehensive amplification of the rpoB gene and detection of rifampin resistance in *Mycobacterium tuberculosis.* J. Clin. Microbiol. **45**:252–254.
20. **Telenti A, et al.** 1993. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis.* Lancet **341**:647–650.
21. **Williams DL, et al.** 1994. Characterization of rifampin-resistance in pathogenic mycobacteria. Antimicrob. Agents Chemother. **38**:2380–2386.
22. **Yang B, et al.** 1998. Relationship between antimycobacterial activities of rifampicin, rifabutin and KRM-1648 and *rpoB* mutations of *Mycobacterium tuberculosis.* J. Antimicrob. Chemother. **42**:621–628.