Table 1: Characteristics of subjects.

	Cases with active TB	Cases without active TB	Total
Nationality (Japanese/Thai)	8/1	95/3	103/4
Gender (Male/Female)	8/1	91/7	99/8
Mean age (range)	53 (39–65)	45 (23–75)	46 (23–75)
Mean and SD of CD4$^+$ T cell count (range)/μL	105 ± 90 (9–270)	226 ± 221 (4–934)	214 ± 215 (4–934)
No. of patients treated with ART	4	44	48 (one interrupted)
TB already treated	0	2	2
Healed TB on chest X-ray	0	2	2
M. kansasii disease	0	1	1
N	9	98	107

not return after 48 hours to have their TST read. Thus, the TST was placed for only 26 (24%) and the final results obtained for 23 (21.5%). All subjects with a TST result were Japanese and had been vaccinated with BCG. Of them, 6 had active TB. The TST was positive in 7/23 (30%) patients and negative in 16/23 (70%) (Table 2). The TST positive rate was 4/12 (33%) for those with CD4$^+$ T cell count <200/μL, compared with 3/11 (27%) for those with CD4$^+$ T cell count more than 200/μL (difference nonsignificant, P for Fisher's exact test = 1.00).

Six of the 9 HIV-infected patients with active TB had a TST result, and 3 (50%) were positive. One of these TST positive patients had CD4$^+$ T cell count less than 50/μL. Of the 17 subjects without TB and with a TST result, 4 were positive, equating to a specificity of 76% (=13/17).

3.3. Relationship between CD4$^+$ T Cell Count, Presence of Active TB, and QFT-G Results. QFT-G results were available for all of the 107 HIV-infected subjects, and of them 6 (6%) were positive, 92 (86%) negative, and 9 (8%) indeterminate (Table 3). Indeterminate results were significantly associated with very low CD4$^+$T cell count, with frequency of indeterminate tests being 25% (8/32) in those with CD4$^+$ T cell count less than 50/μL, compared with 1% (1/75) in those with CD4$^+$ T cell count greater than 50/μL (Fisher's P < .0001).

For the 9 patients with active TB, 5 (56%) were positive by QFT-G and 1 (11%) indeterminate. There were 3 TB patients with CD4$^+$ T cell count less than 50/μL, and QFT-G was negative for two and indeterminate for the other. In contrast, all 5 HIV-TB patients with a CD4$^+$ T cell count between 50/μL and 199/μL were QFT-G positive, and the one patient with a CD4$^+$ T cell count between 200/μL and 499/μL was negative but the response value was near the cutoff. Of the 98 HIV positive subjects without active TB, one was positive by QFT-G.

If limiting analysis to those HIV patients with a CD4$^+$ T cell count more than 50/μL, the sensitivity of QFT-G for TB infection as seen in TB patients as surrogates of the infected was 83% (5/6), and specificity was 99% (68/69). QFT-G was negative in the two subjects with chest X-ray evidence compatible with old TB and positive in the patient with *M. kansasii* infection.

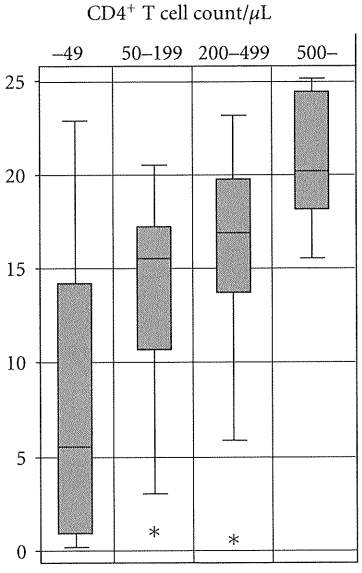

Figure 1: Box plot of the interferon-gamma response to mitogen according to the CD4$^+$ T cell count level. * indicates an outlier.

As for ART status, QFT-G was positive in 3 of 5 active TB patients with ART and 2 of 4 cases without ART. In one patient who developed TB within one month after starting ART, QFT-G was positive.

3.4. Relationship between CD4$^+$ T Cell Count and Positive Control Level in QFT-G. As the QFT-G indeterminate rate was high for HIV patients with CD4$^+$ T cell count less than 50/μL as seen above, we analyzed the relationship between CD4$^+$ T cell count and level of responses to the test's positive control (stimulation with mitogen) for a total of 95 patients excluding those with TB (n = 9) or *M. kansasii* disease (n = 1) and those with negative control response being higher than positive control response (n = 2). As shown in Figure 1 and Table 4, there is a continuous rise in the response level along with the cell count from less than 50/μL up to over 500/μL with statistical significance (Kruskal-Wallis test, P = .0001). There is no significant difference in the level of response between HIV-infected patients with CD4$^+$

Table 2: Relationship between TST results and $CD4^+$ T cell count.

$CD4^+$ T cell count/μL	Presence of active TB disease*	TST positive	TST negative
<200 [<50]	Y ($n = 6$)	3 (50%) [1]*	3 (50%) [2]*
	N ($n = 6$)	1 (17%) [0]*	5 (83%)[3]*
≥200	Y ($n = 0$)	0	0
	N ($n = 11$)	3 (27%)	8 (73%)

Y: present, N: absent.
(%): percentage of Y or N number in each CD4 category.
*[ ] indicates the number of those with $CD4^+$ T cells less than 50.

Table 3: Relationship between QFT-G results and $CD4^+$ T cell count.

Category by $CD4^+$ T cell count/μL	Active TB	Results of QFT-G			
		Negative	Doubtful positive	Positive	Indeterminate+
<50 ($n = 32$)	Y ($n = 3$)	2 (67%)	0	0	1 (33%)
	N ($n = 29$)	22 (76%)	0	0	7 (24%)
50–199 ($n = 29$)	Y ($n = 5$)	0	0	5 (100%)	0
	N ($n = 24$)	23 (96%)	0	1 (4%) a	0
200–499 ($n = 35$)	Y ($n = 1$)	0	1 (10%)	0	0
	N ($n = 34$)	32 (94%)	1 (3%)	0	1 (3%)
≥500 ($n = 11$)	N ($n = 11$)	11(100%)	0	0	0
Total	Y ($n = 9$)	2 (22%)	1 (11%)	5 (56%)	1 (11%)
	N ($n = 98$)	88 (90%)	1 (1%)	1 (1%)	8 (8%)

Y: present, N: absent, and a: *M. kansasii* disease.
(%): percentage of Y or N number in each CD4 category.
+Indeterminate results were significantly associated with $CD4^+$T cell count less than 50/μL, compared with $CD4^+$ T cell count greater than 50/μL (Fisher's $P < .0001$).

T cell count greater than 500/μL and healthy control subjects (Table 4).

4. Discussion

Although the TST has been used as a diagnostic tool for TB infection for many decades, the specificity of the TST is known to be low in not only HIV-infected individuals but also in the general population of Japan where BCG vaccination is widely carried out. Moreover, TST requires two visits of health care providers for administration and measurement of a test with 48-hour time interval. This is a significant barrier for the cooperation of the patients. In fact, only 21.5% of the enrolled patients in our study underwent a TST in the clinical setting of this study, and the number of cases with TST was not enough for thorough evaluation of TST results.

The QFT-G was approved in 2005 in Japan, but there remain several issues to be addressed, such as applicability of QFT-G for children or for immunocompromised populations such as HIV infected individuals [15]. Several reports have been published on QFT-G's performance in the HIV infected [10–12], but the present study is the first report which evaluates the QFT-G performance in a large number of HIV-infected individuals in Japan, one of TB middle-burden countries. The data suggest that QFT-G has high sensitivity for TB infection in HIV coinfected patients who have $CD4^+$ T cell count > 50/μL, but based on a very small sample size of active TB cases, the test had poor sensitivity in patients with very low $CD4^+$ T cell count (<50/μL).

As would be expected for QFT-G, the test was highly specific in the HIV cohort without active TB, with only one of the 69 non-TB patients being QFT-G positive. The one person who was QFT-G positive had *M. kansasii* infection. This is an expected result as *M. kansasii* is one of the few NTM that carry the RD1 gene which encodes the ESAT-6 and CFP-10 proteins used in QFT-G [16]. In contrast, the TST had a poor specificity of 76% (13/17) in the HIV-positive subjects tested, likely due to the effects of BCG vaccination and revaccination in the Japanese population.

Previous studies of QFT-G indeterminate rates for HIV infected reported that their frequency increased with $CD4^+$ T cell count less than 100 or 200/μL [17–20]. Similarly, we found a significant evidence for an increased indeterminate rate in the group of patients with $CD4^+$ T cell count less than 50/μL, although the number of each group was not so large. At the same time we found that there is a clear proportional relationship between T cell count and the level of IFN-gamma response in those with cell count less than 500/μL. This implies that HIV-infected patients with T cell count above 50/μL (and less than 500/μL) have also impaired IFN-gamma response more or less although their QFT-G test results are not "indeterminate". The differences between studies could be due to the relatively small sample sizes, so that cases with slight decrease of response in those with intermediate cell count group could be not judged as

Table 4: Interferon-gamma responses for mitogen according to $CD4^+$ T cell count category in HIV-infected patients without active TB disease and healthy controls.

	<49/μL	50–199/μL	200–499/μL	>500/μL	Healthy controls
Number	27 a	22	35	11	29
Mean	7.48	13.50	16.08	20.40	19.27
S.D.	7.03	5.60	5.17	3.20	5.06
Median	5.55	15.51	16.91	20.21	20.03
Maximum	22.93	20.51	23.19	25.18	26.31
Minimum	0.14	0.67	0.25	15.56	5.12

S.D.: standard deviation.
a. 2 of 29 cases were omitted because negative control value was higher than response to mitogen.

"indeterminate" by chance in a small size of observations. Therefore, care should be taken when we interpret the negative QFT-G test results of such subjects, as is the case with the TST. Of course, in such severely immunosuppressed individuals as with $CD4^+$ T cell count less than 50/μL, no immunologically based test should be considered as definitive and reliable, and clinicians should use all available information in evaluating MTB infection status.

Comparison of the performance of QFT-G and TST in diagnosing MTB infection in HIV positive patients was very limited in our study by the small number of patients for whom TST results were available. QFT-G appeared to have at least as good sensitivity as the TST and significantly better specificity, but the number of subjects was insufficient to make definitive conclusions. Of interest was the very low number of subjects for whom TST results were available (23/107). For most people who were not tested by TST, this was due to the requirement to return 48 hours later to have the test read. This highlights a significant benefit of QFT-G—the fact that only one visit to the clinician is required to obtain a result.

There were some limitations in our study. We used the liquid antigen version of the QFT-G test, which has been replaced by the In-Tube version of the test (QFT-GIT) in most countries worldwide. This makes comparisons of our results with those from other studies difficult as most other studies have used QFT-GIT. Since Harada et al. have shown that QFT-GIT has higher sensitivity than QFT-G with the same high specificity [21], it could be expected that the better performance would be obtained than that obtained in this study. The relatively small number of patients with confirmed active TB limited any detailed analysis of sensitivity and the small number of patients for whom TST results were available limited comparison of test performance.

5. Conclusion

In HIV-infected individuals, sensitivity and specificity of the TST for the diagnosis of TB infection were poor under the influence of BCG vaccination. In contrast, our data suggested that QFT-G had high sensitivity and specificity in HIV-infected populations with $CD4^+$ T cell count greater than 50/μL. However, neither test performed well in HIV-positive patients with $CD4^+$ T cell count less than 50/μL. Therefore, care should be taken when interpreting negative or indeterminate QFT-G results in HIV-infected patients with $CD4^+$ T cell count less than 50/μL. Further studies in HIV-infected people are required to accumulate more QFT-G performance data in active TB patients in developed countries.

Conflict of Interest

We declare that the authors have no conflict of interest.

Acknowledgments

This study was supported by the project research fund of Tokyo Metropolitan Hospitals Group (2006-2007) and the Research Project of Emerging and Re-emerging Diseases (Principal Investigator: S. Kato), funded by Ministry of Health, Labor and Welfare, Japan.

References

[1] CDC, "Prevention and treatment of tuberculosis among patients infected with human immunodeficiency virus: principles of therapy and revised recommendations. Centers for Disease Control and Prevention," *MMWR. Recommendations and Reports*, vol. 47, no. RR-20, pp. 1–58, 1998.

[2] H. Yoshikura, "HIV transmission webs: HIV infection trends in Japan in 1989–2004," *Japanese Journal of Infectious Diseases*, vol. 58, no. 6, pp. S19–S21, 2005.

[3] CDC, "Targeted tuberculin testing and treatment of latent tuberculosis infection. American Thoracic Society.," *MMWR. Recommendations and Reports*, vol. 49, no. RR-6, pp. 1–51, 2000.

[4] T. Mori, T. Yoshiyama, K. Nakata, A. Fujita, and H.. Nagai, "Preventive chemotherapy," in *AIDS- Associated Tuberculosis–Clinical Features and Countermeasures*, T. Kimura and T. Mori, Eds., pp. 127–134, JATA, Tokyo, Japan, 2003 (Japanese).

[5] R. E. Huebner, M. F. Schein, and J. B. Bass Jr., "The tuberculin skin test," *Clinical Infectious Diseases*, vol. 17, no. 6, pp. 968–975, 1993.

[6] 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.

[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," *MMWR. Recommendations and Reports*, vol. 54, no. RR-15, pp. 49–55, 2005.

[8] Prevention Committee and Japanese Society of Tuberculosis, "Guidelines for the use of QuantiFERON®TB-2G," *Kekkaku*, vol. 81, no. 5, pp. 393–397, 2006 (Japanese).

[9] J. E. Kaplan, C. Benson, K. H. Holmes, J. T. Brooks, A. Pau, and H. Masur, "Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents: recommendations from CDC, the National Institutes of Health, and the HIV Medicine Association of the Infectious Diseases Society of America," *MMWR. Recommendations and Reports*, vol. 58, no. RR-4, pp. 1–207, 2009.

[10] M. Bocchino, B. Bellofiore, A. Matarese, D. Galati, and A. Sanduzzi, "IFN-γ release assays in tuberculosis management in selected high-risk populations," *Expert Review of Molecular Diagnostics*, vol. 9, no. 2, pp. 165–177, 2009.

[11] T. Mori, "Usefulness of interferon-gamma release assays for diagnosing TB infection and problems with these assays," *Journal of Infection and Chemotherapy*, vol. 15, no. 3, pp. 143–155, 2009.

[12] H. Nagai, Y. Kawabe, H. Ariga et al., "Usefulness of a whole blood interferon gamma assay (QuantiFERON-TB-2G) for detecting tuberculosis infection in HIV-infected persons," *Kekkaku*, vol. 82, no. 8, pp. 635–640, 2007 (Japanese).

[13] M. Maeda, N. Asami, and T. Murohashi, "Further studies on the potency of purified protein derivatives of tuberculin (PPD-s). 1st Report: comparison of the potency of three preparations of PPD," *Kekkaku*, vol. 35, pp. 563–566, 1960 (Japanese).

[14] N. Harada, K. Higuchi, Y. Sekiya, J. Rothel, T. Kitoh, and T. Mori, "Basic characteristics of a novel diagnostic method (QuantiFERON®TB-2G) of latent tuberculosis infection with a use of Mycobacterium tuberculosis-specific antigens, ESAT-6 and CFP-10," *Kekkaku*, vol. 79, no. 12, pp. 725–735, 2004 (Japanese).

[15] M. Pai, K. Dheda, J. Cunningham, F. Scano, and R. O'Brien, "T-cell assays for the diagnosis of latent tuberculosis infection: moving the research agenda forward," *Lancet Infectious Diseases*, vol. 7, no. 6, pp. 428–438, 2007.

[16] M. Harboe, T. Oettinger, H. G. Wiker, I. Rosenkrands, and P. Andersen, "Evidence for occurrence of the ESAT-6 protein in Mycobacterium tuberculosis and virulent Mycobacterium bovis and for its absence in Mycobacterium bovis BCG," *Infection and Immunity*, vol. 64, no. 1, pp. 16–22, 1996.

[17] I. Brock, M. Ruhwald, B. Lundgren, H. Westh, L. R. Mathiesen, and P. Ravn, "Latent tuberculosis in HIV positive, diagnosed by the *M. tuberculosis* specific interferon-γ test," *Respiratory Research*, vol. 7, article no. 56, 2006.

[18] A. F. Luetkemeyer, E. D. Charlebois, L. L. Flores et al., "Comparison of an interferon-γ release assay with tuberculin skin testing in HIV-infected individuals," *American Journal of Respiratory and Critical Care Medicine*, vol. 175, no. 7, pp. 737–742, 2007.

[19] M. C. Aichelburg, A. Rieger, F. Breitenecker et al., "Detection and prediction of active tuberculosis disease by a whole-blood interferon-γ release assay in HIV-l-infected individuals," *Clinical Infectious Diseases*, vol. 48, no. 7, pp. 954–962, 2009.

[20] N. J. Talati, U. Seybold, B. Humphrey et al., "Poor concordance between interferon-γ release assays and tuberculin skin tests in diagnosis of latent tuberculosis infection among HIV-infected individuals," *BMC Infectious Diseases*, vol. 9, article 15, 2009.

[21] 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.

JCM
Journals ASM.org

Comprehensive Multicenter Evaluation of a New Line Probe Assay Kit for Identification of *Mycobacterium* Species and Detection of Drug-Resistant *Mycobacterium tuberculosis*

Satoshi Mitarai,[a] Seiya Kato,[a] Hideo Ogata,[b] Akio Aono,[b] Kinuyo Chikamatsu,[a] Kazue Mizuno,[b] Emiko Toyota,[c] Akiko Sejimo,[c] Katsuhiro Suzuki,[d] Shiomi Yoshida,[d] Takefumi Saito,[e] Ataru Moriya,[e] Akira Fujita,[f] Shuko Sato,[f] Tomoshige Matsumoto,[g] Hiromi Ano,[g] Toshinori Suetake,[i] Yuji Kondo,[i] Teruo Kirikae,[h] and Toru Mori[a]

Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan[a]; Fukujuji Hospital, Japan Anti-Tuberculosis Association, Tokyo, Japan[b]; Tokyo Hospital, National Hospital Organization, Tokyo, Japan[c]; Kinki-Chuo Chest Medical Center, National Hospital Organization, Osaka, Japan[d]; Ibarakihigashi Hospital, National Hospital Organization, Ibaraki, Japan[e]; Tokyo Metropolitan Tama Medical Center, Tokyo, Japan[f]; Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, Osaka Prefectural Hospital Organization, Osaka, Japan[g]; Research Institute, National Center for Global Health and Medicine, Tokyo, Japan[h]; and Research and Development Laboratory, Nipro Corporation, Shiga, Japan[i]

We evaluated a new line probe assay (LiPA) kit to identify *Mycobacterium* species and to detect mutations related to drug resistance in *Mycobacterium tuberculosis*. A total of 554 clinical isolates of *Mycobacterium tuberculosis* ($n = 316$), *Mycobacterium avium* ($n = 71$), *Mycobacterium intracellulare* ($n = 51$), *Mycobacterium kansasii* ($n = 54$), and other *Mycobacterium* species ($n = 62$) were tested with the LiPA kit in six hospitals. The LiPA kit was also used to directly test 163 sputum specimens. The results of LiPA identification of *Mycobacterium* species in clinical isolates were almost identical to those of conventional methods. Compared with standard drug susceptibility testing results for the clinical isolates, LiPA showed a sensitivity and specificity of 98.9% and 97.3%, respectively, for detecting rifampin (RIF)-resistant clinical isolates; 90.6% and 100%, respectively, for isoniazid (INH) resistance; 89.7% and 96.0%, respectively, for pyrazinamide (PZA) resistance; and 93.0% and 100%, respectively, for levofloxacin (LVX) resistance. The LiPA kit could detect target species directly in sputum specimens, with a sensitivity of 85.6%. Its sensitivity and specificity for detecting RIF-, PZA-, and LVX-resistant isolates in the sputum specimens were both 100%, and those for detecting INH-resistant isolates were 75.0% and 92.9%, respectively. The kit was able to identify mycobacterial bacilli at the species level, as well as drug-resistant phenotypes, with a high sensitivity and specificity.

The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis*, resistant to at least rifampin (RIF) and isoniazid (INH), markedly hinders the control of tuberculosis (8). Nontuberculous mycobacteria (NTM) are also associated with pulmonary diseases (2, 16). Drug resistance in *M. tuberculosis* is due to mutations, including *rpoB* mutations, associated with RIF resistance; mutations in *katG*, the promoter region of the *fabG1-inhA* operon, *fabG1*, *furA*, and *inhA*, associated with INH resistance; *pncA* mutations, associated with pyrazinamide (PZA) resistance; and *gyrA* mutations, associated with resistance to fluoroquinolones (FQ) (47). Hybridization-based line probe assays (LiPAs) detect mutations associated with resistance to RIF (12, 21, 33, 38), INH (3), PZA (42), and FQ (15).

A new LiPA kit was recently developed to identify clinically important *Mycobacterium* species and to detect drug resistance mutations in *M. tuberculosis*. Evaluation of this kit in six independent hospitals in Japan showed that this assay is promising for the rapid detection of drug-resistant tuberculosis and for identification of major NTM.

MATERIALS AND METHODS

Clinical isolates. A total of 554 clinical isolates of *M. tuberculosis* and NTM were obtained between January 2005 and December 2009 from 554 patients with pulmonary tuberculosis or NTM-related disease in the following six hospitals in Japan: Japan Anti-Tuberculosis Association Fukujuji Hospital (hospital A), National Hospital Organization (NHO) Tokyo Hospital (hospital B), NHO Kinki-Chuo Chest Medical Center (hospital C), NHO Ibaraki Higashi Hospital (hospital D), Tokyo Metropolitan Tama Medical Center (hospital E), and Osaka Prefectural Medical Center for Respiratory and Allergic Diseases (hospital F). Each participating hospital provided 79 to 109 isolates, all of which were subjected to species identification and drug susceptibility testing (DST). The *M. tuberculosis* isolates included 160 that were susceptible to all drugs tested and 156 that were resistant to at least one of the drugs tested (see Table S1 in the supplemental material). Of the drug-resistant isolates, 88 were resistant to RIF, 138 were resistant to INH, 58 were resistant to PZA, and 57 were resistant to levofloxacin (LVX) (data not shown). Other isolates included *Mycobacterium avium* ($n = 71$), *Mycobacterium intracellulare* ($n = 51$), and *Mycobacterium kansasii* ($n = 54$), as well as other NTM ($n = 62$) (see Table S1).

Clinical specimens. A total of 163 sputum specimens were obtained from patients suspected to have or previously diagnosed with tuberculosis or NTM disease (one specimen each) in the hospitals during the period from June 2009 to April 2010. These specimens were transported to the National Reference Laboratory of Tuberculosis (RIT) and stored at −80°C until tested. Each specimen was smeared and stained according to the Ziehl-Neelsen method, followed by treatment with an *N*-acetyl-L-cysteine–NaOH solution as described previously (41). Each pretreated specimen was resuspended in 1.5 ml of phosphate buffer (pH 6.8). Ali-

Received 30 August 2011 Returned for modification 9 October 2011
Accepted 19 December 2011
Published ahead of print 28 December 2011
Address correspondence to Teruo Kirikae, tkirikae@ri.ncgm.go.jp.
Supplemental material for this article may be found at http://jcm.asm.org/.

doi:10.1128/JCM.05638-11

quots of 0.2 ml of each suspension were transferred to 1.5-ml tubes and subjected to LiPA. Further aliquots of 0.2 ml of each suspension were transferred to fresh 1.5-ml tubes and subjected to the PCR-based Cobas Amplicor MTB test (Roche Diagnostics, Basel, Switzerland) (9, 26, 31) and the Cobas Amplicor *M. avium* and *M. intracellulare* tests (Roche Diagnostics). Aliquots of 0.1 ml and 0.5 ml of each specimen were inoculated into egg-based modified Ogawa medium (27) containing 2% (wt/vol) KH_2PO_4 and into MGIT broth (Bactec MGIT 960; BD Biosciences, Sparks, MD), respectively, for mycobacterial examination. Aliquots of 1 ml of the suspension for LiPA were centrifuged for 15 min at 13,000 × *g*, and the supernatant was removed with a pipette. Tris-EDTA (TE) buffer (100 μl) was added to the pellet, and the solution was again centrifuged for 15 min at 13,000 × *g*. The pellet was suspended in 50 μl of TE buffer and incubated at 95°C for 30 min. Aliquots of the supernatant (5 μl) were used for each LiPA. The total time for all procedures was about 3 h.

Species identification. *M. tuberculosis* was identified at hospitals A and B by use of TRCRapid M.TB kits (Tosoh Bioscience, Tokyo, Japan), based on the transcription-reverse transcription concerted reaction (13, 44), and at hospitals C to F and RIT by use of the Cobas Amplicor MTB test. *M. avium* and *M. intracellulare* were identified at hospitals C to F and RIT by use of the Cobas Amplicor *M. avium* and *M. intracellulare* tests, respectively. The *M. avium* complex (MAC) was identified at hospitals A and B by use of TRCRapid MAC kits (Tosoh Bioscience); isolates identified as MAC species were heat-killed and transported to RIT for species identification. The other NTM were identified at hospitals A, B, and D to F and at RIT by use of the DNA-DNA hybridization technique (DDH Mycobacteria Kyokuto; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) (28) and at hospital C by using AccuProbe (Gen-Probe, San Diego, CA) (17, 18). NTM isolates that were not identified by commercial kits were subjected to 16S rRNA gene sequencing at RIT.

DST and pyrazinamidase activity assay. DSTs for RIF, INH, PZA, and LVX were performed at each participating hospital. At hospitals A and B, the MGIT AST (BD Biosciences) test was performed to detect RIF, INH, and PZA resistance, and an egg-based Ogawa medium (24) (1% KH_2PO_4) method (Welpack S test; Nihon BCG Inc., Tokyo, Japan) was used to detect RIF, INH, and LVX resistance. At hospital C, MGIT AST, Welpack S, and a broth microdilution method (broth MIC MTB-I; Kyokuto Pharmaceutical Industrial Co.) were performed to detect RIF, INH, and LVX resistance. At hospital D, the egg-based Ogawa medium (1% KH_2PO_4) method (Bit Spectre-SR; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) was performed to detect RIF, INH, and LVX resistance, and a broth method was used to detect PZA resistance (PZA broth; Kyokuto Pharmaceutical Industrial Co.). At hospital E, the MGIT AST test was used to test for RIF, INH, and PZA resistance (LVX resistance was not tested at this hospital). At hospital F, the broth MIC MTB-I test was used to test for RIF, INH, and LVX resistance, and PZA broth (Kyokuto) was used to detect PZA resistance. At RIT, the standard proportion method using Ogawa medium (1% KH_2PO_4) was used to test for RIF, INH, and LVX resistance, and the MGIT AST test was used to test for PZA resistance. Isolates showing discordant results between phenotypic and genotypic DSTs for PZA were transferred to RIT and their pyrazinamidase activities tested (45), except for six isolates that had not been stored at the hospital. The INH resistance levels were as follows: isolates were considered resistant to INH at 0.2 μg/ml when they were resistant to INH at 0.2 μg/ml and susceptible to INH at 1.0 μg/ml; isolates were considered resistant to INH at 1.0 μg/ml when they were resistant to INH at 1.0 μg/ml. All kits for identification of mycobacteria and DSTs used in this study were recommended by the Japanese Society for Tuberculosis and approved as diagnosis reagents by the Ministry of Health, Labor and Welfare, Japan.

LiPA. LiPA was performed as described previously (3, 42), using 121 oligonucleotide probes (see Table S2 in the supplemental material) immobilized onto four strips, called the NTM/MDR-TB, INH, PZA, and FQ strips (Nipro Co., Osaka, Japan). All clinical isolates and all sputum specimens were tested by LiPAs using all four strips, regardless of the results of any particular strip. The NTM/MDR-TB strip was designed to identify four *Mycobacterium* species—*M. tuberculosis*, *M. avium*, *M. intracellulare*, and *M. kansasii*—and to detect mutations associated with RIF and INH resistance in *M. tuberculosis*. The INH, PZA, and FQ strips were designed to detect mutations associated with INH, PZA, and FQ resistance of *M. tuberculosis*, respectively. The corresponding regions and mutations for each probe are shown in Table S2. A probe designed to detect the wild-type sequence of *M. tuberculosis* was designated as S probe, whereas a probe designed to detect a mutant sequence frequently found in drug-resistant *M. tuberculosis* was designated as R probe. On the INH strip, 46 S probes covered various regions of the following *M. tuberculosis* genes: $P_{fabG1\text{-}inhA}$ (*inhA*-1), *inhA* (*inhA*-2), *fabG1* (*fabG1*-1 and -2), *furA* (*furA*-1 and -2), and *katG* (*katG*-1 to -40) (3). The *katG* probes covered 90 mutations related to INH resistance. On the PZA strip, 47 S probes covered regions of *M. tuberculosis pncA* (*pncA*-1 to -47), with 2 probes (*pncA*-16 and -17) containing a silent mutation in *pncA* (42). Probes *inhA*-S6 and -S7 and *katG*-S8 to -S11 on the NTM/MDR-TB strip were the same as *inhA*-1 and -2 and *katG*-20, -22, -23, and -24 on the INH strip, respectively.

Using biotinylated primers, the following products were obtained by nested PCR: *rpoB* (290 bp), $P_{fabG1\text{-}inhA}$ (477 bp), and *katG* (248 bp) for the NTM/MDR-TB strip; $P_{fabG1\text{-}inhA}$ (477 bp), *fabG1* (209 bp), *furA* (256 bp), and *katG* (612 bp, 698 bp, and 907 bp) for the INH strip; *pncA* (641 bp) for the PZA strip; and *gyrA* (379 bp) for the FQ strip. The immobilized probes on each strip were hybridized with the PCR products at 62°C for 30 min and then incubated with streptavidin labeled with alkaline phosphatase at room temperature for 30 min. Color was developed by incubation with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and nitroblue tetrazolium.

The presence or absence of bands, i.e., hybridization signals, on all strips was judged independently by three different observers. The results of LiPA were interpreted as follows. For identification of *Mycobacterium* species, when a signal was observed on the NTM/MDR-TB strip with any of the four probes (*rpoB*-AVI, *rpoB*-INT, *rpoB*-KAN, and *rpoB*-TB), the sample was thought to contain the corresponding *Mycobacterium* species. Conversely, when no signals were observed, the sample contained none of these four species. For detection of drug-resistant *M. tuberculosis*, when no signal was observed with any of the S probes, *M. tuberculosis* in the sample was considered resistant to the corresponding drug. In addition, when a signal(s) was observed with any of the R probes, the samples contained drug-resistant *M. tuberculosis* with the corresponding mutation(s). It took about 7 h to complete all procedures of the LiPA method.

DNA sequencing. The PCR products were sequenced. The sequenced samples were as follows: 1 isolate and 1 clinical specimen that showed discrepancies in species identification between conventional methods and LiPA and 40 isolates and 4 clinical specimens that showed discrepancies in drug susceptibility between phenotypic DST and LiPA. DNA sequences were compared with the sequence of *M. tuberculosis* H37Rv by using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan). We also sequenced the 16S rRNA genes of NTM isolates when they could not be identified by conventional identification kits. The sequences of the 16S rRNA genes were analyzed with software for DNA sequence-based diagnosis, published by the Ribosomal Differentiation of Microorganisms Project (RIDOM) (19), or with the Basic Local Alignment Search Tool (BLAST) to identify the species.

Ethical considerations. The study protocol was carefully reviewed and approved by the ethics committee of each participating hospital (hospital A approval date, 29 January 2009; hospital B approval date, 30 April 2009 [approval number 21-02-Da]; hospital C approval date, 14 November 2008 [approval number 20-18]; hospital D approval date, 18 September 2008; hospital E approval date, 28 November 2008; hospital F approval date, 28 March 2009 [approval number 5-84]). All clinical sputum specimens were collected after obtaining written informed consent from the participants.

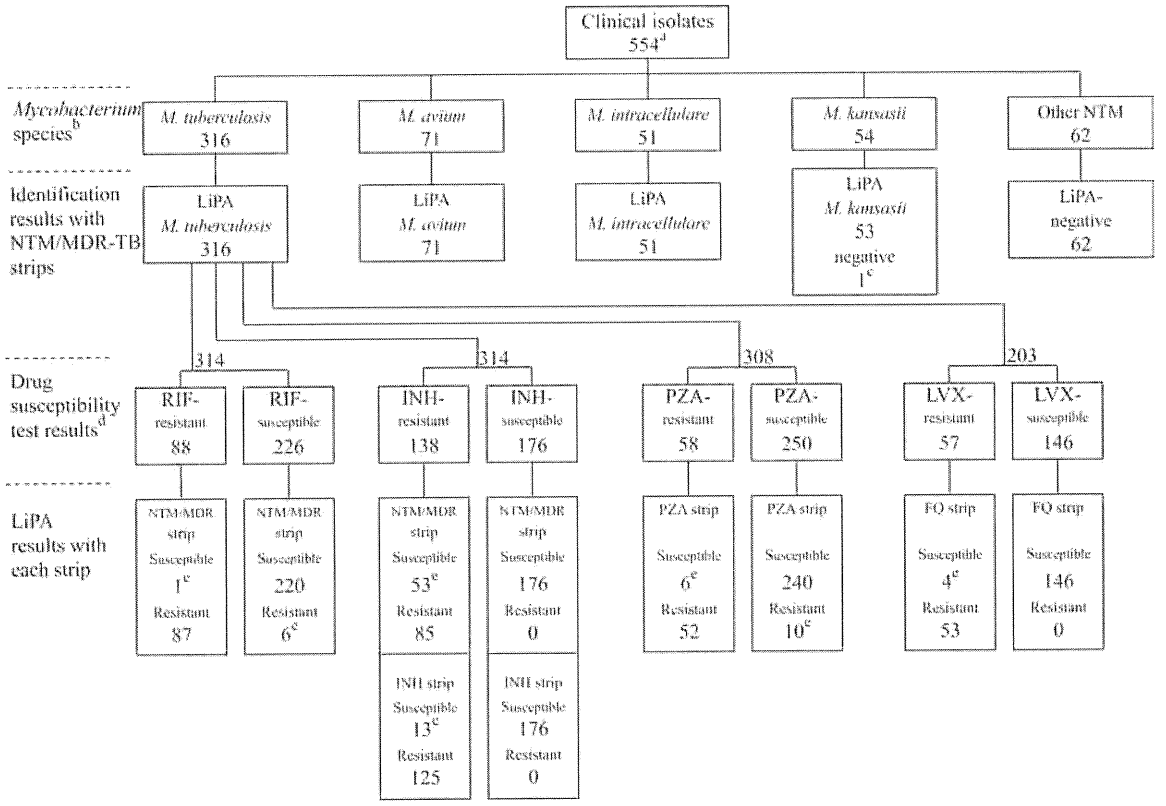

FIG 1 Distribution of LiPA results for 554 clinical isolates. [a], number of clinical isolates. [b], *Mycobacterium* species identified by conventional methods. [c], *M. kansasii* subtype III. [d], drug susceptibility testing and assays for pyrazinamidase activity were performed at each hospital (see the supplemental material). Of 316 *M. tuberculosis* isolates, 314 were subjected to RIF and INH susceptibility testing, 308 to PZA susceptibility testing, and 203 to LVX susceptibility testing. [e], some isolates showed different results between DST and LiPA. DNA sequences of each target gene were determined for these discrepant isolates (see the footnote in Table S4 in the supplemental material).

RESULTS

Identification of clinical isolates. Among 554 isolates, LiPA results for species identified as *M. tuberculosis*, *M. avium*, and *M. intracellulare* showed 100% agreement with those of conventional genetic methods (Fig. 1; see Table S3 in the supplemental material). Of 54 *M. kansasii* isolates, 53 were identified as *M. kansasii* by the LiPA kit. The one discrepant isolate of *M. kansasii* carried an *rpoB* sequence identical to that of subtype III of the seven subtypes of *M. kansasii*, defined by sequence polymorphisms in *hsp65* (1, 43). LiPA results were negative for 62 isolates of other NTM species.

Correlation between conventional DST and LiPA results. LiPA results were compared with those of DST (Table 1; see Table S4 in the supplemental material).

(i) RIF resistance and *rpoB* mutations. LiPA identified 98.9% (87/88 isolates) of RIF-resistant isolates and 97.3% (220/226 isolates) of RIF-susceptible isolates when *M. tuberculosis* isolates were tested using NTM/MDR-TB strips designed to detect *rpoB*

TABLE 1 Diagnostic performance of LiPA in comparison with drug susceptibility testing

Antituberculosis drug (strip used in LiPA[c])	Clinical isolates		Clinical samples (sputa)	
	Sensitivity[a]	Specificity[b]	Sensitivity[a]	Specificity[b]
RIF (NTM/MDR-TB strip)	98.9 (87/88)	97.3 (220/226)	100 (3/3)	100 (52/52)
INH (INH strip)	90.6 (125/138)	100 (176/176)	75.0 (3/4)	92.9 (39/42)
INH (NTM/MDR-TB strip)	61.6 (85/138)	100 (176/176)	50.0 (3/6)	97.8 (45/46)
PZA (PZA strip)	89.7 (52/58)	96.0 (240/250)	100 (4/4)	100 (52/52)
LVX (FQ strip)	93.0 (53/57)	100 (146/146)	100 (7/7)	100 (48/48)

[a] Data are percentages (no. of drug-resistant samples by LiPA/no. of drug-resistant samples by DST).

[b] Data are percentages (no. of drug-susceptible samples by LiPA/no. of drug-susceptible samples by DST).

[c] LiPA was performed using four strips, namely, NTM/MDR-TB, INH, PZA, and FQ strips (see the supplemental material). The NTM/MDR-TB strip was designed to identify four *Mycobacterium* species and to detect mutations associated with RIF resistance and INH resistance (C-15T and T-8C mutations in $P_{fabG1\text{-}inhA}$ and S315T and S315N mutations in *katG*). The INH, PZA, and FQ strips were designed to detect mutations associated with INH, PZA, and FQ resistance of *M. tuberculosis*, respectively. The corresponding regions and mutations for each probe are shown in Table S1 in the supplemental material. The INH strip covered 46 regions of the following *M. tuberculosis* genes: $P_{fabG1\text{-}inhA}$, *inhA*, *fabG1*, *furA*, and *katG* (3). The PZA strip covered *pncA* (40), and the FQ strip covered *gyrA* (4).

mutations (Table 1). Of all the isolates tested, seven showed discrepancies between DST and LiPA for RIF susceptibility testing. One isolate, identified as RIF resistant by DST but RIF susceptible by LiPA, had an I572F substitution. The remaining six were identified as RIF susceptible by DST but RIF resistant by LiPA. Of these, three had an H526S substitution, while the other three had an L511P mutation, a D516Y mutation, and a silent mutation at codon 516 (GAC → GAT). Of these six isolates, the three with the H526S mutation and the one with the L511P mutation were reported by hospital C as RIF susceptible by the MGIT AST and Welpack S tests but as RIF "intermediate" (MICs of 0.25 mg/liter and 0.5 mg/liter, respectively) by the broth MIC MTB-I test.

(ii) INH resistance and mutations of $P_{fabG1\text{-}inhA}$, *fabG1*, *furA*, and *katG*. The INH strip was designed to detect mutations associated with INH resistance in *M. tuberculosis*, including mutations in $P_{fabG1\text{-}inhA}$ (C-15T and T-8C), *fabG1* (G609A [L203L]), *furA* (C41T [A14V]), and *katG* (see Table S2 in the supplemental material). The strips identified 90.6% (125/138 isolates) of INH-resistant isolates and 100% (176/176 isolates) of INH-susceptible isolates (Table 1). Thirteen isolates were found to be INH resistant by DST but INH susceptible by LiPA (see Table S4). Of these, 10 had no mutations in the amplified regions for the INH strip, while the other 3 had S17N, G206S, and E340Q substitutions in *katG*.

The NTM/MDR-TB strip was designed to detect mutations of $P_{fabG1\text{-}inhA}$ (C-15T and T-8C) and *katG* (S315T and S315N) (see Table S2 in the supplemental material) which are frequently detected in INH-resistant clinical isolates (41, 47). NTM/MDR-TB strips identified 61.6% (85/138 isolates) of INH-resistant isolates and 100% (176/176 isolates) of INH-susceptible isolates (Table 1). Fifty-three isolates were identified as INH resistant by DST but INH susceptible by LiPA using NTM/MDR-TB strips. Of these, 13 isolates and the remaining 40 isolates were identified as INH susceptible and INH resistant, respectively, by LiPA using INH strips (see Table S4). Of these 40 INH-resistant isolates, 21 were resistant to INH at 1.0 μg/ml, and the remaining 19 isolates were resistant to INH at 0.2 μg/ml. Of the 21 isolates resistant to INH at 1.0 μg/ml, 5 showed no hybridization with the *fabG1*-1 probe (G609A [L203L]); 12 showed no hybridization with any of the *katG* probes, including *katG*-1 (1 isolate with a Δ152A mutation [frameshift]), *katG*-5 (1 isolate with an A338C [Y113S] mutation), *katG*-6 (2 isolates with a Δ367G mutation [frameshift]), *katG*-8 (1 isolate with a G412T [N138Y] mutation), *katG*-9 (1 isolate with an A425G [D142G] mutation), *katG*-10 (1 isolate with an A454C [K152Q] mutation), *katG*-11 (1 isolate with a G487A [D163N] mutation), *katG*-15 (1 isolate with a T571G [W191G] mutation), *katG*-29 (1 isolate with an A1382C [Q461P] mutation), *katG*-37 (1 isolate with a G1795T [G599stop] mutation), and *katG*-39 (1 isolate with a T2093C [F698S] mutation); 2 showed no hybridization with two *katG* probes, either *katG*-21 and *katG*-25 (A922C [T308P] and G1037C [S346T] mutations) or *katG*-39 and *katG*-40 (Δ1991-2173 [frameshift] mutation); 1 showed no hybridization with *katG*-26 to -40 (the DNA sequence was not determined); and 1 showed no hybridization with the *fabG1*-1 (G609A [L203L] mutation) and *katG*-6 (G378T [M126I] mutation) probes. Of the 19 isolates resistant to INH at 0.2 μg/ml, 12 showed no hybridization with the *fabG1*-1 probe (G609A [L203L] mutation), 6 showed no hybridization with the *katG*-28 probe (G1255C [D419H] mutation), and 1 showed no hybridization with the *katG*-28 and -34 probes (sequence not determined).

(iii) PZA resistance and *pncA* mutations. The PZA strip was designed to detect *pncA* mutations associated with PZA resistance in *M. tuberculosis* (42). The LiPA test identified 89.7% (52/58 isolates) of PZA-resistant and 96.0% (240/250 isolates) of PZA-susceptible isolates (Table 1). Sixteen isolates showed discrepancies between DST and LiPA results (see Table S4 in the supplemental material). Six isolates found to be PZA resistant by DST but PZA susceptible by LiPA had no mutations in *pncA*. Ten other isolates were PZA susceptible by DST but PZA resistant by LiPA. Of these 10 isolates, 4 had G162S, 2 had G17D, 2 had T168I, 2 had G132D, and 2 had V147I substitutions.

(iv) FQ resistance and *gyrA* mutations. The FQ strip was designed to detect *gyrA* mutations associated with FQ resistance in *M. tuberculosis* (see Table S2 in the supplemental material). FQ strips identified 93.0% (53/57 isolates) of LVX-resistant and 100% (146/146 isolates) of LVX-susceptible isolates (Table 1). Four isolates found to be LVX resistant by DST but LVX susceptible by LiPA had no mutations in *gyrA*. These isolates also had no mutations in *gyrB*.

Direct identification of *Mycobacterium* species and detection of drug-resistant *M. tuberculosis* in sputum specimens. A total of 163 sputum specimens were collected from patients who had been diagnosed with or were suspected to have pulmonary tuberculosis or NTM diseases.

(i) Detection and identification of *Mycobacterium* species in sputum specimens. Direct application of the LiPA kit to sputum samples for species identification showed high degrees of consistency and efficiency that were comparable with those of conventional methods. The sensitivity of LiPA with NTM/MDR-TB strips was 90.2% (74/82 specimens) for *M. tuberculosis*, 84.6% (11/13 specimens) for *M. avium*, 54.5% (6/11 specimens) for *M. intracellulare*, and 80.0% (4/5 specimens) for *M. kansasii* (see Table S5 in the supplemental material). One specimen, which was misidentified by LiPA, was found to be *Mycobacterium rhodesiae* by DNA sequence analysis. The overall sensitivity of LiPA with NTM/MDR-TB strips for detection of target species was 85.6% (95/111 specimens) (see Table S5). Eighteen samples were LiPA negative despite being PCR and/or culture positive (7 smear-positive and 11 smear-negative samples) (Fig. 2), whereas 14 samples were LiPA positive despite being PCR and culture negative (6 smear-positive and 8 smear-negative samples) (Fig. 2).

(ii) Correlation of conventional DST and LiPA results for sputum specimens. Among 163 samples, 49 smear-positive and 10 smear-negative samples were culture positive for *M. tuberculosis* (Fig. 3). For the 49 smear-positive samples, LiPA results for any drug susceptibility ranged from 89.8% (44/49 specimens) to 100% (49/49 specimens); for the 10 smear-negative samples, LiPA results ranged from 20% (2/10 specimens) to 70% (7/10 specimens). LiPA results were even obtained for some culture-negative samples, although these results could not be compared with those of DST. LiPA results were obtained for 11 to 16 of 45 smear-positive and *M. tuberculosis* culture-negative samples and for 7 to 11 of 59 smear-negative and *M. tuberculosis* culture-negative samples (Fig. 3). Direct application of the LiPA kit to sputum samples showed high sensitivities and specificities for detection of resistance to RIF, PZA, and LVX, whereas LiPA for detection of INH resistance showed a relatively low sensitivity (Table 1; see Table S6 in the supplemental material). However, its sensitivity and specificity were improved by using the INH strip.

Eight specimens showed discordance between DST and LiPA results (see Table S6 in the supplemental material). Four showed

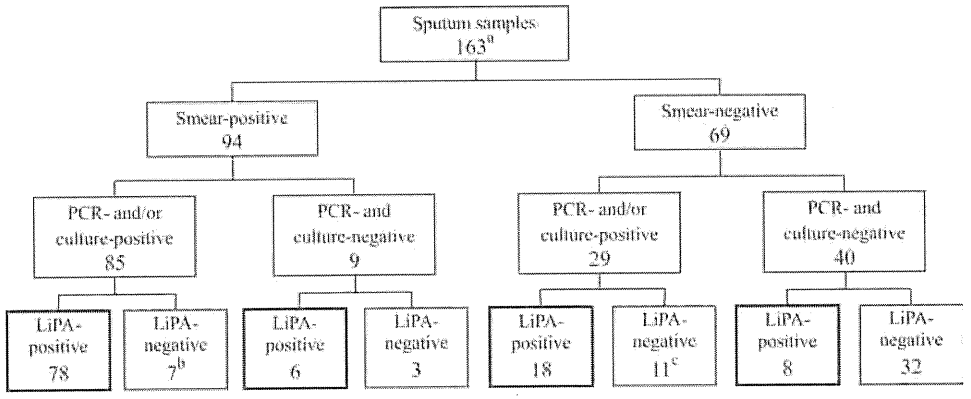

FIG 2 Distribution of LiPA results obtained with NTM/MDR-TB strips for the detection of target species in 163 sputum samples. [a], number of clinical samples. [b], one of these isolates was *Mycobacterium fortuitum*. [c], one of these isolates was *Mycobacterium abscessus*.

discordance between DST results for INH susceptibility and LiPA results obtained using INH strips. Of these, isolates from two specimens that were INH resistant by LiPA had a *fabG1* (G609A [L203L]) mutation, and one had a $P_{fabG1\text{-}inhA}$ (C-15T) mutation. Four specimens showed discordance between DST results for INH susceptibility and LiPA results obtained using NTM/MDR-TB strips (see Table S6). Of these, two specimens indicated as INH susceptible with NTM/MDR-TB strips were identified as INH resistant with INH strips, and isolates from these two specimens had *katG* mutations, i.e., G1795T (G599stop) and T2093C (F698S) mutations. One specimen was also identified as INH susceptible with the INH strip, and DNA sequencing revealed that an isolate from the specimen had two *katG* mutations (T571C [W191R] and G1079A [G360D]). One specimen showing INH resistance with the NTM/MDR-TB strip had a $P_{fabG1\text{-}inhA}$ (C-15T) mutation.

As shown in Fig. 3, for the culture-negative specimens, LiPA results were obtained for 26 specimens (15 smear-positive and 11 smear-negative specimens) for RIF susceptibility, 18 specimens (11 smear-positive and 7 smear-negative specimens) for INH susceptibility with INH strips, 23 specimens (15 smear-positive and 8 smear-negative specimens) for INH susceptibility with NTM/MDR-TB strips, 23 specimens (15 smear-positive and 8 smear-negative specimens) for PZA susceptibility, and 24 specimens (16 smear-positive and 8 smear-negative specimens) for LVX susceptibility. Of these, no specimens were found to be RIF resistant, three were INH resistant, one was PZA resistant, and none were LVX resistant (data not shown).

DISCUSSION

The newly developed LiPA kit successfully identified important *Mycobacterium* species, including *M. kansasii*, except for subtype III of *M. kansasii*. The *rpoB*-KAN probe on the NTM/MDR-TB strip is compatible with the *rpoB* genes of subtypes I, II, IV, and V but not III and VI, perhaps explaining why this LiPA kit was unable to identify a subtype III *M. kansasii* isolate (Fig. 1; see Table S3 in the supplemental material). Among the isolates of *M. kansasii* obtained in four European countries, the majority belonged to subtypes I (68%) and II (31%), with only 1% belonging to subtype III (1). Similar distributions of subtypes were reported in Switzerland (subtype I, 67%; subtype II, 21%; subtype III, 8%; and other subtypes, 4%) (43) and in Catalonia, Spain (subtype I, 98%; subtype VI, 2%) (39). These epidemiological results indicate that subtype III of *M. kansasii* causes significantly fewer human infections than subtypes I and II. Although the LiPA kit showed a signifi-

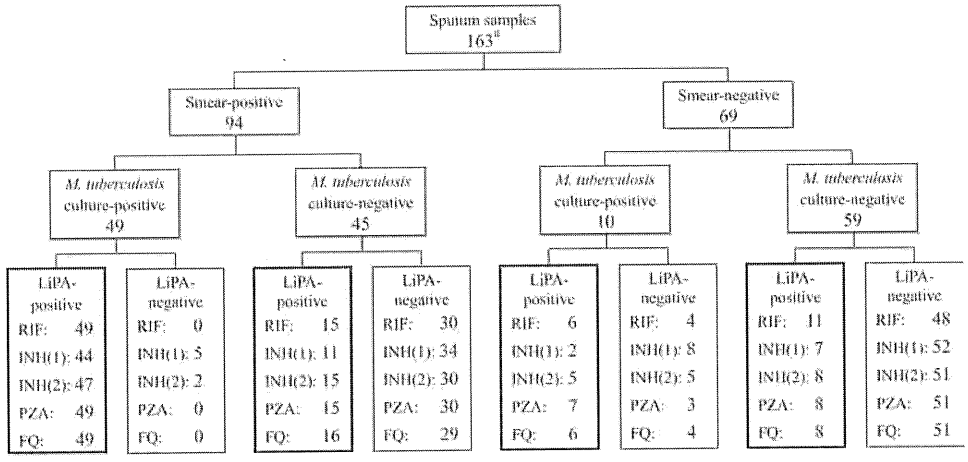

FIG 3 Distribution of LiPA results obtained with four strips for detection of a mutation(s) associated with drug resistance in 163 sputum samples. [a], number of clinical samples. RIF, RIF susceptibility with NTM/MDR-TB strips; INH(1), INH susceptibility with INH strips; INH(2), INH susceptibility with NTM/MDR-TB strips; PZA, PZA susceptibility with PZA strips; FQ, LVX susceptibility with FQ strips.

cantly efficient performance, improvements are required for detection of subtype III.

Seven isolates showing discrepancies between DST and LiPA for detection of RIF resistance had an I572F, D516D (a silent mutation), L511P, D516Y, or H526S mutation in *rpoB*. With the exception of the D516D mutation, these mutations are associated with RIF resistance. The I572F mutation has been reported to be associated with RIF resistance (46), although this mutation was not covered by the probes on the NTM/MDR-TB strip. The L511P and D516Y mutations have been reported to be associated with RIF resistance (25). RIF-resistant isolates have been reported to possess at least 11 mutations in codon 526, resulting in amino acid mutation of H to C, D, E, G, L, N, P, R, Q, T, or Y but not to S (47). An H526S mutation would be associated with RIF resistance.

LiPA using NTM/MDR-TB strips to detect mutations associated with INH resistance showed a low sensitivity (61.6%) among the isolates, although the strips were able to detect the most frequent mutations found in INH-resistant isolates, including S315T and S315N mutations in *katG* and C-15T and T-8C mutations in the promoter region of *inhA* (34, 35, 47). The Genotype MTBDRplus kit (Hain Lifescience, Nehren, Germany), a commercially available LiPA kit that uses a strip to detect these mutations, showed various degrees of sensitivity to INH-resistant *M. tuberculosis* isolates, including MDR isolates, in several countries, i.e., 92% in Germany (20), 82% in Taiwan (23), 73% in Spain (29), 67% in Italy (30), and 66% in Japan (10). The frequency of INH-resistant clinical isolates with S315T and S315N *katG* mutations and C-15T and T-8C mutations in the promoter region of *inhA* depends on the geographical origin of isolates. INH-resistant isolates with these mutations make up relatively small populations in Japan and Italy.

LiPA using INH strips, which covered more mutations, showed greater sensitivity than that with NTM/MDR-TB strips. Thirteen isolates were found to be INH resistant by DST but INH susceptible by LiPA using INH strips (see Table S4 in the supplemental material). No mutations were detected in 10 isolates, indicating that mutations in other genes may be associated with INH resistance. One isolate had a mutation of *katG* (S17N) which has been reported to confer INH resistance (11) but which is located away from the target sites. Of the remaining two isolates, one each had *katG* G206S and *katG* E340Q mutations, neither of which has been reported previously, to our knowledge. Both may be associated with INH resistance.

LiPA using PZA strips to detect mutations associated with PZA resistance showed high sensitivity and specificity. However, discrepancies between LiPA and DST were observed for 16 isolates. Six isolates were identified as PZA resistant by DST but PZA susceptible by LiPA, with none of these having a mutation in *pncA*, although one was positive in the pyrazinamidase test. Pyrazinamidase-positive but PZA-resistant strains are very rare and usually show a low level of resistance (36). The PZA resistance of the pyrazinamidase-positive strain may have been due to a mechanism other than *pncA* mutation (37). Ten isolates were identified as PZA susceptible by DST but PZA resistant by LiPA. Of these, two had *pncA* mutations causing T168I substitution, and one had a V147I mutation. These three isolates were positive in the pyrazinamidase test, suggesting that these mutations are not related to PZA resistance. Four isolates had a *pncA* G162S mutation, two had a G17D mutation, and one had a G132D mutation. These isolates were PZA susceptible by DST, but they were not tested for pyrazinamidase activity. To our knowledge, the G162S mutation has not been reported previously. The G17D and G132D mutations have been reported to confer PZA resistance (22). These discrepancies may have been due to the limited efficiency of DST methods (6).

LiPA with FQ strips for detection of mutations associated with FQ resistance showed high sensitivity and specificity, with only four isolates showing discordant results. None of these four had a mutation in *gyrA*, indicating that the FQ strips could detect all known mutations associated with FQ resistance. These four isolates had no mutation in *gyrB*, which was recently reported to confer FQ resistance in clinical isolates without *gyrA* mutations (7, 14, 32). Alternatively, the results of DST may show false resistance.

Of 163 sputum samples, 14 were LiPA positive but PCR and culture negative (Fig. 2). The results of LiPA for these 14 samples are likely correct assignments, as all came from patients previously diagnosed by culture methods or PCR as having tuberculosis or NTM diseases, showing 100% agreement. However, many of these results could have come from the shedding of nonviable bacilli from previously treated patients. Therefore, nucleic acid amplification methods, including LiPA, need to be interpreted carefully for previously treated tuberculosis patients. LiPA was always performed with a negative control and repeated when the results were in discordance with those of conventional methods. However, the discrepancies may be explained by cross-contamination during LiPA procedures.

The LiPA kit might be useful for rapid diagnosis of MDR tuberculosis, especially in Asian countries, where the genetic characteristics of INH resistance are unique (36). It is also important to detect resistance to PZA and LVX in MDR tuberculosis, as the majority of MDR *M. tuberculosis* isolates have been reported to be resistant to either PZA or LVX in Japan (4, 5).

The LiPA kit reported here is the first genetic diagnosis kit that can simultaneously identify the major clinical isolates of *Mycobacterium* species and detect mutations associated with resistance to INH, RIF, PZA, and FQ. The present study provides a unique perspective for assessing the overall reliability, specificity, and sensitivity of this kit in comparison with conventional tests. The LiPA kit may also be useful in laboratories in developing countries where mycobacterial culture cannot be performed. However, a follow-up culture-based DST is recommended where resources permit.

ACKNOWLEDGMENTS

This study was supported by grants from the Ministry of Health, Labor, and Welfare of Japan (H21-SHINKO-IPPAN-016) and the Nipro Corporation (3R07020).

REFERENCES

1. **Alcaide F, et al.** 1997. Heterogeneity and clonality among isolates of *Mycobacterium kansasii*: implications for epidemiological and pathogenicity studies. J. Clin. Microbiol. 35:1959–1964.
2. **Alvarez-Uria G.** 2010. Lung disease caused by nontuberculous mycobacteria. Curr. Opin. Pulm. Med. **16**:251–256.
3. **Ando H, et al.** 2010. Identification of *katG* mutations associated with high-level isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **54**:1793–1799.
4. **Ando H, et al.** 2011. Evaluation of a line probe assay for the rapid detection of *gyrA* mutations associated with fluoroquinolone resistance in multidrug-resistant *Mycobacterium tuberculosis*. J. Med. Microbiol. **60**: 184–188.
5. **Ando H, et al.** 2010. Pyrazinamide resistance in multidrug-resistant *My-*

cobacterium tuberculosis isolates in Japan. Clin. Microbiol. Infect. **16**: 1164–1168.
6. **Aono A, Hirano K, Hamasaki S, Abe C.** 2002. Evaluation of BACTEC MGIT 960 PZA medium for susceptibility testing of *Mycobacterium tuberculosis* to pyrazinamide (PZA): compared with the results of pyrazinamidase assay and Kyokuto PZA test. Diagn. Microbiol. Infect. Dis. **44**:347–352.
7. **Aubry A, et al.** 2006. Novel gyrase mutations in quinolone-resistant and -hypersusceptible clinical isolates of *Mycobacterium tuberculosis*: functional analysis of mutant enzymes. Antimicrob. Agents Chemother. **50**: 104–112.
8. **Aziz MA, et al.** 2006. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-Tuberculosis Drug Resistance Surveillance): an updated analysis. Lancet **368**:2142–2154.
9. **Bodmer T, Gurtner A, Scholkmann M, Matter L.** 1997. Evaluation of the COBAS AMPLICOR MTB system. J. Clin. Microbiol. **35**:1604–1605.
10. **Chikamatsu K, et al.** 2011. Evaluation of GenoTypeR MTBDRplus for the detection of multi-drug resistant *Mycobacterium tuberculosis* strains. Kekkaku **86**:697–702.
11. **Cockerill FR, III, et al.** 1995. Rapid identification of a point mutation of the *Mycobacterium tuberculosis* catalase-peroxidase (*katG*) gene associated with isoniazid resistance. J. Infect. Dis. **171**:240–245.
12. **Cooksey RC, Morlock GP, Glickman S, Crawford JT.** 1997. Evaluation of a line probe assay kit for characterization of *rpoB* mutations in rifampin-resistant *Mycobacterium tuberculosis* isolates from New York City. J. Clin. Microbiol. **35**:1281–1283.
13. **Drouillon V, et al.** 2009. Multicenter evaluation of a transcription-reverse transcription concerted assay for rapid detection of *Mycobacterium tuberculosis* complex in clinical specimens. J. Clin. Microbiol. **47**:3461–3465.
14. **Duong DA, et al.** 2009. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob. Agents Chemother. **53**:4835–4839.
15. **Giannoni F, et al.** 2005. Evaluation of a new line probe assay for rapid identification of *gyrA* mutations in *Mycobacterium tuberculosis.* Antimicrob. Agents Chemother. **49**:2928–2933.
16. **Glassroth J.** 2008. Pulmonary disease due to nontuberculous mycobacteria. Chest **133**:243–251.
17. **Gonzalez R, Hanna BA.** 1987. Evaluation of Gen-Probe DNA hybridization systems for the identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare.* Diagn. Microbiol. Infect. Dis. **8**:69–77.
18. **Goto M, Oka S, Okuzumi K, Kimura S, Shimada K.** 1991. Evaluation of acridinium-ester-labeled DNA probes for identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* complex in culture. J. Clin. Microbiol. **29**:2473–2476.
19. **Harmsen D, et al.** 2003. RIDOM: comprehensive and public sequence database for identification of *Mycobacterium* species. BMC Infect. Dis. **3**:26.
20. **Hillemann D, Rusch-Gerdes S, Richter E.** 2007. Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J. Clin. Microbiol. **45**:2635–2640.
21. **Hirano K, Abe C, Takahashi M.** 1999. Mutations in the *rpoB* gene of rifampin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. J. Clin. Microbiol. **37**:2663–2666.
22. **Hirano K, Takahashi M, Kazumi Y, Fukasawa Y, Abe C.** 1997. Mutation in *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis.* Tuber. Lung Dis. **78**:117–122.
23. **Huang WL, Chen HY, Kuo YM, Jou R.** 2009. Performance assessment of the GenoType MTBDRplus test and DNA sequencing in detection of multidrug-resistant *Mycobacterium tuberculosis.* J. Clin. Microbiol. **47**: 2520–2524.
24. **Kantor IN, et al.** 1998. Laboratory services in tuberculosis control. III. Culture. World Health Organization, Geneva, Switzerland.
25. **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.
26. **Katila ML, Katila P, Erkinjuntti-Pekkanen R.** 2000. Accelerated detection and identification of mycobacteria with MGIT 960 and COBAS AMPLICOR systems. J. Clin. Microbiol. **38**:960–964.
27. **Kudoh S, Kudoh T.** 1974. A simple technique for culturing tubercle bacilli. Bull. World Health Organ. **51**:71–82.
28. **Kusunoki S, et al.** 1991. Application of colorimetric microdilution plate hybridization for rapid genetic identification of 22 *Mycobacterium* species. J. Clin. Microbiol. **29**:1596–1603.
29. **Lacoma A, et al.** 2008. GenoType MTBDRplus assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* strains and clinical samples. J. Clin. Microbiol. **46**:3660–3667.
30. **Miotto P, et al.** 2006. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. J. Clin. Microbiol. **44**:2485–2491.
31. **Mitarai S, Kurashima A, Tamura A, Nagai H, Shishido H.** 2001. Clinical evaluation of Amplicor *Mycobacterium* detection system for the diagnosis of pulmonary mycobacterial infection using sputum. Tuberculosis (Edinburgh) **81**:319–325.
32. **Mokrousov I, et al.** 2008. Molecular characterization of ofloxacin-resistant *Mycobacterium tuberculosis* strains from Russia. Antimicrob. Agents Chemother. **52**:2937–2939.
33. **Morgan M, Kalantri S, Flores L, Pai M.** 2005. A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*: a systematic review and meta-analysis. BMC Infect. Dis. **5**:62.
34. **Morris S, et al.** 1995. Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis.* J. Infect. Dis. **171**:954–960.
35. **Musser JM.** 1995. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin. Microbiol. Rev. **8**:496–514.
36. **Park H, et al.** 2006. Comparison of a conventional antimicrobial susceptibility assay to an oligonucleotide chip system for detection of drug resistance in *Mycobacterium tuberculosis* isolates. J. Clin. Microbiol. **44**:1619–1624.
37. **Raynaud C, et al.** 1999. Mechanisms of pyrazinamide resistance in mycobacteria: importance of lack of uptake in addition to lack of pyrazinamidase activity. Microbiology **145**:1359–1367.
38. **Rossau R, et al.** 1997. Evaluation of the INNO-LiPA Rif.TB assay, a reverse hybridization assay for the simultaneous detection of *Mycobacterium tuberculosis* complex and its resistance to rifampin. Antimicrob. Agents Chemother. **41**:2093–2098.
39. **Santin M.** 2004. Incidence and molecular typing of *Mycobacterium kansasii* in a defined geographical area in Catalonia, Spain. Epidemiol. Infect. **132**:425–432.
40. **Scorpio A, et al.** 1997. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis.* Antimicrob. Agents Chemother. **41**:540–543.
41. **Sekiguchi J, et al.** 2007. Detection of multidrug resistance in *Mycobacterium tuberculosis.* J. Clin. Microbiol. **45**:179–192.
42. **Sekiguchi J, et al.** 2007. Development and evaluation of a line probe assay for rapid identification of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis* strains. J. Clin. Microbiol. **45**:2802–2807.
43. **Taillard C, et al.** 2003. Clinical implications of *Mycobacterium kansasii* species heterogeneity: Swiss National Survey. J. Clin. Microbiol. **41**:1240–1244.
44. **Takakura S, et al.** 2005. Rapid detection of *Mycobacterium tuberculosis* in respiratory samples by transcription-reverse transcription concerted reaction with an automated system. J. Clin. Microbiol. **43**:5435–5439.
45. **Wayne LG.** 1974. Simple pyrazinamidase and urease tests for routine identification of mycobacteria. Am. Rev. Respir. Dis. **109**:147–151.
46. **Yuen LK, Leslie D, Coloe PJ.** 1999. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia. J. Clin. Microbiol. **37**:3844–3850.
47. **Zang Y, Telenti A.** 2000. Genetics of drug resistance in *Mycobacterium tuberculosis*, p.235–254. *In* G. F. Hatfull, W. R. Jabobs (ed), Molecular genetics of mycobacteria. American Society for Microbiology, Washington, DC.

Supplementary Information

TABLE S1. Clinical isolates used in the present study[a]

Hospital	No. of clinical isolates						
	total	*M. tuberculosis*		*M. avium*	*M. intracellulare*	*M. kansasii*	Other NTM
		susceptible to all drugs tested	resistant to any drugs				
Hospital A	84	20	22	15	5	10	12
Hospital B	92	26	30	12	7	7	10
Hospital C	109	20	50	10	10	9	10
Hospital D	83	24	19	10	10	10	10
Hospital E	107	51	20	14	5	8	9
Hospital F	79	19	15	10	14	10	11
total	554	160	156	71	51	54	62

[a] A total of 554 clinical isolates of *M. tuberculosis* and NTM were obtained at Hospitals A – F in Japan. Each participating hospital provided from 79 to 109 isolates, all of which were subjected to species identification and drug susceptibility testing. The *M. tuberculosis* isolates included 160 that were susceptible to all drugs tested, and 156 resistant to at least one of the drugs tested. Other isolates included *M. avium* ($n = 71$), *M. intracellulare* ($n = 51$), and *M. kansasii* ($n = 54$), as well as other NTM ($n = 62$).

TABLE S2. Oligonucleotide probes designed to cover regions specific to *Mycobacterium* species and mutation(s) associated with anti-tuberculosis drug resistance

NTM/MDR-TB strip		INH strip		PZA strip		FQ strip	
Probe[a]	Amino acid (nucleotide) region covered by probe	Probe	Amino acid (nucleotide) region covered by probe	Probe	Amino acid (nucleotide) region covered by probe	Probe	Amino acid region covered by probe
rpoB-AVI	510-515	*inhA*-1	(-17 to -3)[b]	*pncA*-1	(-17 to 4)[c]	*gyrA*-S1	88-92
rpoB-INT	521-525	*inhA*-2	6-11	*pncA*-2	1-5	*gyrA*-S2	92-97
rpoB-KAN	509-515	*fabG1*-1	202-206	*pncA*-3	4-9	*gyrA*-R1	A90V
rpoB-TB	484-488	*fabG1*-2	194-200	*pncA*-4	7-11	*gyrA*-R2a	D94G
rpoB-S1	510-515	*furA*-1	12-16	*pncA*-5	10-15	*gyrA*-R2b	D94A
rpoB-S2	514-520	*furA*-2	6-12	*pncA*-6	13-18	*gyrA*-R2c	D94G+S95T
rpoB-S3	520-525	*katG*-1	45-51	*pncA*-7	17-21	*gyrA*-R2d	D94A+S95T
rpoB-S4	525-529	*katG*-2	92-97	*pncA*-8	21-26		
rpoB-S5	529-534	*katG*-3	94-99	*pncA*-9	26-34		
rpoB-R2	D516V	*katG*-4	105-111	*pncA*-10	32-37		
rpoB-R4a	H526Y	*katG*-5	112-116	*pncA*-11	34-41		
rpoB-R4b	H526D	*katG*-6	123-127	*pncA*-12	40-47		
rpoB-R5	S531L	*katG*-7	132-137	*pncA*-13	46-52		
inhA-S6	(-17 to -3)[b]	*katG*-8	135-140	*pncA*-14	49-54		
inhA-S7	6-11	*katG*-9	140-145	*pncA*-15	52-56		
katG-S8	294-299	*katG*-10	149-154	*pncA*-16	56-61		
katG-S9	313-318	*katG*-11	160-164	*pncA*-17	61-67		
katG-S10	323-327	*katG*-12	170-174	*pncA*-18	66-70		
katG-S11	326-330	*katG*-13	174-179	*pncA*-19	70-74		
katG-R9a	S315T	*katG*-14	178-183	*pncA*-20	73-78		
katG-R9b	S315N	*katG*-15	190-194	*pncA*-21	76-81		
		katG-16	228-236	*pncA*-22	80-86		
		katG-17	247-252	*pncA*-23	85-90		
		katG-18	256-261	*pncA*-24	91-96		
		katG-19	271-277	*pncA*-25	95-101		
		katG-20	294-299	*pncA*-26	100-105		
		katG-21	305-310	*pncA*-27	105-111		
		katG-22	313-318	*pncA*-28	111-116		
		katG-23	323-327	*pncA*-29	114-120		
		katG-24	326-330	*pncA*-30	119-124		
		katG-25	344-349	*pncA*-31	124-129		
		katG-26	383-387	*pncA*-32	129-134		
		katG-27	389-391	*pncA*-33	133-139		
		katG-28	417-422	*pncA*-34	138-143		
		katG-29	457-462	*pncA*-35	142-147		
		katG-30	479-482	*pncA*-36	145-150		
		katG-31	486-490	*pncA*-37	150-156		
		katG-32	522-528	*pncA*-38	154-159		
		katG-33	539-543	*pncA*-39	156-162		
		katG-34	553-558	*pncA*-40	161-165		
		katG-35	565-569	*pncA*-41	163-168		
		katG-36	591-596	*pncA*-42	166-170		
		katG-37	596-602	*pncA*-43	168-172		
		katG-38	631-635	*pncA*-44	170-175		
		katG-39	695-700	*pncA*-45	172-177		
		katG-40	707-712	*pncA*-46	177-182		
				pncA-47	180-185		

[a] Four probes, *rpoB*-AVI, *rpoB*-INT, *rpoB*-KAN, and *rpoB*-TB, covered regions specific to *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. tuberculosis*, respectively. To detect mutations associated with drug resistance in *M. tuberculosis*, S-probes and R-probes were designed. The S-probes were for detection of wild-type sequences and the R-probes were for detection of mutations frequently found in drug-resistant *M. tuberculosis*. Nine *rpoB* probes, including five S-probes (*rpoB*-S1 to -S5) and four R-probes (*rpoB*-R2, -R4a, -R4b, and -R5), were designed for detection of RIF-resistant isolates. The R-probes, *rpoB*-R2, -R4a, -R4b, and -R5, covered the same regions as the S-probes, *rpoB*-S2, -S4, -S4, and -S5, respectively. Probes for *inhA*, *katG*, *fabG1*, and *furA* were designed for detection of INH-resistant isolates. These were S-probes with the exception of *katG*-R9a and -R9b, which were R-probes. Forty-seven *pncA* S-probes (*pncA*-1 to -47) were designed for detection of PZA-resistant isolates. The *gyrA* probes, including two S-probes (*gyrA*-S1 and -S2) and five R-probes (*gyrA*-R1, -R2a, -R2b, -R2c, and -R2d), were designed for detection of FQ-resistant isolates.

[b] Nucleotide position relative to the initiation codon of *fabG1*.

[c] Nucleotide position relative to the initiation codon of *pncA*.

TABLE S3. Identification of 554 clinical isolates of *Mycobacterium* species using genetic diagnostic methods and LiPA with NTM/MDR-TB strip

Mycobacterium species	No. of isolates identified by conventional methods[a]	No. of strains identified using LiPA with NTM/MDR-TB strip				
		M. tuberculosis	*M. avium*	*M. intracellulare*	*M. kansasii*	negative
M. tuberculosis	316	316				
M. avium	71		71			
M. intracellulare	51			51		
M. kansasii	54				53	1[c]
Other NTM[b]	62					62

[a] Isolates were identified by various genetic diagnostic kits and DNA sequencing, as described in the text.

[b] Including 10 *M. abscessus*, 1 *M. celatum*, 5 *M. chelonae*, 9 *M. fortuitum*, 26 *M. gordonae*, 1 *M. marinum*, 2 *M. nonchromogenicum*, 2 *M. peregrinum*, 1 *M. scrofulaceum*, 3 *M. szulgai*, and 2 *M. triplex*.

[c] *M. kansasii* subtype III

TABLE S4. Diagnostic performance of LiPA in comparison with drug susceptibility testing[a]

Drug susceptibility testing result[b]	No. of *M. tuberculosis* isolates	LiPA result		Sensitivity (%)	Specificity (%)
		No. of resistant	No. of susceptible		
RIF		NTM/MDR-TB strip			
Resistant	88	87	1[c]	98.9	97.3
Susceptible	226	6[c]	220		
INH		INH strip			
Resistant	138	125	13[d]	90.6	100
Susceptible	176	0	176		
INH		NTM/MDR-TB strip			
Resistant	138	85	53[e]	61.6	100
Susceptible	176	0	176		
PZA		PZA strip			
Resistant	58	52	6[f]	89.7	96.0
Susceptible	250	10[f]	240		
LVX		NQ strip			
Resistant	57	53	4[g]	93.0	100
Susceptible	146	0	146		

[a] The diagnostic performance of LiPA with NTM/MDR-TB, INH, PZA, and FQ strips was compared with that of drug susceptibility testing.

[b] Drug susceptibility testing and assays for pyrazinamidase activity were performed at each hospital. A total of 314 isolates were subjected to RIF and INH susceptibility testing, 308 isolates were subjected to PAZ susceptibility testing, and 203 isolates were subjected to LVX susceptibility testing.

[c] One isolate, identified as RIF-resistant by DST but RIF-susceptible by LiPA, had a substitution at I572F. Six isolates were confirmed to be phenotypically RIF-susceptible but were indicated as resistant by LiPA. Of these, 3 had a substitution at H526S, while the other 3 had substitutions at L511P and D516Y and a silent mutation at codon 516 (GAC > GAT).

[d] Thirteen isolates were found to be INH-resistant by DST but INH-susceptible by LiPA using INH strips. No mutations were detected in 10 isolates. One isolate had a mutation of *katG* (S17N). Of the remaining two isolates, one each had

katG (G206S) and *katG* (E340Q) mutation, respectively. *Novel mutations are underlined.

[e] Fifty-three isolates were found to be INH-resistant by DST but INH-susceptible by LiPA using the NTM/MDR-TB strips. Of these, 13 isolates and the remaining 40 were found to be INH-susceptible and INH-resistant by LiPA using the INH strips. Of these 40 isolates, 21 were resistant to INH (1.0 μg/mL) and the remaining 19 isolates were resistant to INH (0.2 μg/mL). Of the 21 isolates resistant to INH (1.0 μg/mL), five showed no hybridization with the *fabG1*-1 probe [G609A(L203L)]; 12 showed no hybridization with any one of the *katG* probes, including *katG*-1 [one isolate with Δ152A (frameshift)], -5 [one, A338C (Y113S)], -6 [two, Δ367G (frameshift)], -8 [one, G412T (N138Y)], -9 [one, A425G (D142G)], -10 [one, A454C (K152Q)], -11 [one, G487A (D163N)], -15 [one, T571G (W191G)], -29 [one, A1382C (Q461P)], -37 [one, G1795T (G599stop)], and -39 [one, T2093C (F698S)]; two showed no hybridization with two *katG* probes, including *katG*-21 and -25 [A922C (T308P) and G1037C (S346T)] and *katG*-39 and -40 [Δ1991-2173 (frameshift)]; one showed no hybridization with *katG*-26 to -40 probes [the DNA sequence was not determined (N.D.)] ; one showed no hybridization with the *fabG1*-1 [G609A(L203L)] and *katG*-6 probes [G378T (M126I)]. Of the 19 isolates resistant to INH (0.2 μg/mL), 12 showed no hybridization with the *fabG1*-1 probe [G609A (L203L)]; 6 showed no hybridization with the *katG*-28 probe [G1255C (D419H)]; one showed no hybridization with *katG*-28 and -34 probes (N.D.).

[f] Six isolates were identified as PZA-resistant by DST but PZA-susceptible by LiPA, and none of these had a mutation in *pncA*. Ten isolates were identified as PZA-susceptible by DST but PZA-resistant by LiPA. Of these, two had *pncA* mutations causing T168I substitution and one had V147I. Four isolates had *pncA* mutations of G162S, two had G17D, and one had G132D. *Novel mutations are underlined.

[g] Four isolates showing discordant results had no mutations in *gyrA* and *gyrB*.

TABLE. S5. Identification of *Mycobacterium* species in 163 sputum specimens using genetic diagnostic methods and LiPA with NTM/MDR-TB strips[a]

Mycobacterium species	No. of specimens identified by conventional methods[b]	No. of specimens identified using LiPA with NTM/MDR-TB strips					Sensitivity[c] (%)
		M. tuberculosis	*M. avium*	*M. intracellulare*	*M. kansasii*	negative	
M. tuberculosis	82	74				8	90.2
M. avium	13		11			2	84.6
M. intracellulare	11			6		5	54.5
M. kansasii	5				4	1	80.0
Other NTM[d]	3			1[e]		2	
Negative	49	7	1	1	5	35	

[a] Of 163 specimens, 94 (57.7%) were smear-positive for acid-fast bacilli, 96 (58.9%) were PCR-positive, 89 (54.6%) were culture-positive, and 110 (67.5%) were positive on LiPA with NTM/MDR-TB strips for the direct detection of target species.

[b] Including culture in MGIT broth and 2% Ogawa, and PCR Amplicor tests.

[c] Overall sensitivity for the direct detection of target species was 85.6% (95/111).

[d] Including one *M. abscessus*, one *M. fortuitum*, and one *M. rhodesiae*.

[e] *M. rhodesiae*

TABLE S6. Comparison of the diagnostic performance of LiPA with drug susceptibility testing in clinical specimens[a]

Drug susceptibility test result[b]	No. of culture-positive *M. tuberculosis* tested	LiPA result[c]		Sensitivity (%)	Specificity (%)
		No. of resistant	No. of susceptible		
RIF		NTM/MDR-TB strip			
Resistant	3	3	0	100	100
Susceptible	52	0	52		
INH		INH strip			
Resistant	4	3	1	75.0	92.9
Susceptible	42	3[d]	39		
INH		NTM/MDR-TB strip			
Resistant	6	3	3[e]	50.0	97.8
Susceptible	46	1[f]	45		
PZA		PZA strip			
Resistant	4	4	0	100	100
Susceptible	52	0	52		
LVX		NQ strip			
Resistant	7	7	0	100	100
Susceptible	48	0	48		

[a] Obtained from 163 patients with confirmed or suspected pulmonary tuberculosis or NTM diseases. The specimens were tested by LiPA using NTM/MDR-TB, INH, PZA, and FQ strips, and inoculated into 2% Ogawa medium and MGIT broth. DST results were obtained from 59 specimens.

[b] Drug susceptibility testing of *M. tuberculosis* isolates was performed at RIT.

[c] Of the 59 specimens from which DST results were obtained, LiPA results were obtained from 55 for RIF with NTM/MDR-TB strips, 46 for INH with INH strips, 52 for INH with NTM/MDR-TB strips, 56 for PZA with PZA strips, and 55 for LVX with FQ strips.

[d] Of the three specimens, two had a mutation of *fabG1* [G609A(L203L)] and one had a mutation of $P_{fabG1\text{-}inhA}^{C\text{-}15T}$.

[e] Of the three specimens, two had a *katG* mutation detectable with the INH strip and one had no mutation in the amplified regions for the INH strip.

[f] This specimen had a mutation of $P_{fabG1\text{-}inhA}^{C\text{-}15T}$.

Vol. 27, No. 6, 2011 化学療法の領域

特集 結核菌と結核を巡る新たな知見

1．ゲノム配列にもとづく結核菌群の発生と進化の理解

和田 崇之* 前田 伸司**

結核菌群（*Mycobacterium tuberculosis* complex）はヒトを含めたさまざまな動物種に感染する菌種群である。その種分類は主として感染宿主によって定義されており，必ずしも分子系統分類に従ったものではなかったが，近年，菌株の遺伝多型解析と系統分析が精力的に進められ，各菌種における分子進化の履歴が徐々に明らかになっている。本稿では，ゲノム配列およびその多型分析から推定される結核菌群のルーツと菌種としての進化を概説し，後半ではヒト結核菌（*Mycobacterium tuberculosis*）にスポットを当ててその遺伝的多様性に関する最新の知見を紹介する。

Key Words：結核菌群／*Mycobacterium tuberculosis*／系統進化／遺伝的多様性

I はじめに

近年の病原体研究は既存の実験室株とモデル宿主を用いて行われてきた基礎研究のみならず，臨床分離株が示す多様な個性を研究対象として，より詳細かつ包括的な理解を求めるベクトルが形成されつつある。このような研究展開は次世代シーケンサーによる個別菌株のゲノム解析が現実的なコストで実現可能となった昨今において，ますます拍車がかかっている。

ヒト結核菌（*Mycobacterium tuberculosis*）ではColeらによってその代表株であるH37Rvの全塩基配列[1]が報告されて10余年が経過した今，世界各地由来の臨床分離株を対象としたゲノム解析が精力的に進められており，近縁種における遺伝系統的な関連性に新たな知見と修正が加わる状況になっている。また，ヒト結核菌の種内系統分岐と地理的分布には密接な相関性が見出され，分子進化，宿主適応，菌株個性分析などの多様な研究展開をみせている。

II 結核菌群の発生

結核菌群は大きくはマイコバクテリウム属（抗酸菌）に属し，さまざまな宿主に感染して生存する病原体である（**表1**）。ヒト結核菌はその中でもヒトを自然宿主として伝播・拡散する菌種であるが，ゲノム配列では他の結核菌群種ときわめて相同性が高く，ほぼ同一菌種と言っても差し支えない。現在，結核菌群として定義されている代表的な菌種は*M. bovis*，*M. canetti*，*M. africanum*などがあげられるが，このうち進化学的に重要なのは*M. canetti*である[2]。

*M. canetti*はコロニーなどの形態学的特徴が他の結核菌群と大きく異なっているものの，分子進化学的には結核菌群と近縁である。これらの分岐は約300万年前に生じたと推定されており[3]，系

Origins and evolution of *Mycobacterium tuberculosis* complex based on comparative genomics

*大阪市立環境科学研究所微生物保健担当 研究主任 Takayuki Wada

**公益財団法人結核予防会結核研究所抗酸菌レファレンス部結核菌情報科 科長 Shinji Maeda