Medical Science Program, the Global Center of Excellence Program, the Establishment of International Collaboration Centers for Zoonosis Control, and the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and in part by grants from the Japan Initiative for Global Research Network on Infectious Diseases MEXT to Y.S. and by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to Y.S. and C.N.

REFERENCES

1. An DD, et al. 2009. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with high-level fluoroquinolone resistance in Vietnam. Antimicrob. Agents Chemother. 53:4835–4839.
2. 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.
3. Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau E. 2004. *Mycobacterium tuberculosis* DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. 48: 1281–1288.
4. Cambau E, Perani E, Guillemin I, Jamet P, Ji B. 1997. Multidrug-resistance to dapsone, rifampicin, and ofloxacin in *Mycobacterium leprae*. Lancet 349:103–104.
5. Cambau E, Carthagena L, Chauffour A, Ji B, Jarlierbl V. 2006. Dihydropteroate synthase mutations in the folP1 gene predict dapsone resistance in replaced cases of leprosy. Clin. Infect. Dis. 42:238–241.
6. 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.
7. Campbell PJ, et al. 2011. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 55:2032–2041.
8. Champoux JJ. 2001. DNA topoisomerases: structure, function, and mechanism. Annu. Rev. Biochem. 70:369–413.
9. Cui Z, Wang J, Lu J, Huang X, Hu Z. 2011. Association of mutation patterns in gyrA/B genes and ofloxacin resistance levels in *Mycobacterium tuberculosis* isolates from east China in 2009. BMC Infect. Dis. 11:78–82.
10. Feuerriegel S, et al. 2009. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob. Agents Chemother. 53:3353–3356.
11. Goto M, et al. 2006. Guideline for the treatment of Hansen's disease in Japan (second edition), Japanese leprosy. Nihon Hansenbyo Gakkai Zasshi 75:191–226. (In Japanese.)
12. Honore N, Cole ST. 1993. Molecular basis of rifampin resistance in *Mycobacterium leprae*. Antimicrob. Agents Chemother. 37:414–418.
13. Hooper DC. 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin. Infect. Dis. 31:S24–S28.
14. Ichihara N. 1984. Phase I study on DL-8280 (OFX). Chemotherapy 32: 118–149.
15. Kai M, et al. 1999. Diaminodiphenylsulfone resistance of *Mycobacterium leprae* due to mutations in the dihydropteroate synthase gene. FEMS Microbiol. Lett. 177:231–235.
16. Kim H, et al. 2011. Impact of the E540V amino acid substitution in GyrB of *Mycobacterium tuberculosis* on quinolone resistance. Antimicrob. Agents Chemother. 55:3661–3667.
17. Kyaw UK, Aye DKS. 2006. A case of multi-drug resistant leprogy-relapse or re-infection, Myanmar J. Curr. Med. Pract. 10:41–43.
18. Levy L, Ji B. 2006. The mouse foot-pad technique for cultivation of *Mycobacterium leprae*. Lepr. Rev. 77:5–24.
19. Maeda S, et al. 2001. Multidrug resistant *Mycobacterium leprae* from patients with leprosy. Antimicrob. Agents Chemother. 45:3635–3639.
20. Matrat S, Cambau E, Jarlier V, Aubry A. 2008. Are all the DNA gyrase mutations found in *Mycobacterium leprae* clinical strains involved in resistance to fluoroquinolones? Antimicrob. Agents Chemother. 52: 745–747.
21. Matrat S, et al. 2007. Expression and purification of an active form of the *Mycobacterium leprae* DNA gyrase and its inhibition by quinolones. Antimicrob. Agents Chemother. 51:1643–1648.
22. Matsuoka M. 2010. The history of *Mycobacterium leprae* Thai-53 strain. Lepr. Rev. 81:137.
23. Matsuoka M, et al. 2007. The frequency of drug resistance mutations in Mycobacterium leprae isolates in untreated and replaced leprosy patients from Myanmar, Indonesia and the Philippines. Lepr. Rev. 78:343–352.
24. Matsuoka M, Kashiwabara Y, Namisato M. 2000. A Mycobacterium leprae isolate resistant to dapsone, rifampin, ofloxacin and sparfloxacin. Int. J. Lepr. Other Mycobact. Dis. 68:452–455.
25. Matsuoka M, Kashiwabara Y, Liangfen Z, Goto M, Kitajima S. 2003. A second case of multidrug resistant Mycobacterium leprae isolated from a Japanese patient with relapsed lepromatous leprosy. Int. J. Lepr. Other Mycobact. Dis. 71:240–243.
26. Matsuoka M, et al. 2010. Possible mode of emerging drug resistant leprosy cases revealed in Mexican samples' analysis. Jpn. J. Infect. Dis. 63: 412–416.
27. Nakashima M, et al. 1995. Pharmacokinetics and tolerance of DU-6859a, a new fluoroquinolone, after single and multiple oral doses in healthy volunteers. Antimicrob. Agents Chemother. 39:170–174.
28. Nakashima M, et al. 1995. Single- and multiple-dose pharmacokinetics of AM-1155, a new 6-fluoro-8-methoxy quinolone, in humans. Antimicrob. Agents Chemother. 39:2635–2640.
29. Norman G, Joseph G, Ebenezer G, Rao PSSS, Job CK. 2003. Secondary rifampin resistance following multi-drug therapy–a case report. Int. J. Lepr. Other Mycobact. Dis. 71:18–21.
30. Ohnishi N, et al. 2005. Safety, pharmacokinetics and influence on the intestinal flora of BAY 12-8039 (moxifloxacin hydrochloride) after oral administration in healthy male subjects. Jpn. Pharmacol. Ther. 33: 1029–1045.
31. Pan XS, Yague G, Fisher LM. 2001. Quinolone resistance mutations in *Streptococcus pneumoniae* GyrA and ParC proteins: mechanistic insights into quinolone action from enzymatic analysis, intracellular levels, and phenotypes of wild-type and mutant proteins. Antimicrob. Agents Chemother. 45:3140–3147.
32. Ramaswamy S, Musser JM. 1998. Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis 1998 update. Tuber. Lung Dis. 79:3–29.
33. Scollard DM, et al. 2006. The continuing challenges of leprosy. Clin. Microbiol. Rev. 19:338–381.
34. Sun Z, et al. 2008. Comparison of *gyrA* gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. Int. J. Antimicrob. Agents 31:115–121.
35. Williams DL, Spring L, Harris E, Roche P, Gillis TP. 2000. Dihydropteroate synthase of *Mycobacterium leprae* and dapsone resistance. Antimicrob. Agents Chemother. 44:1530–1537.
36. World Health Organization. 2004. Multidrug therapy against leprosy. Report WHO/CDS/CPE/CEE/2004.46. World Health Organization, Geneva, Switzerland.
37. World Health Organization. 2011. Leprosy update, 2011. Wkly. Epidemiol. Rec. 86:389–399.
38. Reference deleted.
39. Yin X, Yu Z. 2010. Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant *Mycobacterium tuberculosis* clinical isolates from Guangdong province in China. J. Infect. 61:150–154.
40. You E-Y, Kang TJ, Kim S-K, Lee S-B, Chae G-T. 2010. Mutations in genes related to drug resistance in *Mycobacterium leprae* isolates from leprosy patients in Korea. J. Infect. 50:6–11.

Eur J Clin Microbiol Infect Dis (2012) 31:445–454
DOI 10.1007/s10096-011-1326-7

ARTICLE

Correlation between variable-number tandem-repeat-based genotypes and drug susceptibility in *Mycobacterium avium* isolates

Y. Tatano · C. Sano · K. Yasumoto · T. Shimizu · K. Sato · K. Nishimori · T. Matsumoto · S. Yano · H. Takeyama · H. Tomioka

Received: 17 December 2010 / Accepted: 17 June 2011 / Published online: 12 July 2011

Abstract Little is known about the correlation between genotype and drug susceptibility in *Mycobacterium avium* (Mav) strains isolated from patients with Mav infections. To examine whether drug susceptibility profile of Mav is associated with genotype, we carried out variable-number tandem-repeat (VNTR) typing and drug susceptibility testing for Mav isolates from Japanese with nodular-bronchiectasis (NB)-type and cavitary disease (CA)-type diseases. We performed *M. avium* tandem repeat (MATR)-VNTR typing and drug susceptibility testing by the broth dilution method, using macrolides, rifamycins, ethambutol, isoniazid, aminoglycosides, and quinolones, for Mav isolates from patients with NB and CA-type diseases (NB-Mav and CA-Mav). Based on the VNTR genotyping, the Mav strains were grouped into three clusters. There was no difference with respect to the distribution of NB-Mav and CA-Mav among the clusters. We observed a strong association between VNTR genotype and susceptibility to quinolones (levofloxacin, moxifloxacin, gatifloxacin, sitafloxacin, and garenoxacin) and ethambutol. There was essentially no significant difference in drug susceptibility between NB- and CA-Mav strains, although NB-Mav was somewhat more resistant to fluoroquinolones, especially gatifloxacin, than CA-Mav. There was a significant association between VNTR genotype and susceptibility to quinolones and ethambutol in Mav isolates from Japanese patients.

Y. Tatano · C. Sano · K. Yasumoto · T. Shimizu · H. Tomioka (✉)
Department of Microbiology and Immunology,
Shimane University School of Medicine,
Izumo, Japan
e-mail: tomioka@med.shimane-u.ac.jp

K. Sato
Kobe Women's University,
Kobe, Japan

K. Nishimori
National Institute of Animal Health,
Tsukuba, Japan

T. Matsumoto
Osaka Prefectural Medical Center for Respiratory
and Allergic Diseases,
Habikino, Japan

S. Yano · H. Takeyama
National Hospital Organization Matsue Medical Center,
Matsue, Japan

Introduction

The worldwide increase in the incidence of intractable infections by *Mycobacterium avium* complex (MAC) is a serious concern for physicians engaged in the clinical control of mycobacterial infections [1]. To effectively treat MAC diseases, it is important to investigate recent profiles of change in the drug resistance, particularly in relation to the genetic polymorphisms of MAC isolates from patients with different clinical symptoms, pathologic features, medical histories, etc. A number of reports have described the genotyping of MAC using various methods, including insertion sequence (IS) *1245* and IS*1311* restriction fragment length polymorphism (RFLP)-, pulsed field gel electrophoresis (PFGE)-, and variable-number tandem-repeat (VNTR)-based typing [2, 3]. It has recently been demonstrated that VNTR typing is useful to distinguish and

classify MAC isolates from clinical specimens and environmental sources [4, 5]. Moreover, for VNTR typing of MAC, *M. avium* tandem repeat (MATR)-VNTR and mycobacterial interspersed repetitive units (MIRU)-VNTR typing methods using variation in the combination of targeted tandem repeat loci have been reported [4, 6]. Inagaki et al. reported that three different typing methods, IS*1245*-RFLP typing, MATR-VNTR typing and MIRU-VNTR typing were compared using 70 *M. avium* (Mav) clinical isolates [4]. According to them, MATR-VNTR typing is superior to MIRU-VNTR typing and IS*1245*-RFLP typing in classification of Mav strains [4]. However, few reports have examined the correlation between MAC genotype and drug susceptibility, and no report has described the association between genotype based on a VNTR analysis and drug susceptibility patterns.

Meanwhile, there has been a recent increase in the number of cases of MAC lung disease in patients other than those with apparent risk factors such as males with upper lobe cavitary disease (designated CA-type disease) and a history of alcohol abuse and heavy smoking. The patients are primarily elderly women with no history of smoking and reticulonodular infiltrates and have patchy bilateral bronchiectasis, with routine involvement of the right middle lobe and lingula (designated NB-type disease). The incidence of nodular-bronchiectasis type MAC pulmonary infections (NB-type disease) is increasing worldwide [7–9], especially in Japan [10]. The risk factors for such diseases are primarily host-related, and include severe exposure to MAC pathogens in persons who frequently take showers and anomalies of anatomical structure and physiological function in the lungs [7, 11]. At present, it is unclear whether or not there are MAC populations which cause NB-type lung disease rather than MAC disease, which mimics the tuberculosis (TB) showing CA-type disease frequently encountered in males. Recently, we examined whether or not there are unusual populations of MAC, causing NB-type disease rather than CA-type disease [12]. We compared the virulence of Mav isolates from patients with NB-type (NB-Mav) and CA-type diseases (CA-Mav) based on intracellular growth in various types of human cells. The two types of isolates showed a similar ability to replicate in macrophages and lung epithelial cells, indicating no essential difference in virulence in terms of infectivity to human macrophages and lung cells [12].

We carried out MATR-VNTR typing for Mav strains isolated from Japanese patients with MAC disease and examined whether there was any association between the VNTR type and drug susceptibility of these strains [4]. We also examined profiles of drug susceptibility and genotype-distribution of Mav strains isolated from Japanese with NB-type disease, and compared them with those from Japanese with CA-type disease. For this purpose, 14 NB-Mav strains and 10 CA-Mav strains were compared in terms of susceptibility to various antimycobacterials and MATR-VNTR type.

Materials and methods

Bacterial strains

Test Mav strains with transparent and irregular colonial morphology (SmT colonial variant) were isolated from Japanese patients with NB-type or CA-type disease as follows: Mav isolates from patients with NB-type disease ("strain N", 14 strains) and those from patients with CA-type disease ("strain C", 10 strains). The mean age of enrolled patients with NB-type disease was 76.4±3.0 years old and patients with CA-type disease was 72.7±3.7 years old. These was no significant difference between the two values (P=0.5189, Mann-Whitney's U-test). All patients provided informed consent.

Test bacterial strains were isolated from 24 patients with pulmonary MAC infection, including 20 patients who were newly diagnosed based on clinical, X-ray and CT findings, and four patients with recurrent MAC disease, who had previously received anti-MAC chemotherapy as described in the footnote of Table 1. All test strains were isolated from patients prior to the start of anti-MAC chemotherapy consisting of clarithromycin, rifampicin, ethambutol, isoniazid, streptomycin, or kanamycin during hospitalization at National Hospital Organization Matsue Medical Center. For isolation and preparation of test Mav strains, sputum samples from individual patients were cultured on Ogawa's egg medium and resultant bacterial colonies were inoculated onto Middlebrook 7H11 agar plates. After cultivation, a representative Mav colony with smooth and transparent colony morphology was inoculated into Middlebrook 7H9 medium (100 ml) and cultured for 5 to 7 days. Then, 10 ml each of the resultant culture were inoculated into 7H9 medium (100 ml) and further cultivated for 5 to 7 days. The resultant cultures were combined and centrifuged at 1,500 × g for 15 min. The resultant bacterial pellet was suspended in PBS containing 1% BSA and the final bacterial concentration was adjusted to OD_{540nm}=1.0.

Antimicrobial drugs

The following antimicrobials were used: clarithromycin (Taisho-Toyama Pharmaceutical Co., Tokyo), azithromycin (Sigma Chemical Co., St. Louis, MO., USA), rifampicin (Daiichi Sankyo Co., Tokyo), rifabutin (Pfizer Japan Inc., Tokyo), ethambutol (SIGMA), streptomycin (SIGMA), amikacin (SIGMA), isoniazid (SIGMA), moxifloxacin (Shionogi Pharmaceutical Co., Osaka), levofloxacin (Daiichi Sankyo Co., Tokyo), gatifloxacin (Kyorin Pharmaceutical

Table 1 Test Mav strains used in the present study and drug susceptibility of Mav isolates belonging to the three variable-number tandem-repeat (VNTR) clusters

Strain	Type of disease	Sex	Age[a]	MIC (μg/ml)												
				CLR	AZM	RIF	RFB	EMB	STR	AMK	INH	LVX	MXF	GAT	STX	GNX
Cluster A																
N7	NB	F	78	64	128	64	4	16	32	16	64	32	4	4	4	32
N8	NB	F	72	64	128	4	0.125	32	32	16	16	128	16	16	4	128
N10	NB	F	59	64	>128	128	8	128	128	16	64	128	16	16	8	>128
N11	NB	M	75	16	128	128	2	64	32	32	8	4	1	1	0.5	2
N14[b]	NB	F	85	32	128	4	0.06	32	32	16	16	32	16	16	8	128
C7	CA	M	85	32	128	8	0.25	16	64	32	16	1	1	0.5	0.25	4
C9	CA	M	74	32	128	8	0.25	32	64	32	64	8	2	2	1	16
Average age			75.4±3.4													
MIC range				16-64	≥128	4–128	0.06–8	16–128	32–128	16–32	8–64	1–128	1–16	0.5–16	0.25–8	2 to>128
MIC_{50}				32	128	8	0.25	32	32	16	16	32	4	4	4	32
Cluster B																
N5	NB	M	91	32	128	128	2	32	128	32	>128	4	1	1	0.5	2
N6	NB	F	76	32	128	64	1	16	32	16	2	1	0.5	1	0.5	4
N9	NB	F	88	16	16	64	4	8	8	8	32	4	1	1	0.5	2
N12[b]	NB	F	57	8	128	64	2	32	32	32	8	1	1	1	0.25	1
N13	NB	F	68	8	64	128	4	16	16	16	2	1	0.5	0.5	0.25	1
N15	NB	F	69	32	128	64	4	16	16	16	16	2	0.5	0.25	0.5	1
C1	CA	F	81	32	128	8	0.25	32	32	16	128	4	2	1	0.5	4
C2	CA	F	66	64	128	128	4	32	64	16	4	2	1	0.5	0.5	4
C4	CA	M	76	16	128	4	0.125	32	64	32	64	1	0.5	0.25	0.125	1
C5	CA	F	78	32	64	128	4	32	64	16	16	1	0.5	0.5	0.25	2
C6	CA	M	74	16	32	128	2	16	16	8	4	0.25	0.125	0.125	0.06	0.5
C10	CA	F	46	16	64	128	4	32	32	16	16	4	1	1	0.5	8
C11[b]	CA	F	84	8	64	64	2	32	32	32	8	2	0.5	0.5	0.5	2
Average age			73.4±3.4													
MIC range				8–64	16–128	4–128	0.125–4	8–32	8–128	8–32	2 to >128	0.25–4	0.125–2	0.125–1	0.06 – 0.5	0.5–8
MIC_{50}				16	128	64	2	32	32	16	16	2	0.5	0.5	0.5	2

Table 1 (continued)

Strain	Type of disease	Sex	Age[a]	MIC (μg/ml)											
				CLR	AZM	RIF	RFB	EMB	STR	AMK	INH	LVX	MXF	GAT	STX	GNX
Cluster C																
N1	NB	F	82	32	128	8	0.25	32	64	32	8	4	2	2	1	16
N3[b]	NB	F	76	16	128	64	1	64	64	32	128	32	8	8	4	64
N4	NB	F	94	32	128	128	1	64	64	16	1	4	1	1	0.5	2
C3	CA	M	63	32	128	4	0.125	64	32	16	128	128	64	64	16	>128
Average age			78.8±6.4													
MIC range				16–32	128	4–128	0.125–1	32–64	32–64	16–32	1–128	4–128	1–64	1–64	0.5–16	2 to >128
MIC_{50}				32	128	8	0.25	64	64	16	8	4	2	2	1	16

NB nodular-bronchiectasis-type, *CA* tuberculosis-type with upper lobe cavitary disease, *M* male, *F* female, *CLR* clarithromycin, *AZM* azithromycin, *RIF* rifampicin, *RFB* rifabutin, *EMB* ethambutol, *STR* streptomycin, *AMK* amikacin, *INH* isoniazid, *LVX* levofloxacin, *MXF* moxifloxacin, *GAT* gatifloxacin, *STX* sitafloxacin, *GNX* garenoxacin, *KAN* kanamycin

[a] The mean age of enrolled patients with NB-type disease was 76.4±3.0 years old and patients with CA-type disease was 72.7±3.7 years old. There was no significant difference between the two values (P=0.5189, Mann-Whitney's U-test).

[b] These strains were isolated from patients with recurrent MAC disease, who had previously received mono or multi-drug anti-MAC chemotherapy as follows: N3, RIF/EMB/STR; N12 and N14, RIF/EMB/INH; C11, CLR.

Co., Tokyo), sitafloxacin (Daiichi-Sankyo Co.), and garenoxacin (Taisho-Toyama Pharmaceutical Co.).

VNTR genotyping

Genotyping by a MATR-VNTR typing was performed according to Inagaki et al. [4]. Briefly, template DNA was extracted from each test strain using InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA, USA) and subjected to PCR using primer kits described by them. The resultant amplicon DNAs were then subjected to electrophoresis, and the numbers of repetitions of 15 VNTR loci were determined in each test strain according to the amplified fragment sizes determined by Kodak EDAS 290 (Electrophoresis Documentation Analysis System) and Kodak 1D Image software Ver. 3.5.2 (Kodak, Rochester, NY) for fragment size calculation as well as by the results obtained with a previously reported allele-calling table by Inagaki et al. [4].

The dendrogram of the test strains was constructed by the Fitch-Margoliash analysis using PHYLIP software (Version 3.69). This method seeks the tree that best predicts a set of pairwise distances among species or strains, and uses a weighted least squares analysis for clustering based on genetic distance [13]. Closely related sequences are given more weight in the tree construction process in order to correct for the increased inaccuracy in measuring distances between distantly related sequences. In this method, the genotypic diversity of test strains of a given bacterial species is assessed on the basis of Manhattan distance as a parameter of the diversity of genetic profiles between each set of test strains. In our MATR-VNTR typing for Mav isolates, the Manhattan distance between strain x and strain y is calculated using the formula,

$$\sum_{n=1}^{15} |x_n - y_n|$$

where x_n and y_n are the number of repeat units in the nth MATR locus [14]. The Manhattan distances used as input to the algorithm are normalized to prevent large artifacts in computing relationships between closely related and distantly related groups. The least-squares criterion applied to these distances is more accurate but less efficient than the neighbor-joining methods.

Drug susceptibility testing

MICs of test drugs were determined by the broth dilution method using 7HSF medium, a broth medium with the same composition as 7H11 agar without malachite green. The bacterial suspension in 7HSF medium (0.1 ml) containing 1×10^5 CFU was inoculated into 0.1 ml of

7HSF medium containing test drugs in microculture wells. After cultivation at 37°C for 14 days, MICs were read as minimum concentrations of drugs completely inhibiting visible growth of organisms. It has been reported that MIC results determined by this type of the broth dilution method showed good agreement with results obtained by the agar proportion method [15].

Statistical analysis

Bonferroni's multiple *t*-test was used for comparison of the mean $\log_2$ values of MICs of test drugs for Mav strains belonging to the three VNTR clusters and the average patient age in the three VNTR-genotype groups. Mann-Whitney U-test was used for comparison of the mean $\log_2$ values of MICs of test drugs between NB- and CA-Mav and for comparison of the mean patient ages of enrolled patients with NB-type disease and CA-type disease. Two-tailed Fisher's exact test was used for test of significant difference in the distribution pattern of drug susceptibility for Mav strains belonging to the three VNTR clusters or between the two clinical types (NB- and CA-Mav).

Results

Genotyping and clustering of Mav isolates from patients with NB-type and CA-type diseases

To assess the association between genotype and drug susceptibility, we determined the VNTR profiles of each Mav isolate by estimating the numbers of repeat units at 15 MATR loci (Fig. 1a). The profiling data were determined for 24 Mav strains isolated from 14 and 10 patients with NB-type and CA-type MAC diseases, respectively. Genotypic diversity in VNTR profiles was calculated as the Manhattan distance between each pair of Mav isolates and analyzed using a Fitch-Margoliash algorithm. In the phylogenetic tree, showing relationships among VNTR profiles, the Mav isolates separated into three major groups on MATR-VNTR-based genotyping: cluster A containing strains N7, N8, N10, N11, N14, C7, and C9; cluster B comprising N5, N6, N9, N12, N13, N15, C1, C2, C4, C5, C6, C10, and C11; and cluster C containing N1, N3, N4, and C3 (Fig. 1a and b). The characteristics of these clusters are summarized below, although some exceptions exist. It appears that the discriminatory MATR loci are 1, 2, 3, 7, 9, 11 and 14. The numbers of tandem repeat in each loci of cluster A: xx, 0, 4/5, 1–7, 2, xx, 1–4; cluster B: xx, 0–3, 1/2, 1, 3, 1, 2/3; cluster C: 1–3, 0, xx, 2, 2, 2, 2.

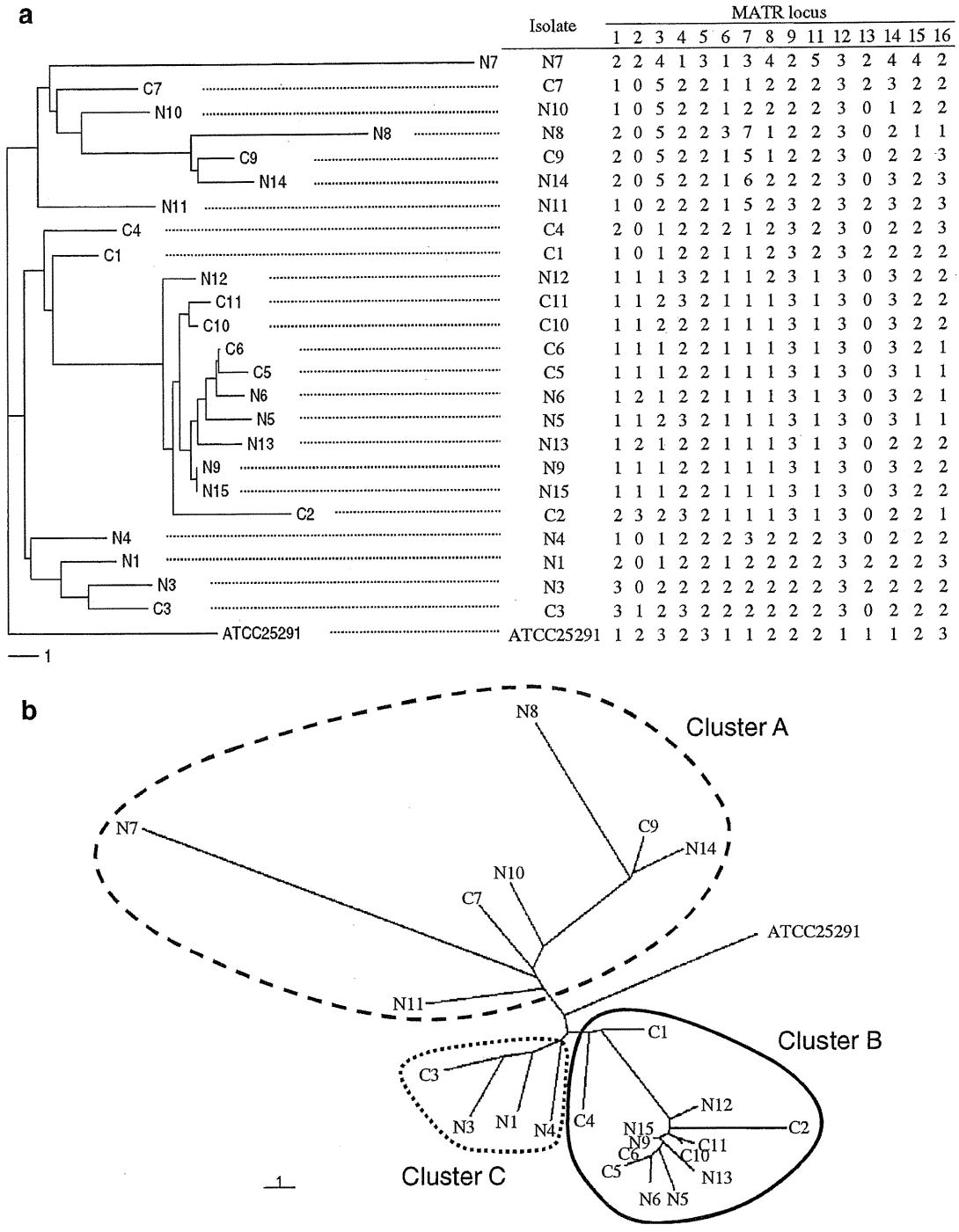
Notably, there was no obvious difference with respect to the distribution of NB-Mav and CA-Mav in the two genotype groups particularly in clusters A and B, although CA-Mav strains were more likely to be grouped in cluster B. This finding indicates essentially no association of the NB-Mav and CA-Mav strains with the phylogenetic tree based on VNTR genotyping. Thus, it appears that distinguishing NB-Mav from CA-Mav isolates has no meaning in terms of the evolutionary history of Mav organisms.

Association between VNTR genotype and drug susceptibility

We next determined the drug susceptibility profiles of Mav isolates belonging to the three VNTR clusters using two macrolides (clarithromycin and azithromycin), two rifamycins (rifampicin and rifabutin), ethambutol, two aminoglycosides (streptomycin and amikacin), isoniazid, four fluoroquinolones (levofloxacin, moxifloxacin, gatifloxacin, and sitafloxacin), and des-F(6)-quinolone (garenoxacin) (Table 1). Table 2 shows the mean $\log_2$ values (± SEM) of MICs of individual drugs for Mav strains belonging to clusters A to C. The following findings were obtained: (1) Log_2MICs of the five quinolones were much smaller for Mav strains belonging to cluster B (designated "cluster B-Mav strains") than cluster A- and cluster C-Mav strains (P=0.0012–0.018: Bonferroni's multiple *t*-test), (2) Log_2MICs of macrolides (clarithromycin and azithromycin) were moderately smaller for cluster B-Mav than cluster A-Mav strains (P=0.066 to 0.078), (3) Log_2MICs of ethambutol were significantly smaller for cluster B-Mav than cluster C-Mav strains (P=0.039), and (4) Log_2MICs of rifamycins (rifampicin and rifabutin) tended to be larger for cluster B-Mav strains than cluster A-Mav strains (P=0.26 to 0.32). The assessment was confirmed using the two-tailed Fisher's exact test as follows (data not shown). Between cluster A and cluster B there was significant difference in the distribution pattern of drug susceptibility to the five quinolones (P<0.01), and between cluster B and cluster C there was significant difference in the distribution mode of drug susceptibility to rifabutin (P< 0.05), ethambutol (P<0.01), and these quinolones (P<0.01 or P<0.05). These findings indicate a significant association between VNTR genotype and susceptibility to certain drugs, especially quinolones.

Association between NB-Mav or CA-Mav isolates and drug susceptibility

Next, we assessed the association of the origins of the Mav isolates, i.e., patients with NB-type or CA-type disease, with drug susceptibility profiles. The following were observed. (1) The Log_2MICs of gatifloxacin tended to be smaller for CA-Mav than NB-Mav (P=0.063, Mann-Whitney U-test). Notably, the two-tailed Fisher's exact test indicated a significant difference between MICs of gatifloxacin for NB-Mav and CA-Mav isolates (P=0.032). (2) No such



Isolate	MATR locus														
	1	2	3	4	5	6	7	8	9	11	12	13	14	15	16
N7	2	2	4	1	3	1	3	4	2	5	3	2	4	4	2
C7	1	0	5	2	2	1	1	2	2	2	3	2	3	2	2
N10	1	0	5	2	2	1	2	2	2	2	3	0	1	2	2
N8	2	0	5	2	2	3	7	1	2	2	3	0	2	1	1
C9	2	0	5	2	2	1	5	1	2	2	3	0	2	2	3
N14	2	0	5	2	2	1	6	2	2	2	3	0	3	2	3
N11	1	0	2	2	2	1	5	2	3	2	3	2	3	2	3
C4	2	0	1	2	2	2	1	2	3	2	3	0	2	2	3
C1	1	0	1	2	2	1	1	2	3	2	3	2	2	2	2
N12	1	1	1	3	2	1	1	2	3	1	3	0	3	2	2
C11	1	1	2	3	2	1	1	1	3	1	3	0	3	2	2
C10	1	1	2	2	2	1	1	1	3	1	3	0	3	2	2
C6	1	1	1	2	2	1	1	1	3	1	3	0	3	2	1
C5	1	1	1	2	2	1	1	1	3	1	3	0	3	1	1
N6	1	2	1	2	2	1	1	1	3	1	3	0	3	2	1
N5	1	1	2	3	2	1	1	1	3	1	3	0	3	1	1
N13	1	2	1	2	2	1	1	1	3	1	3	0	2	2	2
N9	1	1	1	2	2	1	1	1	3	1	3	0	3	2	2
N15	1	1	1	2	2	1	1	1	3	1	3	0	3	2	2
C2	2	3	2	3	2	1	1	1	3	1	3	0	2	2	1
N4	1	0	1	2	2	2	3	2	2	2	3	0	2	2	2
N1	2	0	1	2	2	1	2	2	2	2	3	2	2	2	3
N3	3	0	2	2	2	2	2	2	2	2	3	2	2	2	2
C3	3	1	2	3	2	2	2	2	2	2	3	0	2	2	2
ATCC25291	1	2	3	2	3	1	1	2	2	2	1	1	1	2	3

Fig. 1 Variable-number tandem-repeat (VNTR) genotyping of 24 Mav strains, including 14 NB-Mav and 10 CA-Mav isolates. **a**. Dendrogram and allele profiles constructed from MATR-VNTR typing. The dendrogram was created from distance matrix files by Fitch-Margoliash analysis according to MATR-VNTR markers. The Manhattan distance is indicated at the bottom of this figure. **b**. Cluster analysis of Mav isolates based on VNTR profiles. The Manhattan distance between each pair of isolates was calculated and analysed using a Fitch-Margoliash algorithm

difference in susceptibility profiles between NB- and CA-Mav was noted for the other antimycobacterial drugs. These findings suggest that the susceptibility of NB-Mav and CA-Mav to the drugs tested including macrolides, rifamycins, ethambutol, aminoglycosides, isoniazid and quinolones, did not differ significantly. However, NB-Mav isolates tended to

Table 2 The mean log_2 values of MICs of test drugs for Mav strains belonging to the three variable-number tandem-repeat (VNTR) clusters

Drug	Log_2MIC (mean ± SEM)			*P* value for difference in log_2MICs between[a] :		
	Cluster A	Cluster B	Cluster C	Clusters A vs. B	Clusters A vs. C	Clusters B vs. C
CLR	5.29±0.29	4.31±0.26	4.75±0.25	0.0660	>0.900	>0.900
AZM	7.14±0.14	6.31±0.26	7.00±0.00	0.0783	>0.900	0.3558
RIF	4.29±0.87	5.92±0.45	4.50±1.19	0.2604	>0.900	0.6423
RFB	−0.72±1.02	0.85±0.45	−1.25±0.75	0.3198	>0.900	0.2322
EMB	5.14±0.40	4.54±0.18	5.75±0.25	0.3423	0.6873	0.0393
STR	5.57±0.30	5.00±0.30	5.75±0.25	0.6231	>0.900	0.5292
AMK	4.43±0.20	4.15±0.19	4.50±0.29	>0.900	>0.900	>0.900
INH	4.71±0.47	3.85±0.61	4.25±1.70	>0.900	>0.900	>0.900
LVX	4.14±0.99	0.69±0.33	4.00±1.23	0.0027	>0.900	0.0183
MXF	2.14±0.71	−0.62±0.27	2.50±1.32	0.0039	>0.900	0.0075
GAT	2.00±0.79	−0.85±0.27	2.50±1.32	0.0048	>0.900	0.0063
STX	1.00±0.76	−1.62±0.27	1.25±1.11	0.0048	>0.900	0.0114
GNX	4.86±1.01	0.92±0.31	4.75±1.49	0.0012	>0.900	0.0093

Abbreviations of test drugs are the same as in Table 1

[a] Significance of the difference in Log_2MIC values between indicated clusters (Bonferroni's multiple *t*-test)

be more resistant to gatifloxacin and azithromycin than CA-Mav isolates.

Discussion

RFLP typing with detection of polymorphisms by hybridization to IS*1245* (IS*1245*-RFLP) or IS*1311* (IS*1311*-RFLP) has widely been used to type Mav isolates. However, this method has some disadvantages as follows [3, 4, 6, 16]. This method is time consuming and technically demanding as in the case of PFGE typing. In addition, VNTR requires analysis of complex banding patterns, causing low reproducibility, and it has limited discriminatory power. Therefore, this method is not suitable for epidemiological study among multiple facilities. On the other hand, VNTR typing, especially MATR-VNTR typing, has the following advantages [3, 4, 6, 16]. First, this method is a simple and highly reproducible procedure that can be performed with very little DNA and regardless of whether the bacteria are living or dead. Second, results are digital from the outset, thereby simplifying the comparison of large numbers of strains. Such digitization of data enables easy comparison among facilities. Third, VNTR typing has a high discriminatory power.

In this context, a recent study by Killgore et al. [17] indicated that seven techniques including multilocus VNTR analysis (MLVA), restriction endonuclease analysis (REA), PFGE, PCR-ribotyping, multilocus sequence typing (MLST), amplified fragment length polymorphism (AFLP), and surface layer protein A gene sequence typing (*slpA*ST) were capable of typing and clustering international epidemic strains of *Clostridium difficile* in essentially the same fashion. In this case, the discrimination index scores for these techniques ranged from 0.96 to 0.63 in the following order: MLVA, REA, PFGE, *slpA*ST, PCR-ribotyping, MLST, and AFLP. Thus, it is thought that multilocus VNTR analysis is highly useful in epidemiological studies of clinical isolates and is powerful in clustering test bacterial strains. Notably, also in epidemiological studies on Mav isolates, the MATR-VNTR method, which was used in our study, has been demonstrated to exhibit good discriminatory power, yielding a Hunter-Gaston discriminatory index of 0.990, which is greater than those of IS*1245* RFLP typing and the MIRU-VNTR method, at 0.960 and 0.949, respectively [4]. Similar findings have been reported for molecular epidemiological analysis of *M. avium* subsp. *paratuberculosis* isolates [6]. Incidentally, MIRUs of Mav are identified as homologous regions with MIRU motifs that were derived from consensus of previously described MIRUs from *M. tuberculosis* [6, 18, 19]. MATR-VNTR is the VNTR analysis method using variable tandem repeat loci containing not only MIRU regions but also the regions having exact tandem repeat (ETR) [20], Queen's University Belfast (QUB) [21, 22], etc. Hence, MATR-VNTR and MIRU-VNTR typing methods are based on the same principle; however, MATR-VNTR differs from MIRU-VNTR in the combination of VNTR loci used. As described above, Inagaki et al. typed Mav strains according to IS*1245*-RFLP patterns, MATR-VNTR patterns and MIRU-VNTR patterns, using the MIRUs reported by Thibault et al. [4, 6]. Therefore, they proposed the usefulness of MATR-VNTR typing analysis.

The present study indicated the following with respect to the drug susceptibility profiles of Mav strains isolated from patients with NB-type and CA-type diseases. The MATR-VNTR-based genotypic characteristics of the Mav strains tested were associated with drug susceptibility profiles,

particularly for quinolones and ethambutol. As shown in Table 2, the MICs of quinolones (levofloxacin, moxifloxacin, gatifloxacin, sitafloxacin, and garenoxacin) had a distribution with significantly smaller values for cluster B-Mav than cluster A-Mav or C-Mav strains ($P<0.01$ or $P<0.05$). In addition, MICs of ethambutol were significantly smaller for cluster B-Mav than cluster C-Mav strains ($P<0.05$ or $P<0.01$). The average age of the three VNTR-genotype groups of patients, from whom cluster A, B and C Mav strains were as follows: cluster A, 75.4±3.4 years old; cluster B, 73.4±3.4 years old; cluster C, 78.8±6.4 years old. There was no significant difference in the average age among these three groups of patients ($P=0.427$–0.711; Bonferroni's multiple t-test). Therefore, it appears that the difference in the drug susceptibility profiles among Mav strains belonging to cluster A, B, and C is not related to the age of patients from whom the Mav strains were isolated.

Dvorska et al. found that there was no relation between IS*1311* and IS*1245*-based RFLP genotypes or serotypes and drug susceptibility for MAC isolates obtained from a HIV-negative patient during the course of treatment for TB [23]. Ohkusu et al. reported no apparent correlation between RFLP patterns and antibiograms among Mav strains isolated from HIV-infected patients with disseminate MAC disease [24]. As far as we know, these are the only two reports to have examined the relation between the genotypes and drug susceptibility of MAC strains. The present findings based on VNTR-genotyping are inconsistent with these observations. However, Wu et al. reported a clear relationship between high drug resistance and genetic relatedness based on PFGE genotyping in *Mycobacterium kansasii* isolates from a university hospital in Taiwan [25]. They reported that *M. kansasii* strains belonging to cluster 1 of the nine differentiated PFGE clusters were closely associated with isoniazid and rifampicin resistance and treatment difficulty [25]. Furthermore, it is also known that a close relationship between genotype and drug resistance exists in the case of a characteristic spoligotype W-Beijing family of *M. tuberculosis* [26–29]. These reports support the present finding that VNTR-based genotypic characteristics of Mav strains were associated with susceptibility to certain drugs. Therefore, genetic background may influence the acquisition of particular determinants of resistance by Mav as well as MTB, presumably by facilitating bacterial survival under drug pressure in hosts.

There was no obvious difference in the distribution of VNTR-genotypes, clusters A, B and C, between the NB-Mav and CA-Mav isolates. We previously compared the virulence of NB-Mav and CA-Mav strains based on intracellular growth in various human cells. NB- and CA-Mav isolates showed a similar ability to replicate in THP-1 macrophages, Mono Mac-6 macrophages, U-937 macrophages, A-549 alveolar epithelial cells, and NL-20 tracheal epithelial cells [12]. Moreover, NB-Mav and CA-Mav strains showed a similar ability to induce the production of reactive nitrogen intermediates and reactive oxygen intermediates by THP-1 macrophages [12]. Therefore, it appears that there is no essential difference in virulence in terms of infectivity to human macrophages and lung cells between Mav strains isolated from patients with NB-MAC disease and those from patients with CA-MAC disease. Taken together, the results indicate that there is no particular Mav genotype/phenotype which causes NB-type MAC diseases.

It has been reported that clarithromycin-resistant strains were more frequently isolated from patients with NB-type MAC than CA-type MAC infections, because the former had been treated with high-dose clarithromycin [30]. However, in the present study, we observed no such tendency. In addition, MIC of clarithromycin for strain C11 isolated from one patient who had been given 2-month chemotherapy with clarithromycin alone was 8 μg/ml (Table 1). Meanwhile, MICs of clarithromycin for 20 strains isolated from patients without preceding clarithromycin-treatment ranged from 8 to 64 μg/ml (median = 32 μg/ml) (Table 1). Thus, in the present study, resistance to clarithromycin did not emerge during the course of treatment with clarithromycin. This result is consistent with the finding that MAC strains from a patient with NB-type disease were susceptible to clarithromycin despite long-term chemotherapy that involved clarithromycin [31].

Overall, the present study indicated a significant association between VNTR genotype and susceptibility to certain antimycobacterial drugs including quinolones, macrolides, and ethambutol. Mycobacterial resistance to these drugs is primarily caused by mutations of genes encoding bacterial drug targets, for example, for quinolones: *gyrAB* genes (especially quinolone-resistant-determining region) encoding DNA gyrase; macrolides: 23S rRNA gene; and ethambutol: *embABC* genes encoding arabinosyl transferases [32–35]. Therefore, it is of interest to examine the possibility that mutations or polymorphism in these genes cause mild resistance of certain VNTR clusters to these drugs in the following cases: clusters A and C vs. quinolones; cluster A vs. macrolides; cluster C vs. ethambutol. However, it is noteworthy that, in cases of quinolone resistance of *M. tuberculosis*, the alternative mechanisms, such as mutations in the *mfpA* gene, encoding a DNA gyrase-binidng protein MfpA, and *Rv2686c-Rv2687c-Rv2688c* operon, encoding an ATP-binding cassette transpoter, are associated with lower levels of quinolone resistance [36, 37]. Similarly, mutations of the *embR* gene encoding the EmbR regulatory protein with ATPase activity may be responsible for mild resistance of *M. tuberculosis* to ethambutol [38]. Thus, it is also interesting to examine the linkage of mutations of these genes, engaged in the alternative mechanisms of mycobacterial resistance to these drugs, with the present VNTR

genotyping of Mav isolates. Further study to clarify the molecular ecological reason for the association between VNTR genotype and mild resistance to these drugs is currently under way.

Acknowledgements This study was supported in part by a grant (18590850) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. We thank Taisho-Toyama Pharmaceutical Co., Daiichi Sankyo Co., Pfizer Japan Inc., Shionogi Pharmaceutical Co., and Kyorin Pharmaceutical Co. for providing antimicrobial drugs used in this study. We thank Ms. S. Yamabe for her kind assistance with this study.

References

1. Kasperbauer SH, Daley CL (2008) Diagnosis and treatment of infections due to *Mycobacterium avium* complex. Semin Respir Crit Care Med 29:569–576
2. Inglis N, McFadden J (1999) Strain typing of the *Mycobacterium avium* complex. J Infect 38:151–156
3. Radomski N, Thibault VC, Karoui C et al (2010) Determination of genotypic diversity of *Mycobacterium avium* subspecies from human and animal origins by mycobacterial interspersed repetitive-unit variable-number tandem-repeat and IS*1311* restriction fragment length polymorphism typing methods. J Clin Microbiol 48:1026–1034
4. Inagaki T, Nishimori K, Yagi T et al (2009) Comparison of a variable-number tandem-repeat (VNTR) method for typing *Mycobacterium avium* with mycobacterial interspersed repetitive-unit-VNTR and IS*1245* restriction fragment length polymorphism typing. J Clin Microbiol 47:2156–2164
5. Tirkkonen T, Pakarinen J, Rintala E et al (2010) Comparison of variable-number tandem-repeat markers typing and IS*1245* restriction fragment length polymorphism fingerprinting of *Mycobacterium avium* subsp. *hominissuis* from human and porcine origins. Acta Vet Scand 52:21
6. Thibault VC, Grayon M, Boschiroli ML et al (2007) New variable-number tandem-repeat markers for typing *Mycobacterium avium* subsp. *paratuberculosis* and *M. avium* strains: comparison with IS*900* and IS*1245* restriction fragment length polymorphism typing. J Clin Microbiol 45:2404–2410
7. Iseman MD (1989) *Mycobacterium avium* complex and the normal host: the other side of the coin. N Engl J Med 321:896–898
8. Reich JM, Johnson RE (1992) *Mycobacterium avium* complex pulmonary disease presenting as an isolated lingular or middle lobe pattern. The Lady Windermere syndrome. Chest 101:1605–1609
9. Glassroth J (2008) Pulmonary disease due to nontuberculous mycobacteria. Chest 133:243–251
10. Harada S, Harada Y, Ochiai S et al (2003) A clinical study on cases with pulmonary *M. avium* complex (MAC) disease followed up for more than 10 years. Kekkaku 78:517–523
11. Falkinham JO 3rd, Iseman MD, de Haas P et al (2008) *Mycobacterium avium* in a shower linked to pulmonary disease. J Water Health 6:209–213
12. Tatano Y, Yasumoto K, Tomioka H et al (2010) Comparative study for the virulence of *Mycobacterium avium* isolates from patients with nodular-bronchiectasis- and cavitary-type diseases. Eur J Clin Microbiol Infect Dis 29:801–806
13. Fitch WM, Margoliash E (1967) Construction of phylogenetic trees. Science 155:279–284
14. Kikuchi T, Watanabe A, Gomi K et al (2009) Association between mycobacterial genotypes and disease progression in *Mycobacterium avium* pulmonary infection. Thorax 64:901–907
15. Yajko DM, Madej JJ, Lancaster MV et al (1995) Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J Clin Microbiol 33:2324–2327
16. Castellanos E, Romero B, Rodríguez S et al (2010) Molecular characterization of *Mycobacterium avium* subspecies *paratuberculosis* types II and III isolates by a combination of MIRU-VNTR loci. Vet Microbiol 144:118–126
17. Killgore G, Thompson A, Johnson S et al (2008) Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol 46:431–437
18. Bull TJ, Sidi-Boumedine K, McMinn EJ et al (2003) Mycobacterial interspersed repetitive units (MIRU) differentiate *Mycobacterium avium* subspecies *paratuberculosis* from other species of the *Mycobacterium avium* complex. Mol Cell Probes 17:157–164
19. Romano MI, Amadio A, Bigi F et al (2005) Further analysis of VNTR and MIRU in the genome of *Mycobacterium avium* complex, and application to molecular epidemiology of isolates from South America. Vet Microbiol 110:221–237
20. Frothingham R, Meeker-O'Connell WA (1998) Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. Microbiology 144:1189–1196
21. Skuce RA, McCorry TP, McCarroll JF et al (2002) Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. Microbiology 148:519–528
22. Roring S, Scott A, Brittain D et al (2002) Development of variable-number tandem repeat typing of *Mycobacterium bovis*: comparison of results with those obtained by using existing exact tandem repeats and spoligotyping. J Clin Microbiol 40:2126–2133
23. Dvorska L, Bartos M, Ostadal O et al (2002) IS*1311* and IS*1245* restriction fragment length polymorphism analyses, serotypes, and drug susceptibilities of *Mycobacterium avium* complex isolates obtained from a human immunodeficiency virus-negative patient. J Clin Microbiol 40:3712–3719
24. Ohkusu K, Bermudez LE, Nash KA et al (2004) Differential virulence of *Mycobacterium avium* strains isolated from HIV-infected patients with disseminated *M. avium* complex disease. J Infect Dis 190:1347–1354
25. Wu TS, Leu HS, Chiu CH et al (2009) Clinical manifestations, antibiotic susceptibility and molecular analysis of *Mycobacterium kansasii* isolates from a university hospital in Taiwan. J Antimicrob Chemother 64:511–514
26. Anh DD, Borgdorff MW, Van LN et al (2000) *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 6:302–305
27. Bifani PJ, Plikaytis BB, Kapur V et al (1996) *Mycobacterium tuberculosis* clone family. JAMA 275:452–457
28. Portaels F, Rigouts L, Bastian I (1999) Addressing multidrug-resistant tuberculosis in penitentiary hospitals and in the general population of the former Soviet Union. Int J Tuberc Lung Dis 3:582–588
29. Cox HS, Niemann S, Ismailov G et al (2007) Risk of acquired drug resistance during short-course directly observed treatment of tuberculosis in an area with high levels of drug resistance. Clin Infect Dis 44:1421–1427
30. Kuwabara K, Tsuchiya T (2007) Clinical features and treatment history of clarithromycin resistance in *M. avium-intracellulare* complex pulmonary disease patients. J Jap Respir Soc 45:587–592
31. Kuwabara K, Watanabe Y, Wada K et al (2004) Relations between clinical subtypes of *Mycobacterium avium* pulmonary disease and

polyclonal infections detected by IS*1245* based restriction fragment length polymorphism analysis. Kekkaku 79:39–46
32. Zhang Y, Yew WW (2009) Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 13:1320–1330
33. Takiff HE, Salazar L, Guerrero C et al (1994) Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother 38:773–780
34. Meier A, Kirschner P, Springer B et al (1994) Identification of mutations in 23S rRNA gene of clarithromycin-resistant *Mycobacterium intracellulare*. Antimicrob Agents Chemother 38:381–384
35. Belanger AE, Besra GS, Ford ME et al (1996) The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci USA 93:11919–11924
36. Hegde SS, Vetting MW, Roderick SL (2005) A fluoroquinolone resistance protein from *Mycobacterium tuberculosis* that mimics DNA. Science 308:1480–1483
37. Pasca MR, Guglierame P, Arcesi F et al (2004) Rv2686c-Rv2687c-Rv2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 48:3175–3178
38. Sharma K, Gupta M, Krupa A et al (2006) EmbR, a regulatory protein with ATPase activity, is a substrate of multiple serine/threonine kinases and phosphatase in *Mycobacterium tuberculosis*. FEBS J 273:2711–2721

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.

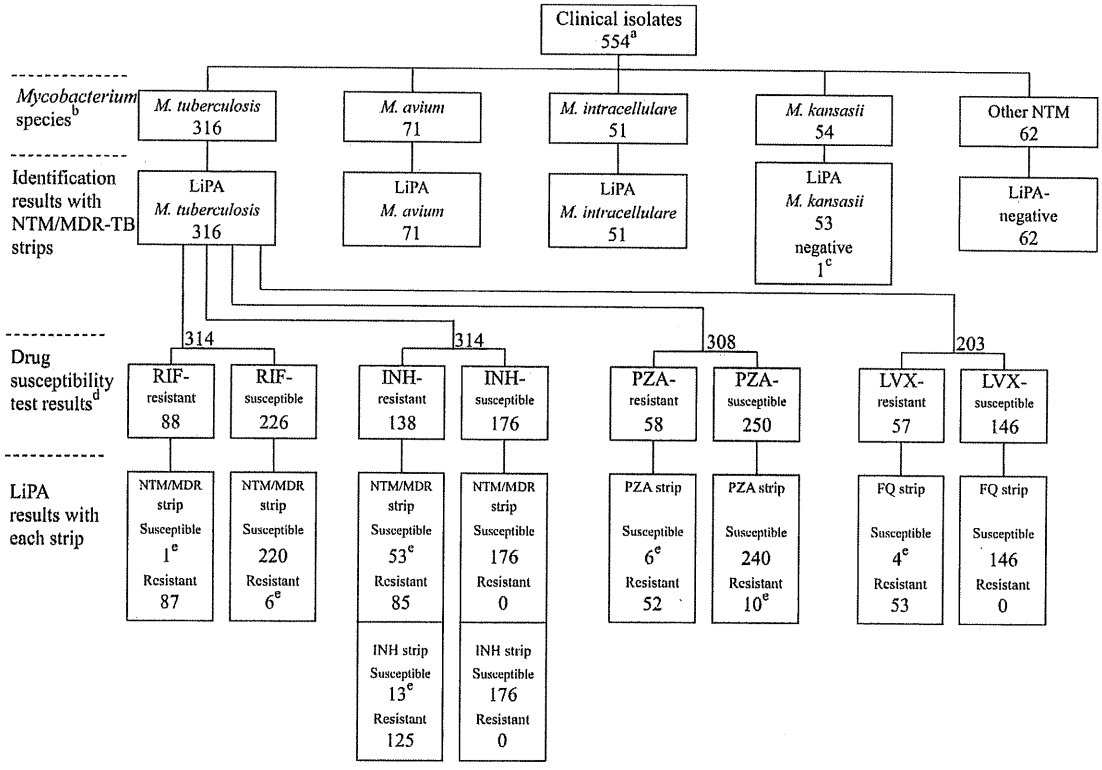


FIG 1 Distribution of LiPA results for 554 clinical isolates. [a], number of clinical isolates. [b], *Mycobacterium* species identified by conventional methods. [c], *M. kansasii* subtype III. [d], drug susceptibility testing and assays for pyrazinamidase activity were performed at each hospital (see 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

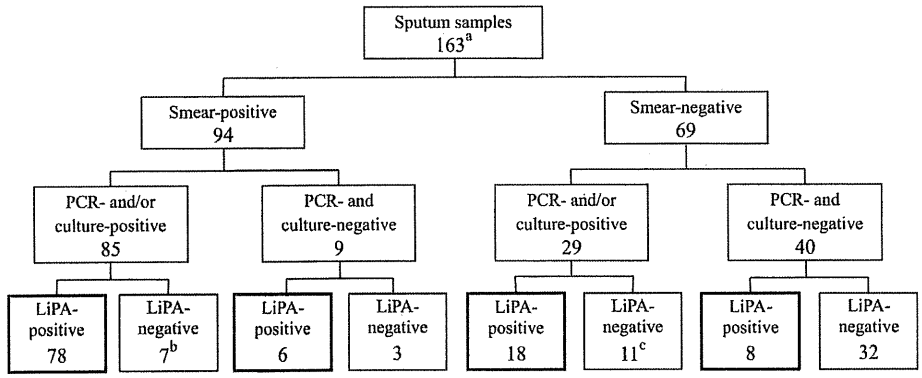


FIG 2 Distribution of LiPA results obtained with NTM/MDR-TB strips for the detection of target species in 163 sputum samples. [a], number of clinical samples. [b], one of these isolates was *Mycobacterium fortuitum*. [c], one of these isolates was *Mycobacterium abscessus*.

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-

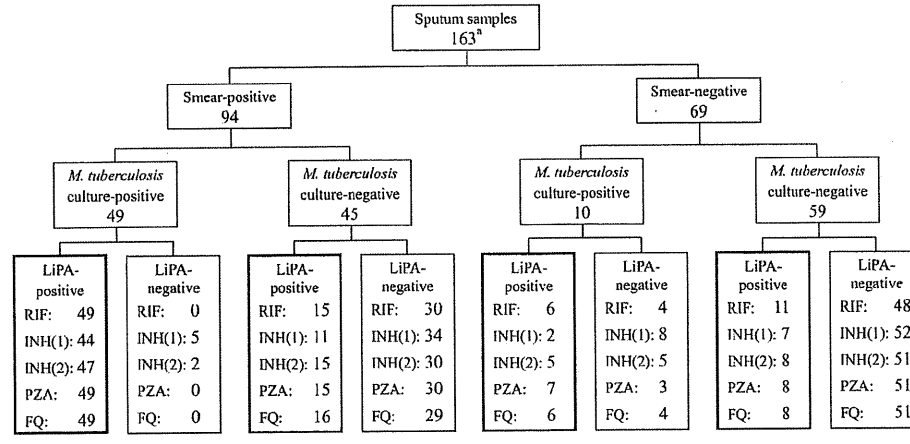


FIG 3 Distribution of LiPA results obtained with four strips for detection of a mutation(s) associated with drug resistance in 163 sputum samples. [a], number of clinical samples. RIF, RIF susceptibility with NTM/MDR-TB strips; INH(1), INH susceptibility with INH strips; INH(2), INH susceptibility with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

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 33, pp. 27743–27752, August 10, 2012

A Novel Mechanism of Growth Phase-dependent Tolerance to Isoniazid in Mycobacteria*

Received for publication, December 13, 2011, and in revised form, May 27, 2012 Published, JBC Papers in Press, May 30, 2012, DOI 10.1074/jbc.M111.333385

Makoto Niki[‡], Mamiko Niki[‡1], Yoshitaka Tateishi[‡§], Yuriko Ozeki[‡¶], Teruo Kirikae[‖], Astrid Lewin[**], Yusuke Inoue[‡‡], Makoto Matsumoto[‡‡], John L. Dahl[§§], Hisashi Ogura[¶¶], Kazuo Kobayashi[‖‖], and Sohkichi Matsumoto[‡2]

From the [‡]Departments of Bacteriology and [¶¶]Virology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan, the [§]Department of Internal Medicine, National Hospital Organization Toneyama National Hospital, 5-1-1 Toneyama, Toyonaka, Osaka 560-8552, Japan, the [¶]Department of Food and Nutrition, Sonoda Women's University, 7-29-1 Minamitsukaguchi-cho, Amagasaki, Hyogo 661-8520, Japan, the [‖]National Center for Global Health and Medicine, 1-21-1 Toyama, Shinjuku, Tokyo 162-8655, Japan, the [**]Robert Koch Institute, Nordufer 20, 13353 Berlin, Germany, the [‡‡]Microbiological Research Institute, Otsuka Pharmaceutical, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan, the [§§]Department of Biology, University of Minnesota Duluth, Duluth, Minnesota 55812, and the [‖‖]Department of Immunology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Background: The mechanism underlying mycobacterial phenotypic tolerance to isoniazid is unknown.
Results: MDP1, a mycobacterial histone-like protein, down-regulates KatG expression.
Conclusion: Down-regulation of KatG by MDP1 causes growth phase-dependent phenotypic tolerance to isoniazid in mycobacteria.
Significance: Understanding the mechanism by which mycobacteria acquire tolerance to isoniazid is important for developing novel therapies.

Tuberculosis remains one of the most deadly infectious diseases worldwide and is a leading public health problem. Although isoniazid (INH) is a key drug for the treatment of tuberculosis, tolerance to INH necessitates prolonged treatment, which is a concern for effective tuberculosis chemotherapy. INH is a prodrug that is activated by the mycobacterial enzyme, KatG. Here, we show that mycobacterial DNA-binding protein 1 (MDP1), which is a histone-like protein conserved in mycobacteria, negatively regulates *katG* transcription and leads to phenotypic tolerance to INH in mycobacteria. *Mycobacterium smegmatis* deficient for MDP1 exhibited increased expression of KatG and showed enhanced INH activation compared with the wild-type strain. Expression of MDP1 was increased in the stationary phase and conferred growth phase-dependent tolerance to INH in *M. smegmatis*. Regulation of KatG expression is conserved between *M. smegmatis* and *Mycobacterium tuberculosis* complex. Artificial reduction of MDP1 in *Mycobacterium bovis* BCG was shown to lead to increased KatG expression and susceptibility to INH. These data suggest a mechanism by which phenotypic tolerance to INH is acquired in mycobacteria.

Tuberculosis is a disease caused by infection with *Mycobacterium tuberculosis* complex and remains a serious threat to health around the world. Approximately one-third of the world's population is infected with *M. tuberculosis*. The current World Health Organization report shows that 8.8 million new tuberculosis cases arose, and 1.4 million people died from tuberculosis in 2010. Although medications are indispensable for treating infectious diseases, one of the predominant problems in tuberculosis chemotherapy is the prolonged treatment duration. The current treatment of tuberculosis with first-line antitubercular agents including isoniazid (isonicotinic acid hydrazide, INH)[3], rifampin, pyrazinamide, streptomycin, and ethambutol requires at least six months to cure the acute disease, yet there is still a relapse rate of 2 to 3% (1). For latent tuberculosis, the standard treatment takes six to nine months using INH alone.

The relatively long duration of tuberculosis chemotherapy is not only due to reduced metabolism based on the slow growth rates of the pathogens but also the emergence of drug-resistant cells. There are two possible mechanisms by which *M. tuberculosis* acquires drug resistance. First, spontaneous chromosomal mutations in genes related to drug resistance can result from irregular drug supply, inappropriate drug prescriptions, and poor patient adherence to treatment (2). Secondly, *M. tuberculosis* can acquire phenotypic drug resistance in the absence of genotypic alterations in drug-target genes (3). In particular, it is well known that INH tolerance is acquired by *M. tuberculosis*

* This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology, the Ministry of Health, Labor, and Welfare (Research on Emerging and Re-emerging Infectious Diseases, Health Sciences Research Grants), The Japan Health Sciences Foundation, and The United States-Japan Cooperative Medical Science Program against Tuberculosis and Leprosy.

[1] To whom correspondence may be addressed: Dept. of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel.: 81-6-6645-3745; Fax: 81-6-6645-3746; E-mail: myoshi@med.osaka-cu.ac.jp.

[2] To whom correspondence may be addressed: Dept. of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel.: 81-6-6645-3745; Fax: 81-6-6645-3746; E-mail: sohkichi@med.osaka-cu.ac.jp.

[3] The abbreviations used are: INH, isoniazid; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; MDP1, mycobacterial DNA-binding protein 1; MIC, minimum inhibitory concentration; NBT, Nitroblue tetrazolium; ADC, albumin-dextrose-catalase; qRT-PCR, quantitative RT-PCR; ANOVA, analysis of variance.

during the stationary phase, and requires prolonged tuberculosis chemotherapy.

INH is one of the key drugs used to control tuberculosis (4). It is critical in tuberculosis therapy because of its potent bactericidal activity against organisms growing actively in the pulmonary cavities, whereas the sterilizing activity of INH is reduced nearly 1,000-fold in *M. tuberculosis* cells during the stationary phase of growth (5).

The mode of action of this drug is complicated. INH is a prodrug that is converted into the active form by the mycobacterial catalase-peroxidase, KatG (6). The expression of KatG is regulated by an iron-containing transcription factor, *furA*, which is situated immediately upstream of *katG* (7). In its active form, INH inhibits both InhA, which is a primary target of INH (8), and an enoyl-acyl carrier protein reductase of the fatty acid synthase II (9, 10).

DNA sequencing of INH-resistant clinical isolates has revealed several mutations associated with resistance to INH. In addition to mutations in *katG* and *inhA*, *ahpC* (coding for alkyl hydroperoxide) (11) and *ndh* (coding for NADH dehydrogenase) (12) have also been reported to be associated with INH resistance. Mutations in *kasA*, which encodes ketoacyl acyl carrier protein synthase, are involved in INH resistance (13). However, later studies showed that overexpression of KasA did not lead to INH resistance in *M. tuberculosis* (14), and mutations in *kasA* are not likely to participate in INH resistance (4). Among these mechanisms, INH resistance in *M. tuberculosis* is most commonly associated with mutations in *katG*. In addition, a recent study by Ando *et al.* (15) revealed that mutations in the intergenic region of *furA* and *katG* also affected *katG* expression and conferred INH resistance. Although the reason why mycobacteria in the stationary phase acquire INH tolerance has not been fully elucidated, it is likely that the regulated expression of genes involved in the action of this drug is responsible for INH tolerance.

Transcriptional regulators are thought to play important roles in growth phase-dependent bacterial adaptive responses, including drug tolerance. Histone-like proteins are possible candidate transcriptional regulators in such responses. Recently, it was reported that Lsr2, a histone-like protein highly conserved in mycobacteria, inhibits a wide variety of DNA-interacting enzymes to regulate genes induced by antibiotics and those associated with inducible multidrug tolerance (16). Mycobacterial DNA-binding protein 1 (MDP1) is another histone-like protein in mycobacteria that binds to genomic DNA at guanine and cytosine residues. MDP1 is generally a negative regulator of gene expression (17) that participates in the slow growth rate of mycobacteria (18). Expression of this protein is enhanced in both stationary and dormant mycobacteria (19, 20).

In this study, we describe for the first time that MDP1 negatively regulates KatG expression, which, in turn, causes phenotypic tolerance to INH. The current study describes a novel molecular mechanism by which phenotypic drug tolerance is acquired in mycobacteria. This mechanism may strongly impact our understanding of phenotypic tolerance to INH in *M. tuberculosis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Antimicrobial Agents—Mycobacterium smegmatis mc^2155 (WT) and its histone-like protein/MDP1-deficient strain (MDP1-KO) were kindly provided by Dr. Thomas Dick (Novartis Institute for Tropical Diseases). An MDP1-complemented strain (MDP1-Comp) was generated previously (21). All *M. smegmatis* strains were cultured in Luria-Bertani (LB) broth or on LB agar plates (Sigma) aerobically at 37 °C on a magnetic stirrer set to rotate at 130 rpm. *Mycobacterium bovis* BCG strains were cultured in 7H9 broth base (Becton Dickinson and Company) supplemented with glycerol, 10% albumin-dextrose-catalase (ADC), and 0.05% Tween 80 (7H9-ADC-Tween), or on 7H11 agar plates supplemented with oleic acid, albumin, dextrose, and catalase (OADC). Ethambutol, rifampin, levofloxacin, and INH were purchased from Sigma. Rifampin was dissolved in ethanol, whereas levofloxacin, INH, and ethambutol were dissolved in distilled water. Stock solutions of each drug were filter-sterilized through 0.2-μm pore-size polyethersulfone membrane filters (Iwaki) except for rifampin, which was dissolved in ethanol.

Broth Microdilution—For the estimation of the minimum inhibitory concentrations (MICs) of each antibiotic, we used the broth microdilution method as previously described (22). Briefly, serial 2-fold dilutions of compounds were added to LB broth (for *M. smegmatis*) or 7H9-ADC-Tween (for BCG) to achieve final concentrations ranging from 128–0.125 μg/ml. The diluted antibiotic was then dispensed into the wells of microdilution plates at 0.1 ml per well. Aliquots of mycobacterial cells were then inoculated to a final concentration of $\sim 10^4$ CFU/well. After incubation at 37 °C for 4 days (*M. smegmatis*) or 14 days (BCG), the MICs were determined as the lowest concentrations of compound that prevented visible growth.

Cell Viability against INH—M. smegmatis and BCG cells were grown aerobically at 37 °C in liquid medium under appropriate conditions for 2–6 days. At each time point, an aliquot of each culture was withdrawn and adjusted to an optical density at 600 nm (A_{600}) of 0.1 and subsequently diluted 1:100 in fresh medium. After addition of INH solution to a final concentration of 6.25 μg/ml (for *M. smegmatis*) or 0.125 μg/ml (for BCG), cells were grown aerobically at 37 °C for 24 h (*M. smegmatis*) or for 48 h (BCG). Serial 10-fold dilutions of the cell suspensions were plated on agar plates to estimate the number of viable bacteria in the inoculum. After incubation for 4 days (*M. smegmatis*) or 14 days (BCG) at 37 °C, colonies were counted, and the proportion growing in the presence of various drug concentrations was compared with the total number of viable bacteria in the inoculum.

RNA Extraction—Cells were suspended in 1 ml of TRIzol reagent (Invitrogen) and disrupted using a Mini-BeadBeater. After incubation for 5 min at room temperature, 0.2 ml of chloroform was added, and the samples were shaken vigorously for 15 s. Cell lysates were centrifuged at 12,000 × *g* for 10 min at 4 °C, and the colorless upper aqueous phases were transferred to fresh tubes. Total RNA in the aqueous phase was precipitated by mixing samples with isopropyl alcohol followed by centrifugation. The pellets were washed with 75% ethanol, dried, and resuspended in 100 μl of diethylpyrocarbonate-treated dH_2O.