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Five-antituberculosis Drug-resistance Genes Detection Using Array System

Yasuo Shimizu[1,*], Kunio Dobashi[2], Yoko Yoshikawa[3], Shigeki Yabe[4], Seiichi Higuchi[1], Yoko Koike[1], Yoshihiro Mita[1], Mitsuyoshi Utsugi[1], Katsuaki Endou[1], Kouichi Takahashi[5], Satoru Watanabe[6], Ryusei Saito[6], and Masatomo Mori[1]

[1]*Department of Medicine and Molecular Science, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan*
[2]*Gunma University School of Health Sciences, Maebashi, Gunma 371-8511, Japan*
[3]*Research & Development Center, Nisshinbo Industry, Inc., Chiba, Chiba 267-0056, Japan*
[4]*Department of Laboratory Medicine and Clinical Laboratory Center, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan*
[5]*Matsuida Hospital, Gunma, Annaka, Gunma 379-0021, Japan*
[6]*Nishigunma National Hospital, Shibukawa, Gunma 377-0027, Japan*

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Summary **Detection of resistance to drugs for *Mycobacterium tuberculosis* takes about two months from the sample collection using culture-based methods. To test a rapid method for detection of resistance for five antituberculosis drugs using DNA microarray and to examine its potential for clinical use, we employed a DNA microarray for detection of seven mutations genes related to resistance of five kinds of antituberculous drugs using *Mycobacterium tuberculosis* DNA isolated from sputum. The results of microarray analysis were compared with the results of a standard culture method of Lowenstein-jensen drug sensitivity testing system. DNA microarray analysis showed a high sensitivity (>90%) for all five drugs. Specificity of rifampicin and ethambutol were nearly 90%, however specificity of isoniazid (60%) and kanamycin (67%) were not enough. The amount of *Mycobacterium tuberculosis* DNA required for microarray analysis corresponded to at least 1–9 Acid-Fast Bacilli per 10 fields by carbolfuchsin staining. DNA microarray analysis appears to be useful for estimation of drug resistances, nevertheless its limitations. To minimize misunderstanding, it is necessary to confirm the number of bacilli in the sputum, and culture method is needed for comparison when use the PCR-based array system.**

Key Words**: multidrug resistance, rapid diagnosis, *Mycobacterium tuberculosis*, DNA microarray**

Introduction

The prevalence of drug-resistant tuberculosis continues to increase around the world. According to a survey of resistance to first-line antituberculous drugs performed in 1994 by the World Health Organization and the International Union Against Tuberculosis and Lung Disease, multi drug-resistant tuberculosis (MDR-TB) was found in all 35 countries investigated. In 2000, MDR-TB was detected in 72 countries, and MDR-TB was also serious problem on immuno-compromised host [*1*]. With current methods based on culture, it takes about two months after collection of sputum to obtain the result of drug resistance test, i.e. after sample collection, the tuberculosis organisms needed to growth in culture, and then, the part of organisms grown on

*To whom correspondence should be addressed.
Tel: +81-27-220-8123 Fax: +81-27-220-8136
E-mail: yasuos@med.gunma-u.ac.jp

its culture are put on another drug sensitivity test culture. At the start of therapy, empirical therapy with several antituberculous drugs are needed, and if failed to cure due to drug resistance of TB, the duration of hospitalization would be quite long. Therefore, it would be beneficial to treat drugs resistant-TB more effectively. In particular, the rapid detection of drug resistance would be expected to overcoming this problem. Several new technologies have been developed for the rapid assessment of drug resistance using high density DNA probe [*2*], solid-phase sequence scanning [*3*] and invader assay using real-time PCR [*4*]. Although these methods have proven effective, these method need for an expensive microarray scanner to detect fluorescence, or sequencer. The INNO-LiPA Rif. TB® (LiPA) is a commercial line probe assay using nitrocellulose paper strips, and adapted to clinical setting in developed countries [*5*]. This system is used for detect genetic mutation in the rpoB gene region related to Rifampicin (RFP) resistance. There is no method for simultaneously rapid detection of five kinds of antituberculosis drug resistances in practical medicine. Also, the current rapid diagnosis systems have not yet become widely available, and the rapid detection of drug resistances remains a major public health problem.

From the study of *M. tuberculosis* DNA, it has become clear that missense mutations play a role in drug resistance. Mutations of the *inhA* [*6*] and *KatG* [*7*] genes of *M. tuberculosis* are involved in about 80% of resistance to isoniazid (INH), mutations of the *rpoB* gene control about 95% of resistance to rifampicin (RFP) [*8*], mutations of the *rrs* and *rpsL* genes are concerned with about 80% of resistance to streptomycin (SM), mutations of the *rrs* gene cause 70% of resistance to kanamycin (KM), and mutations of the *embB* gene produce about 70% of resistance to ethambutol (EB) [*9*]. On the basis these seven genes related to antituberculous drug resistance, we developed a DNA microarray that could detect mutations of all seven genes rapidly in one day and simply. Array signals were obtained by a peroxidase reaction, and were detected easily using an office scanner, as previously reported [*10*]. There have been no reports about detection of resistance to five antituberculous drugs at once.

In this study, we tested the DNA microarray for detection of mutations of the seven genes mediating resistance to the above-mentioned five drugs using *M. tuberculosis* DNA isolated from sputum. The results of microarray analysis were compared with the drug resistance data obtained using culture method, and we assessed the accuracy of detecting of drug resistance with the microarray, and also we try to clarify the limitations of DNA microarray use in clinical setting.

Method

Sample collection and extraction of M. tuberculosis DNA

Sputum samples (n = 48) were obtained from 48 tuberculosis patients with pulmonary tuberculosis. The diagnosis of *M. tuberculosis* infection was based on radiography, smear of sputum, and PCR using the Amplicore method for *M. tuberculosis* (Roche Diagnostic Systems, Somerville, NJ), and culture-based method of Lowenstein-jensen standard culture-based drug sensitivity testing system raised in recommended drug concentrations for drug susceptibility testing as described [*11*]. Sputum samples were homogenized with semi-alkaline proteinase (Sputazyme, Kyokuto Pharmaceutical, Co. Ltd., Japan) according to the manufacturer's instructions. Homogenized sputum was treated by the NALC-NaOH method [*11*], and then DNA was isolated using a specimen preparation kit (COBAS AMPLICORE™, Roche Diagnostics, NJ). This study was conducted according to the Declaration of Helsinki, and all patients gave written informed consent before enrollment, and was approved by the Human Research Committee of Gunma University.

DNA amplification

Fragments of drug resistance-related genes (*rpoB*, *inhA*, *katG*, *rpsL*, *rrs*, and *emb B*) containing drug-sensitive or drug-resistant polymorphisms were amplified by multiplex PCR using 18 sets of primers. The *rrs* gene was also amplified in the same tube to distinguish *M. tuberculosis* from *M. avium*. The target genes, amplicon sizes, and primer sequences are listed in Table 1a, and the relationship between target gene, primer, and mutation site is shown in Table 1b. These 18 primers detect known mutations in the followings of the *rpoB*, *inhA*, *katG*, *rpsL*, *rrs*, and *emb B* genes (Table 2). PCR amplification was performed by the following procedure. The reaction mixture (19 μl) contained 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 nM of each dNTP, 500 nM of each primer, 2.5 U of AmpliTaq Gold (Applied Biosystems Japan Ltd., Japan), and 1 μl of genomic DNA. The reaction mixture was preheated at 95°C for 9 min, followed by 50 cycles of denaturation at 94°C for 30 s, annealing and extension at 64°C for 1 min, with post-cycling extension at 72°C for 10 min using a DNA engine thermal cycler (MJ Research Inc., Waltham, MA).

DNA microarray

DNA oligomers about 20 to 24 bp long were immobilized on glass slides [*10*]. The arrangement of mutation detection sites on the DNA microarray is shown in Table 1c. The microarray field was largely divided into isoniazid (INH), RFP, SM, KM, and EB resistance detection fields, as well as a *M. tuberculosis* and *M. avium complex* detection field. The oligomers spotted in the extreme left hand lane contained the wild-type DNA sequences, while the other lanes contained the mutants. If there was no signal in the *M. tuberculosis* detection field, the sample did not contain *M. tuberculosis* and the test was invalid even if positive

Table 1a. Forward and Reverse Primers for Drug Resistance Genes.

amplicon size	target gene	primer	forward primer sequence (5'-3')	primer	reverse primer sequence (5'-3')
71 bp	inhA	1F	CGT GGA CAT ACC GAT TTC G	1R	5'biotin-TCA GTG GCT GTG GCA GTC A
186 bp	KatG	2F	GGT CGC GAC CAT CGA CGT TG	2R	5'biotin-AAC CGC TGC ATG CCG C
79 bp	KatG	3F	GCT TAA CAG CAG GCC CGA C	3R	5'biotin-CTT GCC GTA CTT CTT CTT GAC C
142 bp	KatG	4F	GGT CAA GAA GAA GTA CGG CAA G	4R	5'biotin-AAT AgA CCT CAT Cgg gCT C
50 bp	KatG	5F	GCG GTC ACA CTT TCG GTA	5R	5'biotin-GAC CAG ATC GGC CGG G
119 bp	KatG	6F	AAG AGC TCG TAT GGC ACC	6R	5'biotin-TCG CCG TAC AGG ATC T
102 bp	KatG	7F	GCC GAG ATT GCC AGC CTT	7R	5'biotin-GCT ACC ACG GAA CGA CGA
93 bp	KatG	8F	GAA TCC TTT GCC GTG CTG GAG	8R	5'biotin-GTC GAG CAG CAT GTA CTC
158 bp	negative control	9F	CTC TTC GGA GAT ACT CGA GTG	9R	5'biotin-CCG CGG GCT CAT CCC AC
124 bp	rpoB	10F	GCC GCG ATC AAG GAG TTC	10R	5'biotin-CAC GTG ACA GAC CGC CGG
153 bp	rrs	11F	GGT TCT CTC GGA TTG ACG GTA G	11R	5'biotin-AGC CGT GAG ATT TCA CGA ACA AC
99 bp	rrs	12F	GGT TTC CTT CCT TGG GAT C	12R	5'biotin-GGC CCC CGT CAA TTC CTT
63 bp	rpsL	13F	TAT GCA CCC GCG TGT ACA	13R	5'biotin-GGG CAA CCT TCC GAA GCG
106 bp	rpsL	14F	GAG GTC ACG GCG TAC ATT	14R	5'biotin-TCT TGT AGC GCA CAC CAG
65 bp	rrs	15F	CTT GTA CAC ACC GCC CGT	15R	5'biotin-CGA GGG TTA GGC CAC TGG
76 bp	rrs	16F	CCA GTG GCC TAA CCC TCG	16R	5'biotin-CGG CTA CCT TGT TAC GAC
92 bp	embB	17F	GTG GTG ATA TTC GGC TTC CT	17R	5'biotin-TGG TCG GCG ACT CGG GC
80 bp	embB	18F	CAA CTA TTT CCG CTG GTT CG	18R	5'biotin-TGA CAT GGG TCA TCA GCG

Drug resistance genes were *inhA* and *KatG* (INH), *rpoB* (RFP), *rrs* and *rpsL* (SM), *rrs* (KM), and *embB* (EB).

Table 1b. Relations between Primer Numbers, Drug Resistance Genes and Mutation Sites.

primer	target gene	mutation
1	inhA	1022A/G, 1023C/T, 1030T/A, 1030T/G
2	KatG	281A/C, 322, 324CC/GG, 369G/C
3	KatG	412A/C, 413A/G, 419G/A, 425A/C
4	KatG	479C/T, 514G/A, 539C/A
5	KatG	823A/G
6	KatG	944G/A, 944G/C, 944, 945GC/CA, 982T/G
7	KatG	1431G/A
8	KatG	1778G/A
9	negative control	—
10	rpoB	180C/A, 180C/G, 180C/T, 181A/C, 190A/T, 191C/A, 201C/T, 208C/T, 218, 220, 222CAA/GCC, 219C/A, 219C/G, 219C/T, 220A/C, 220A/G, 221C/A, 221C/G, 235C/G, 235C/T, 241T/C
11	rrs	505C/T, 526C/T, 527A/C, 527A/T, 530C/T
12	rrs	920C/A, 920C/G, 921A/G, 893G/A
13	rpsL	134A/G, 134A/C
14	rpsL	269A/C, 269A/G, 269A/T
15	rrs	1423A/G, 1424C/A, 1424C/T
16	rrs	1516G/T
17	embB	7868A/G, 7868A/C, 7870G/A, 7870G/T, 7870G/C
18	embB	7940T/G

Drug resistance genes were *inhA* and *KatG* (INH), *rpoB* (RFP), *rrs* and *rpsL* (SM), *rrs* (KM), and *embB* (EB).

signals were detected in the drug resistance fields. If the wild-type oligomer did not show a signal and mutants yielded signals on the DNA microarray, drug resistance was diagnosed.

Hybridization and signal detection

The PCR product (5 µl) was mixed with 20 µl of hybridization solution, and then the mixture was denatured at 98°C for 5 min and chilled on ice for 1 min before being used as biotin-labeled DNA probes. Subsequently, the mixture was

Table 1c. Arrangements on Microarray for Drug Resistance Genes

B	W	1022A/G	1023C/T		INH	B	B	W	180C/A	180C/G	180C/T	181A/C					RFP	B
	W	1030T/A	1030T/G					W	190A/T	191C/A								
	W	281A/C						W	201C/T									
	W	322,324						W	208C/T									
		CC/GG																
	W	369G/C						W	218, 220, 222	219C/A	219C/G	219C/T	220A/G	220A/G	221C/A	221C/G		
									CAA/GCC									
	W	412A/C	413A/G	419G/A				W	234C/G	234C/T	240T/C							
	W	425A/C					B	W	505C/T								SM	B
	W	479C/T						W	526C/T	527A/C	527A/T	530C/T						
	W	514G/A						W	920C/A	920C/G	921A/G							
	W	539C/A						W	893G/A									
	W	823A/G						W	134A/G	134A/C								
	W	944G/A	944G/C	944, 945				W	268A/C	269A/C	269A/T							
				GC/CA														
	W	982T/G					B	W	1423A/G	1424C/A	1424C/T						KM	B
	W	1431G/A						W	1516G/T									
	W	1700T/C					B	W	7868A/G	7868A/C	7870G/A	7870G/T	7870G/C				EB	B
	W	1778G/A					B	W	7940T/G									B
B	Tub-P	Tub-N	avi-P	avi-N	acid-fast	B												

Drug resistance genes were *inhA* and *KatG* (INH), *rpoB* (RFP), *rrs* and *rpsL* (SM), *rrs* (KM), and *embB* (EB). Tub-p, Tub-N, Avi-P and Avi-N indicates tuberculosis positive, negative, avidin positive, negative. Acid-fast indicates non-tuberculosis of acid fast. The site of B indicates position marker of each drugs mutation detection field on array. The microarray field is devided into INH, RFP, SM, KM, and EB resistance detection fields. When there are positive signal in the Tub-P and Avi-P spots, the sample contains *M. tuberculosis* and the DNA microarray test is valid.

Table 2. Antituberculous Drug Resistance Genes and the Role of them in the Resistance of *M. tuberculosis*.

anti-TB drug	Drug resistance-related genes	Importance of each gene in anti-TB drug resistance	DNA microarray coverage of resistance genes
INH	*inhA*, *katG*	80%	100%
RFP	*rpoB*	95%	100%
SM	*rrs*, *rpsLL*	80%	100%
KM	*rrs*	70%	100%
EB	*embB*	70%	100%

Oligomers on the DNA microarray were arranged to detect all mutations of *rpoB*, *inhA*, *KatG*, *rrs*, *rpsL*, *rrs*, and *emb B*.

applied to a DNA microarray slide, covered with a coverslip, and hybridized at 42°C for 60 min. After hybridization, the DNA microarray was washed in washing buffer A at 42°C for 20 min to remove excess biotin-labeled DNA probes. Hybridization signals were developed as black spots by the peroxidase method. The binding solution (1.4 ml) was prepared from a kit according to the manufacturer's instructions, and was applied to the oligonucleotide spots on the DNA microarray, after which the microarray was incubated for 30 min at room temperature. Then the slide was washed twice with the coloring buffer for 5 min at room temperature. Next, 1.4 ml of coloring solution was prepared from the same kit according to the manufacturer's instructions, drops of the solution were added to the DNA microarray, and incubation was done at room temperature for 30 min. Subsequently, the microarray was rinsed with distilled water and air-dried. All of the solutions and buffers for hybridization were supplied by Nisshinbo Industries, Inc. Black hybridization signals on the microarray were scanned using a standard OA scanner (GT-8700F, Epson, Tokyo, Japan) to provide signal intensity data for subsequent computer analysis.

Drug sensitivity test using broth culture

Homogenized sputum was treated with NALC-NaOH [*12*], and then inoculated into a Lowenstein-jensen standard culture-based drug sensitivity testing system raised in recommended drug concentrations for drug susceptibility testing as described [*8*] according to the manufacturer's instructions (Mycosensitive spectrum-SR, Kyokuto, Tokyo,

Table 3. Sensitivity and Specificity of DNA Microarray Analysis in *M. tuberculosis* Sputum Sample.

anti-TB drug	sensitivity (95% CI)	specificity (95% CI)
INH	0.91 (0.77–0.97)	0.60 (0.30–0.88)
RFP	0.97 (0.82–1.0)	0.95 (0.75–1.0)
SM	0.93 (0.80–1.0)	0.73 (0.4–0.94)
KM	0.96 (0.85–1.0)	0.67 (0.22–1.0)
EB	0.98 (0.87–1.0)	0.89 (0.52–1.0)

The sensitivity and specificity were calculated by assuming that the culture-based method of Lowenstein-jensen drug sensitivity test was correct. The number of samples tested for each drug (wild/mutant) was as follows: INH (38/10), RFP (28/20), SM (40/8), KM (44/4), and EB (39/9).

Japan). The cut-off values for each drug were as follows: INH (0.2 μg/ml), RFP (40.0 μg/ml), SM (10 μg/ml), KM (20 μg/ml), and EB (2.5 μg/ml) [*11*]. The result of drug resistant patient's number based on culture method were followings (wild/mutant); INH (38/10), RFP (28/20), SM (40/8), KM (44/4) and EB (39/9).

Grading of sputum smears

To grade the quantity of *M. tuberculosis* bacilli in sputum smears, carbolfuchsin staining was performed. Stained smears were examined by microscopy (Olympus BX50F4) of high power field (HPF) of ×200, and scored according to the quantitation scale for AFB smears. Criteria for grading the quantity of *M. tuberculosis* bacilli were according to the method reported in the official statement of the ATS and CDC [*11*].

Amplification of H37Rv M. tuberculosis DNA

DNA from the laboratory strain *M. tuberculosis* H37Rv was isolated using phenol-chloroform and ethanol, and was employed as the positive control on gel electrophoresis. *M. tuberculosis* DNA from sputum samples and H37Rv was diluted (1, 10, 100, 1000, 10000, 10000) in TE buffer, and the 85A gene (a *M. tuberculosis*-specific gene) was amplified by PCR. The PCR master mix contained 2 μl of 10 × PCR buffer with 15 mM $MgCl_2$, 1.6 μl of 2.0 mM dNTP, 2 μl of 25 mM $MgCl_2$, 7.6 μl of distilled water, and 0.2 μl of AmpliTaqGold. Then 1 μl of HRv37 DNA was added, as well as 100 pmol of the primers for the 85A gene (U; 5' CCGCGGGGGCATTTTC 3', L; 5' GCTCCCGCG-TAGACGAACT 3'), and amplification was done. PCR started with denaturation for 9 min at 95°C, followed by 50 cycles of 30 s at 94°C and 1 min at 64°C, as well as 10 min at 72°C for extension using an ABI prism 3300 (PE Biosystems Ltd.).

Statistical analysis

The accuracy of the DNA microarray for detecting resistance was calculated from the sensitivity (true positive rate) and specificity (true negative rate) of 95% confidence intervals (CI). Specimens that were positive by both methods were defined as true positive and specimens negative by both methods were defined as true negative.

Results

The drug resistances of *M. tuberculosis* isolated from sputum samples were evaluated by culture-based method of Lowenstein-jensen drug sensitivity testing system and DNA microarray analysis, and both results were compared. DNA microarray analysis of resistance to RFP, SM, and KM is shown in Figure 1a and 1b. The test was performed on 48 samples, and the result of the sensitivity of DNA microarray diagnosis was high (>90%) for all five antituberculous drugs. The specificity for RFP and EB was also high, being >95.0% and 89.0%, respectively, by DNA microarray diagnosis. However, specificity for INH, SM, and KM was only 60.0%, 73.0%, and 67.0%, respectively.

To investigate the reasons for the discrepancy between culture-based and DNA microarray methods, we performed sequencing of sputum DNA samples. Sequence analysis was performed as previously [*10*]. Mutant type by DNA microarray analysis on sputum sample showed mutant by sequence analysis (data not shown). Colonies samples from these sputum samples showed wild type by cultured-based drug resistance test. In addition, we harvested the colonies grown in culture of the sputum specimens, and purified DNA from those colonies was subjected to DNA microarray analysis. When the colonies samples were used for DNA microarray after cultured (i.e., not sputum), the result of culture based method and DNA microarray analysis were corresponded. Thus, after growth on culture, microarray analysis of colonies were corresponded with the culture-based method, appeared that the bacteria were supposed to be heterogeneous from those in the sputum as described [*13*].

Among the specimens that were mutant by the culture-based method but wild-type by microarray analysis, there were several samples with a low number of acid-fast bacilli (AFB) on smears of less than 1-9 AFB per 100 fields using carbolfuchsin stain. To determine the limit of detection by

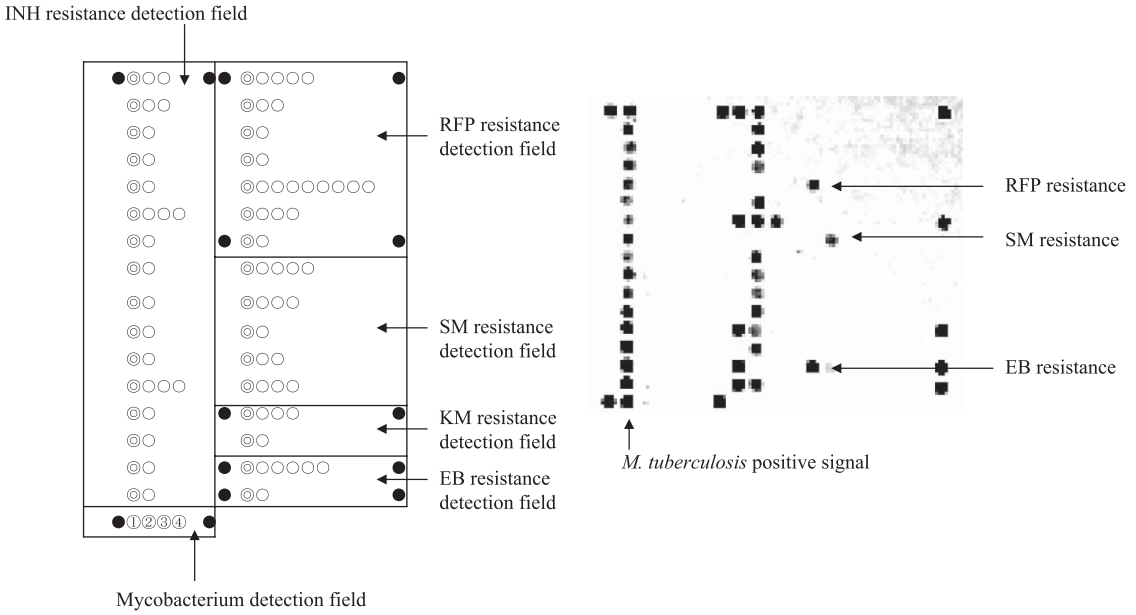

Fig. 1. a. Explanation of the DNA microarray. The array was divided into six fields. Black circles are position markers for biotinylated oligomers in each fields. Double circles are wild-type and single circles are mutant. b. The result of DNA microarray analysis of sputum with RFP, SM, and KM resistance.

DNA microarray analysis for smear grades, we performed PCR for the 85A gene of *M. tuberculosis* obtained from sputum samples and the pure genome of H37Rv *M. tuberculosis* strain. A PCR for the 85A gene showed that the limit of DNA microarray analysis was 10-20 AFB per 100 fields in sputum samples including AFB and 10^4 bacilli for the H37Rv genome. These results showed that at least 1-9 AFB per 10 fields on carbofuchsin staining was needed.

Discussion

The present study revealed that a DNA microarray method showed good sensitivity in five drugs of INH, RFP, SM, KM and EB (> 90%) and specificity in RFP (95%) and EB (89%) for distinguishing wild-type from mutant *M. tuberculosis* in patient sputum samples. Signals on the DNA microarray could be easily viewed with an office scanner, however, at least 1-9 AFB (i.e., *M. tuberculosis bacilli*) per 10 fields on carbolfuchsin staining of amount of bacilli were needed to prevent misunderstandings in microarray.

There were several possible reasons for the difference in results between the culture-based method and the DNA microarray. DNA microarray could detect mutant correctly, when the *M. tuberculosis* DNA after growing in culture was applied to DNA microarry, that is, the results of drug resistant analyzed by culture-based method and the result of DNA microarry was corresponded in analysis from the *M. tuberculosis* DNA after growing in culture. However, in several *M. tuberculosis* DNA samples isolated from sputum, the results of drug resistant analyzed by culture-based method and the result of DNA microarray were not corresponded. Possible reason was reported that heterogeneity of resistance in sputum sample could be affected the discrepancy between culture-based method and DNA microarray analysis [*13*]. The meta-analysis of accuracy of LiPA showed that LiPA was a highly sensitive and specific test for the detection of RIF resistances in culture isolates. However, the test was also reported that it appeared to have lower sensitivity when used directly on clinical specimens as our DNA microarray [*5*]. Prediction of drug-resistant genotypes of mycobacterium found after cultivation not always give a good reflection of those in the original clinical sample. Further analysis was needed whether this matter occurred or not in our study. From the analysis of present study of needed amount of *M. tuberculosis* bacilli using H37Rv genome and sputum sample genome from *M. tuberculosis* patients, to minimize misunderstandings in microarray, we found that at least 1-9 AFB (i.e., *M. tuberculosis* bacilli) per 10 fields on carbofuchsin staining were needed to in our microarray analysis. There was another possible reason for the culture-based method showing mutant DNA and the microarray showing the wild-type. Present DNA microarray was not prepared with primers and spots for mutations of *KasA* and *ahpC* (INH) or *embA* and *embC* (EB) [*9*]. If *M. tuberculosis* contained these genes, they would not be amplified by PCR of DNA directly isolated from sputum, and these genes would confer resistance in the culture-based method. This specimen possibly

shows mutant by the cultured-based method, nevertheless wild-type by the DNA microarray method. To improve specificity, it will be necessary to devise suitable primers and add spots for these genes to the DNA microarray in the future.

Taken together, to minimize misunderstandings on DNA microarray, it will be necessary to obtain bacteria equivalent to at least 1-9 AFB per 10 fields on carblofuchsin staining, as well as using the culture-based method for confirmation.

In conclusion, drug resistance genes detection using DNA microarray appeared to be useful, however, direct application of *M. tuberculosis* DNA from the sputum to DNA microarray has a risk of misdiagnosis. It is thus necessary to carefully select the conditions for use of such methods.

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