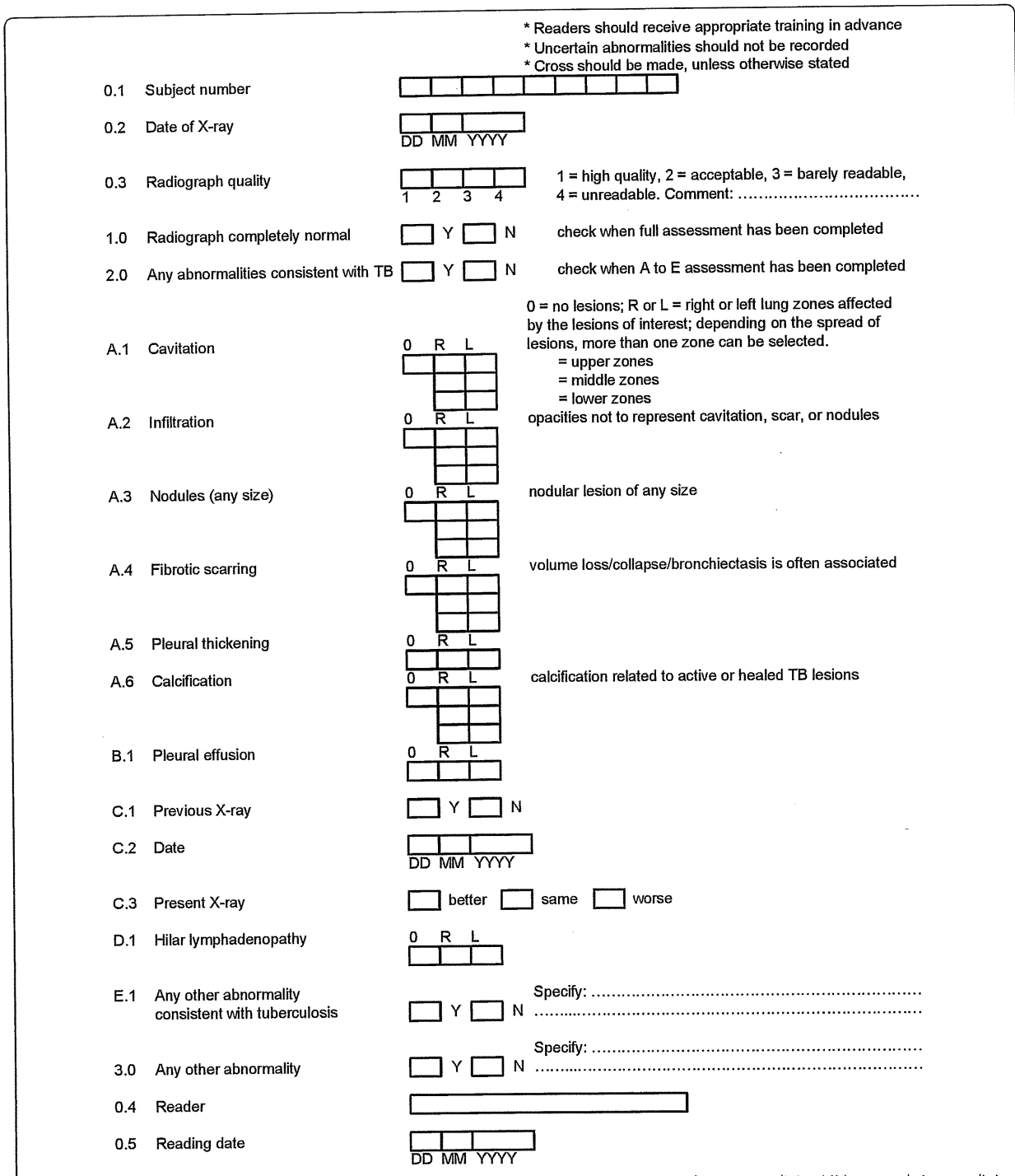

Figure 1 Chest X-ray coding: JVCS. JVCS = Japan-Vietnam chest X-ray coding system; DD = date in two digits; MM = month in two digits; YYYY = year in four digits; Y = yes; N = no; R = right; L = left.

similarities in frequencies of positive findings between groups of raters (marginal homogeneity). No symmetry or non-marginal homogeneity was considered to be significant when $P < 0.05$.

Results

Follow-up after TB prevalence survey

In 2004, one year after the prevalence survey, 204 (64.4%) of the 317 individuals who presented with

radiographic findings of suspected TB underwent a sputum smear test, one of whom tested positive. The initial CXR film of this case showed infiltrates, fibrosis/scarring, and calcification. The follow-up radiograph in 2006 showed improvement after treatment.

In the follow-up in 2006, 93 individuals were checked, one of whom was diagnosed by smear and culture as TB positive (Figure 2). Besides calcification, which was seen in the initial CXR film, infiltrates were present in the follow-up film. All raters evaluated this case as "worse" based on the radiographic findings.

In total, five individuals were reported to have active TB during the 3-year follow-up period. Two were diagnosed bacteriologically and three were diagnosed based on self-reported TB episodes. All the films were randomly mixed in the study set.

Inter-rater agreement on CXR findings

Using the two coding systems, four raters assessed the 258 films taken during the 2003 prevalence survey; two raters assessed the 93 films taken in the 2006 follow-up. A total of 2,436 readings were conducted (Figure 2).

Agreement levels regarding overall parenchymal abnormalities assessed by CRRS varied. Their kappa values were interpreted as fair to good, ranging from 0.24 to 0.63, from the following six comparisons: a comparison between the two Japanese raters (JP-JP); four comparisons between Japanese and Vietnamese raters (JP-VN (1) to (4)); and a comparison between the two Vietnamese raters (VN-VN) (Table 1). Agreement levels regarding calcification also varied. They were considered as fair to good with JVCS and slight to fair with CRRS. Kappa values for pleural effusion with JVCS were interpreted as moderate to good, ranging from 0.54 to 0.77, indicating high level of agreement irrespective of country or rater.

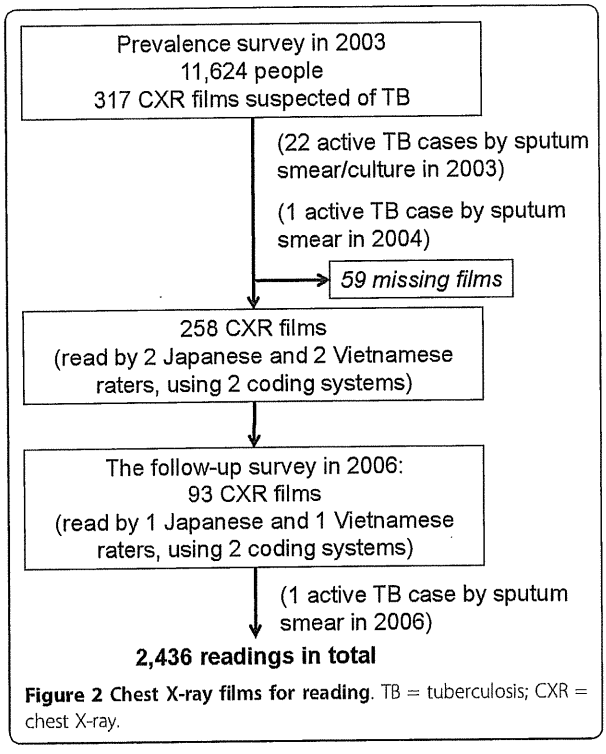

Figure 2 Chest X-ray films for reading. TB = tuberculosis; CXR = chest X-ray.

Major parenchymal findings, cavity, fibrosis/scarring, infiltrates, and nodules were assessed in a similar way, as shown in Table 2. Agreement levels regarding primary and secondary cavities in CRRS were rather low (kappa values ranged from -0.02 to 0.36) except for relatively high agreement levels regarding a primary cavity between the Japanese raters (kappa = 0.60), and a secondary cavity between the Vietnamese raters (kappa = 0.43). Cavitation was, thus, mainly classified as a primary lesion by the Japanese raters and as a secondary lesion by the Vietnamese raters.

Although agreement levels relating to fibrosis/scarring were also low, kappa values for secondary fibrosis/scarring with CRRS revealed fair levels of agreement between raters from the same country (kappa = 0.28 [JP-JP] and 0.22 [VN-VN]), but revealed only slight agreement between raters from different countries (kappa = 0.11 to 0.20 [JP-VN]). Among all Japanese-Vietnamese pairs, the Vietnamese raters specified secondary fibrosis/scars more frequently than the Japanese raters ($P = 0.0001$ or $P < 0.0001$ by McNemar test). The frequency of positive findings of secondary fibrosis with CRRS by both Vietnamese raters was 26/255 (10.2%), whereas that by both Japanese raters was only 7/245 (2.9%) (Table not shown). The frequency of positive findings of fibrosis/scarring with JVCS by both Vietnamese raters (56/255 = 22.0%) also tended to be higher than that by both Japanese raters (42/245 = 17.1%). GEE further confirmed the significant difference in frequencies of fibrosis/scarring between raters from different countries ($P = 0.0082$).

Agreement levels regarding infiltrates between the two raters from the same country were considered as moderate (kappa = 0.49 [JP-JP] and 0.57 [VN-VN]) and as fair between two raters from different countries (kappa = 0.21 to 0.30 [JP-VN]) according to JVCS (Table 2). The Japanese raters detected infiltrates more frequently than the Vietnamese raters ($P < 0.0001$ by McNemar test) in all comparisons. The frequency of positive findings of primary infiltrates with CRRS by both Japanese raters was 68/245 (27.8%), whereas that by both Vietnamese raters was only 22/255 (8.6%) (Table not shown). The frequency of positive findings of infiltrates with JVCS by both Japanese raters (119/245 = 48.6%) also tended to be higher than that by both Vietnamese raters (46/255 = 18.0%). The different frequencies of positive

Table 1 Inter-rater agreement with respect to general and parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement Kappa with 95% confidence interval and the absolute number of films (−/−+/+−/++)					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number of tested films	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Parenchymal abnormality	JVCS	NA	NA	NA	NA	NA	NA
	CRRS	0.63 [0.51-0.75] (32/21/7/185)	0.24 [0.16-0.32] (9/44/0/192)	0.50 [0.38-0.62] (24/29/6/187)	0.25 [0.16-0.34] (7/32/2/205)	0.58 [0.46-0.70] (22/17/8/198)	0.27 [0.17-0.37] (6/3/24/222)
Calcification*	JVCS	0.62 [0.49-0.75] (188/13/14/30)	0.47 [0.35-0.59] (187/14/22/23)	0.21 [0.12-0.30] (198/2/38/7)	0.55 [0.42-0.68] (190/12/18/25)	0.30 [0.21-0.39] (201/0/34/9)	0.26 [0.17-0.35] (215/2/31/7)
	CRRS	0.28 [0.15-0.41] (219/10/11/5)	0.35 [0.23-0.47] (206/23/6/10)	0.15 [0.04-0.26] (226/4/14/2)	0.36 [0.25-0.47] (208/23/5/10)	0.36 [0.25-0.47] (228/2/11/4)	0.17 [0.09-0.25] (219/2/30/4)
Pleural effusion	JVCS	0.58 [0.45-0.71] (222/8/5/10)	0.77 [0.64-0.90] (226/5/2/13)	0.66 [0.54-0.78] (222/8/3/12)	0.64 [0.51-0.77] (221/6/6/12)	0.54 [0.41-0.67] (217/9/7/11)	0.73 [0.61-0.85] (230/6/4/15)
Pleural thickening	JVCS	0.35 [0.23-0.47] (158/39/19/29)	0.22 [0.14-0.30] (88/110/2/46)	0.45 [0.33-0.57] (161/36/14/34)	0.28 [0.18-0.38] (84/93/6/62)	0.45 [0.32-0.58] (148/28/26/42)	0.31 [0.22-0.40] (87/4/95/69)
Pleural abnormalities	CRRS	0.30 [0.20-0.40] (130/73/6/36)	0.16 [0.09-0.23] (83/120/3/39)	0.46 [0.34-0.58] (159/44/7/36)	0.45 [0.33-0.57] (76/60/10/100)	0.54 [0.42-0.66] (124/12/42/67)	0.32 [0.22-0.42] (81/6/91/77)
Pleural effusion/ thickening**	CRRS	0.48 [0.36-0.60] (176/35/6/28)	0.34 [0.22-0.46] (168/43/11/23)	0.55 [0.43-0.67] (189/22/9/26)	0.49 [0.36-0.62] (156/26/23/41)	0.67 [0.55-0.79] (176/6/22/41)	0.48 [0.36-0.60] (173/14/33/35)

* In CRRS, calcification here indicates calcified granuloma only

**In CRRS, pleural effusion and thickening are combined

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters; *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters, (−/−+/+−/++) (negative findings by both raters/positive findings only by the second rater/positive findings only by the first rater/positive findings by both raters)

infiltrate readings between the raters from the two countries were also confirmed by using GEE ($P < 0.0001$).

The levels of inter-rater agreement were considered slight to fair for nodules, irrespective of the raters' home country or the coding system used.

Table 2 Inter-rater agreement with respect to parenchymal findings for each coding system (n = 258)

Item	Coding system	Inter-rater agreement Kappa with 95% confidence interval					
		JP-JP	JP-VN				VN-VN
			[1]	[2]	[3]	[4]	
Total number of tested films	JVCS	(245)	(246)	(245)	(245)	(244)	(255)
	CRRS	(245)	(245)	(246)	(246)	(245)	(255)
Cavity	JVCS	0.44 [0.32-0.56]	0.36 [0.25-0.47]	0.47 [0.34-0.64]	0.30 [0.20-0.40]	0.50 [0.38-0.62]	0.52 [0.40-0.64]
	CRRS primary *	0.60 [0.48-0.72]	0.10 [0.03-0.17]	0.28 [0.18-0.38]	0.06 [-0.02-0.14]	0.36 [0.25-0.47]	0.15 [0.04-0.26]
	CRRS secondary	-0.02 [-0.14-0.10]	0.04 [0.00-0.08]	0.06 [0.01-0.11]	0.00 [-0.05-0.05]	0.04 [-0.03-0.11]	0.43 [0.32-0.54]
Fibrosis/scar	JVCS	0.30 [0.17-0.43]	0.19 [0.07-0.31]	0.34 [0.22-0.46]	0.18 [0.05-0.31]	0.34 [0.23-0.45]	0.31 [0.20-0.42]
	CRRS primary	0.31 [0.18-0.44]	0.02 [-0.02-0.06]	0.27 [0.14-0.40]	-0.02 [-0.07-0.03]	0.15 [0.02-0.28]	0.03 [-0.01-0.07]
	CRRS secondary	0.28 [0.16-0.40]	0.20 [0.10-0.30]	0.11 [0.02-0.20]	0.14 [0.03-0.25]	0.16 [0.06-0.26]	0.22 [0.10-0.34]
Infiltrate	JVCS	0.49 [0.37-0.61]	0.30 [0.20-0.40]	0.27 [0.18-0.36]	0.22 [0.13-0.31]	0.21 [0.13-0.29]	0.57 [0.45-0.69]
	CRRS primary	0.33 [0.21-0.45]	0.24 [0.15-0.33]	0.31 [0.19-0.43]	0.15 [0.08-0.22]	0.22 [0.12-0.32]	0.41 [0.30-0.52]
	CRRS secondary	-0.05 [-0.18-0.08]	0.13 [0.03-0.23]	-0.02 [-0.12-0.08]	-0.04 [-0.14-0.06]	-0.02 [-0.12-0.08]	0.02 [-0.03-0.07]
Nodule	JVCS	0.27 [0.14-0.40]	0.11 [0.05-0.17]	0.26 [0.14-0.38]	0.09 [0.03-0.15]	0.31 [0.20-0.42]	0.19 [0.11-0.27]
	CRRS primary	0.37 [0.25-0.49]	0.13 [0.06-0.20]	0.40 [0.28-0.52]	0.09 [0.03-0.15]	0.24 [0.12-0.36]	0.21 [0.13-0.29]
	CRRS secondary	0.22 [0.10-0.34]	0.22 [0.11-0.33]	0.14 [0.02-0.26]	0.13 [0.03-0.23]	0.29 [0.16-0.42]	0.22 [0.12-0.32]

* Primary and secondary lesions are described in CRRS

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *TB* tuberculosis, *NA* not applicable, *JP-JP* a comparison between the Japanese raters, *JP-VN* [1] to [4] comparisons between Japanese-Vietnamese raters, *VN-VN* a comparison between the Vietnamese raters

An overall assessment of CXR changes after 3 years was conducted by one of the two raters from each country. Agreement was moderate for both coding systems (weighted kappa = 0.47 and 0.40). The Japanese rater indicated deterioration more frequently than the Vietnamese rater (Table 3); this difference was considered highly significant for both JVCS and CRRS by the symmetry test (P = 0.0002 and 0.0008, respectively, by Bowker's test). When assessing changes in specific findings after 3 years, the Japanese rater detected infiltrates more frequently than the Vietnamese rater (P < 0.0001; Figure 3). Among 55 cases of infiltrates, 12 (22%) were assessed as "further spread" by the Japanese rater while 2 (8%) out of 25 cases of infiltrates were assessed as "further spread" by the Vietnamese rater (data not shown).

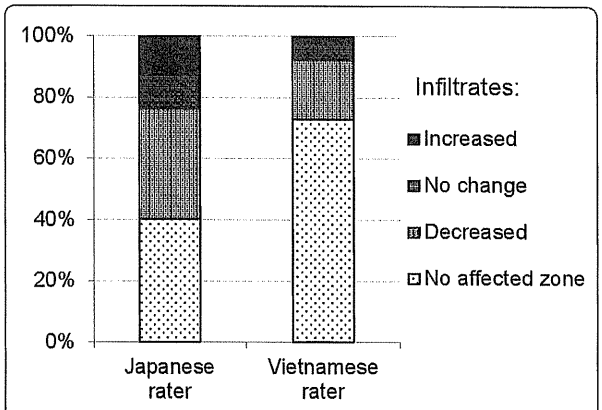

Figure 3 Infiltrates on chest X-rays after a 3-year interval. Evaluation of infiltrates on chest X-ray films between 2003 and 2006 using JVCS codes shows changes in the number of affected areas (upper, middle, and lower zones of each side of the lung). "No affected zone" indicates that the rater did not indicate the presence of infiltrates in either film. The Japanese rater detected infiltrates more commonly than did the Vietnamese rater (P < 0.0001), which corresponds with the results of a greater proportion of "increased" and a smaller proportion of "no detected zone" readings after 3 years. JVCS = Japan-Vietnam chest X-ray coding system

Discussion

Our study confirmed that the readings of CXR findings of suspected TB vary significantly among the raters. Differences in the backgrounds of the raters and different coding systems were considered potential factors affecting the levels of agreement. We found the following two patterns of marked tendency toward inconsistency in the CXR findings: 1) disagreement presumably attributed to the raters' home country and typically observed for infiltrates and secondary fibrosis/scarring and 2) disagreement observed for nodules, irrespective of the rater background. Through discussions conducted with the four raters after the trial, we identified some possible causes of this disagreement, though pre-existing problems were not disclosed when the standard films were checked prior to commencement of the study.

First, it is likely that this disagreement was partly caused by differences between countries regarding the definition of pulmonary lesions. For example, the Vietnamese raters limited the definition of infiltrates to relatively homogenous opacities greater than 10 mm in size, whereas the Japanese raters also included groups of smaller-sized scattered lesions with unclear margins in this classification. As a result, positive findings of infiltrates were more frequently reported by the Japanese raters.

Second, spontaneously cured mild TB resulting in parenchymal fibrosis or scarring, which is commonly seen in countries with high prevalence of TB, is a probable reason for the more frequent detection of these lesions by the Vietnamese raters. In addition, CT scans are compared with plain CXRs more commonly in Japan than in Viet Nam. This practice in TB diagnosis and management might affect the interpretations of the Japanese raters.

Disagreement between the raters from the two Asian countries could be attributed to many background factors, including the medical educational systems and on-the-job training imparted after graduation. In Japan, plain CXR films are read predominantly by clinicians, while in Viet Nam, radiologists also perform this role. Such differences are likely to affect the reading and should be taken into consideration in international studies. Even within a single country, inter-rater agreement depends on the experience of the raters [7,10,12] and is relatively low between raters in different centers [10].

Table 3 Overall assessment of radiographic findings after 3 years

JVCS					CRRS				
JP	VN			Total	JP	VN			Total
	Better	Same	Worse			Better	Same	Worse	
Better	23	6	0	29	Better	28	6	0	34
Same	18	21	0	39	Same	16	18	1	35
Worse	7	7	7	21	Worse	4	10	5	19
Total	48	34	7	89	Total	48	34	6	88
	Weighted kappa = 0.40 [0.22-0.57]					Weighted kappa = 0.47 [0.31-0.63]			

JVCS Japan-Vietnam chest X-ray coding system, *CRRS* chest radiograph reading and recording system, *JP* Japanese rater, *VN* Vietnamese rater

The tested coding systems had both advantages and disadvantages in the context of our study. With CRRS, parenchymal abnormalities are classified into primary and secondary lesions, and it is not easy for raters to differentiate between the two. The Japanese raters emphasized on cavitation and presence of infiltrates as primary lesions of active TB, but the Vietnamese raters objectively judged the primary lesions on the basis of the size of lesions and proportion of the lung involved.

Although fairly reproducible, a disadvantage of JVCS is that it cannot provide any information regarding the significance of active lesions. Thus, CRRS is more informative. Activity, however, is a subjective term and the reproducibility of this description apparently worsens when included in a coding system. This implies the limitations of the plain CXR as a classic imaging tool. It may be assumed that defining necessary medical terms carefully through training and in-depth discussion prior to actual reading would minimize misunderstandings, even with a detailed coding system. However, this was not effective in our study, possibly because of language barriers, different medical backgrounds, and insufficient recognition of the problems. Collectively, our results support the concept of reproducibility of a simplified coding system [6,7,19], which may be critical when a system is shared by raters from different countries, such as even Asian countries.

On comparing CXR findings 3 years after the prevalence survey, Japanese raters detected deterioration in more cases than Vietnamese raters. The fact that the Japanese raters more frequently detected infiltrates may partly explain this discrepancy, because infiltrates generally signify active lesions, though unknown factors may also have affected their readings. This should be considered when CXRs are used for follow-up because the radiological appearance of lesions will not provide sufficient information for monitoring TB unless patient history and bacteriological examination are combined [8,10,19].

Our study has several limitations. First, caution should be exercised when extrapolating the results to describe the way CXRs are generally read in the two Asian countries. Although different medical backgrounds in the countries were obvious after reviewing and discussing the results, the raters' qualifications should also be considered. Second, in the present study, the overall sensitivity and specificity of CXR-based diagnosis of tuberculosis were not determined because the number of active TB cases detected in our cohort study was rather small (< 10%) and because these parameters would be influenced more by individual raters' skills and experiences than by the coding system used. Third, the coverage rate of the radiographic follow-up study after 3 years was not high, one of the reasons being the rapid speed of urbanization and an increasingly mobile population in Hanoi, which caused difficulties when tracing particular individuals. Nevertheless, our findings present an important point to be considered in international studies of TB using a CXR coding system.

Conclusions

In our study, CXR findings of suspected TB were inconsistent between raters with different backgrounds, presumably because of differences in medical practice and education between the two countries. Although each coding system has its advantages and disadvantages, a simplified classification system is suitable for maintaining sufficient agreement between raters from different countries. To improve the quality of future international collaborative studies, harmony could be obtained between raters of different nationalities by thorough discussion regarding the possible causes of disagreement in CXR readings, using standard films and descriptions of major findings.

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Authors' contributions

SS and NTLH participated in supervising the on-site implementation of the study, drafting the paper, and substantially revising it. ET, LDH, PTC, and NKo read the chest X-ray films. LTL and PHT participated in the conception, design, and supervision of the study. PTNB participated in on-site implementation of the study. NI supervised and performed statistical analysis. NKe participated in the conception and design of the study, analysis and interpretation of data, drafting of the paper, and substantially revising it. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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PLOS | NEGLECTED TROPICAL DISEASES

Impact of Amino Acid Substitutions in B Subunit of DNA Gyrase in *Mycobacterium leprae* on Fluoroquinolone Resistance

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Abstract

Background: Ofloxacin is a fluoroquinolone (FQ) used for the treatment of leprosy. FQs are known to interact with both A and B subunits of DNA gyrase and inhibit supercoiling activity of this enzyme. Mutations conferring FQ resistance have been reported to be found only in the gene encoding A subunit of this enzyme (*gyrA*) of *M. leprae*, although there are many reports on the FQ resistance-associated mutation in *gyrB* in other bacteria, including *M. tuberculosis*, a bacterial species in the same genus as *M. leprae*.

Methodology/Principal Findings: To reveal the possible contribution of mutations in *gyrB* to FQ resistance in *M. leprae*, we examined the inhibitory activity of FQs against recombinant DNA gyrases with amino acid substitutions at position 464, 502 and 504, equivalent to position 461, 499 and 501 in *M. tuberculosis*, which are reported to contribute to reduced sensitivity to FQ. The FQ-inhibited supercoiling assay and FQ-induced cleavage assay demonstrated the important roles of these amino acid substitutions in reduced sensitivity to FQ with marked influence by amino acid substitution, especially at position 502. Additionally, effectiveness of sitafloxacin, a FQ, to mutant DNA gyrases was revealed by low inhibitory concentration of this FQ.

Significance: Data obtained in this study suggested the possible emergence of FQ-resistant *M. leprae* with mutations in *gyrB* and the necessity of analyzing both *gyrA* and *gyrB* for an FQ susceptibility test. In addition, potential use of sitafloxacin for the treatment of problematic cases of leprosy by FQ resistant *M. leprae* was suggested.

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Introduction

Leprosy is one of the oldest human infectious diseases and remains a public health problem. At the beginning of 2011, the number of registered leprosy cases was 192,246, and that of new cases reported during 2010 was 228,474, mainly from Asian, Latin American, and African countries [1]. Multibacillary leprosy is usually treated by administering dapsone (DDS), clofazimine (CLF), and rifampicin (RIF) in combination, where single skin lesion paucibacillary leprosy is recommened to be treated by administering RIF, ofloxacin (OFX), and minocycline (MIN) [2]. Since the late 1990s, multi-drug resistant (MDR) isolates of *M. leprae*, resistant to RIF and DDS, have emerged and the importance of OFX has been a focus for the treatment of MDR-leprosy [3]; however, their use not only for leprosy but also for other infectious diseases including tuberculosis has already led to OFX resistance in *M. leprae* [4–8]. Hence, early prediction of FQ resistance seems to be essential for the proper treatment of leprosy.

OFX is a fluoroquinole (FQ) and FQs inhibit type II DNA topoisomerases, including DNA gyrase and topoisomerase IV [9]. FQ resistance is given mainly by amino acid substitutions in the quinolone resistance-determining regions (QRDRs) located on the N- and C-terminal domains of A (GyrA) and B (GyrB) subunits of DNA gyrase and, less prominently, amino acid substitution in the QRDR on the N- and C-terminal domains of A (ParC) and B (ParE) subunits of topoisomerase IV has been reported [10]. *M. leprae* has only DNA gyrase [11], which is therefore the sole target of FQs. Genetic analysis of *M. leprae* clinical isolates revealed reduced FQ sensitivity associated with amino acid substitutions only at position 89 or 91 and 205 in GyrA and GyrB, respectively [4–8,12]. In the latter study, the contribution of amino acid

Author Summary

Leprosy is one of the oldest human infectious diseases, which remains a public health problem with more than 200,000 new cases every year worldwide. Since the late 1990s, multi-drug resistant leprosy, resistant to rifampicin and dapsone, has emerged and the importance of ofloxacin has increased. However, their use for leprosy and other infectious diseases has already elicited ofloxacin resistant leprosy cases. Hence, early detection of ofloxacin resistance is essential for proper treatment. This study, by utilizing recombinant technology, predicted the future emergence of ofloxacin resistant *Mycobacterium leprae* with mutations that have not yet been reported. The data are useful for predicting ofloxacin resistance and, hence, able to contribute to the proper treatment of leprosy through suggesting the importance of analyzing gene mutations for FQ susceptibility testing.

substitution in GyrA at position 89 or 91 to reduced FQ sensitivity was confirmed by an *in vitro* analysis [13]. In addition, the effect of amino acid substitution at position 95 in GyrA was predicted [14]. In contrast, amino acid substitution in GyrB at position 205, reported by You et al. [8], was revealed not to affect FQ sensitivity by an *in vitro* study [13]. Reduced FQ sensitivity associated with amino acid substitutions has been frequently reported in GyrA in *M. tuberculosis*; however, those in GyrB have been reported less frequently (Figure 1) [10,15]. According to the reports, important residues of GyrB in *M. tuberculosis* were thought to be at codon 461, 499 and 501 (with a counting system proposed by Maruri et al. [10]). Notably, amino acid substitutions at position 499 and 501 in *M. tuberculosis* showed a correlation with reduced FQ susceptibility by an *in vitro* assay [15–18]. Lack of the detection of FQ-resistant *M. leprae* carrying GyrB amino acid substitutions is due to the low number of FQ resistant cases analyzed. Hence, it is highly important to elucidate the contribution of amino acid substitutions

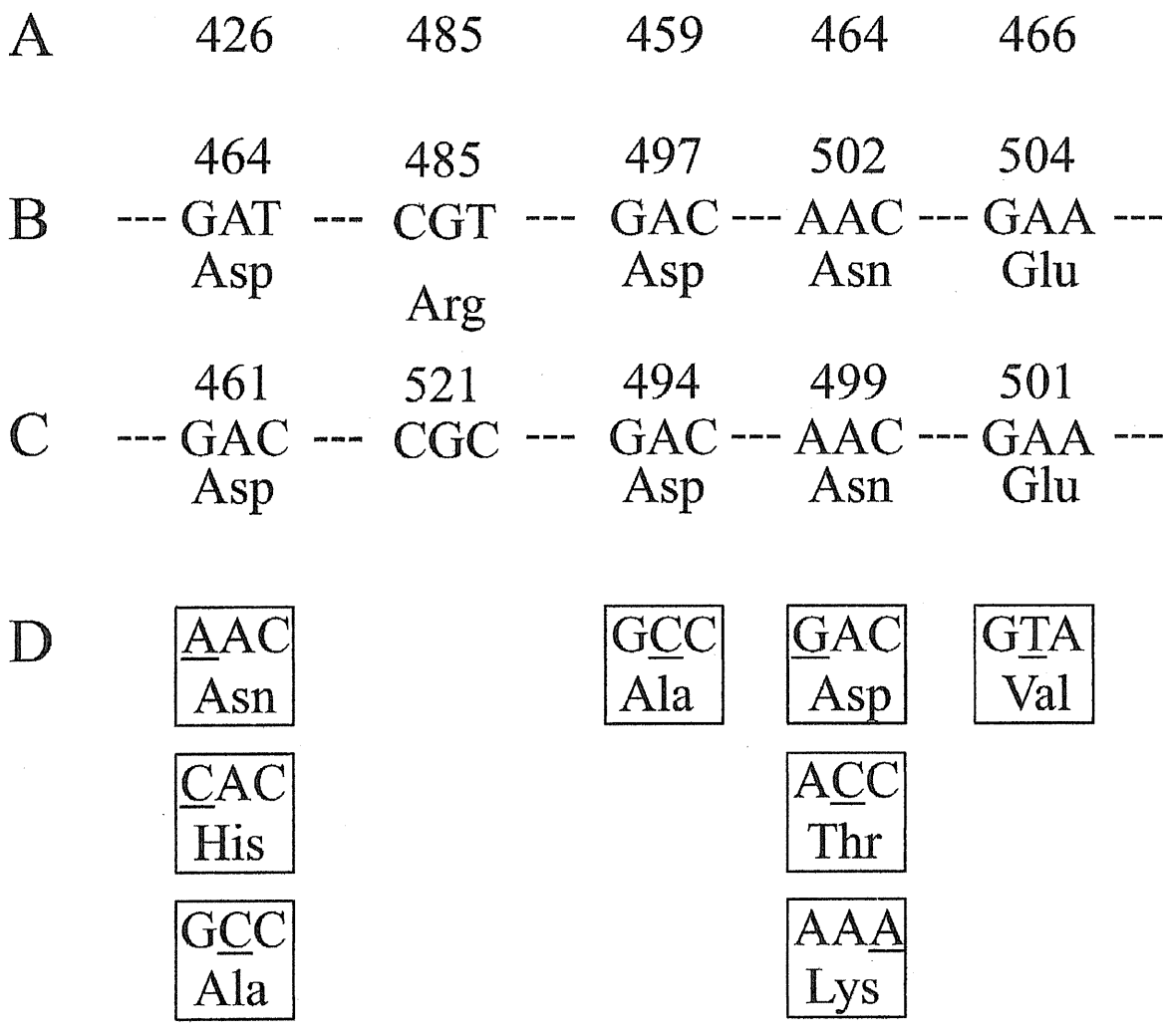

Figure 1. Nucleotide and amino acid sequences of QRDR of *M. leprae* and *M. tuberculosis gyrB* and mutations found in FQ-resistant isolates. (A) amino acid number of GyrB in *E. coli*, (B) Amino acid number, nucleotide sequences and amino acid sequence of WT *M. leprae* GyrB QRDR, (C) Amino acid number, nucleotide sequences and of WT *M. tuberculosis* GyrB QRDR, (D) Altered amino acids and corresponding nucleotide substitutions found in higher rate in FQ-resistant *M. tuberculosis* isolates.
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in GyrB to FQ resistance utilizing recombinant technology and *in vitro* assay.

On the basis of reports on *M. tuberculosis*, we selected target amino acid substitutions at position 464, 502 and 504 in *M. leprae* GyrB, equivalent to position 461, 499 and 501 in *M. tuberculosis*, to reveal the significance of these amino acid substitutions for reduced FQ sensitivity, and conducted the FQ-inhibited supercoiling assay and FQ-mediated DNA cleavage assay using recombinant DNA gyrase.

Methods

Drugs and kits

Ofloxacin (OFX), ciprofloxacin (CIP) and levofloxacin (LVX) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada); sitafloxacin (SIT) was from Daiichi-sankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan); ampicillin and kanamycin were purchased from Meiji Seika Pharma Ltd. (Tokyo, Japan). Oligonucleotide primers were synthesized by Life Technologies Corp. (Carlsbad, CA). Restriction enzymes were obtained from New England Biolabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Bacterial strains and plasmid

The Thai-53 strain of *M. leprae* [19], maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp.), Rosetta-gami 2, and BL21 (DE3) pLysS (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. pET-20b (+) (Merck KGaA) vector was used to construct expression plasmids for *M. leprae* DNA gyrases.

Construction of expression plasmids

Wild-type (WT) recombinant GyrA and GyrB expression plasmids were constructed as described previously [14,16]. Mutations were introduced into the WT *gyrB* gene by PCR using pairs of complementary primers containing the mutations of interest (Table 1). All PCR reactions were carried out in a thermal cycler (Life Technologies Corp.) under the following conditions: pre-denaturation at 95°C for 2 min; 35 cycles of denaturation at 95°C for 10 s, annealing at 50–60°C for 15 s, and extension at 68°C for 1 to 3 min, and then a final extension at 68°C for 5 min. The *gyrB* C-terminal cassettes with base substitutions were digested with *Pml* I and *Xho* I, ligated into WT *gyrB* expression plasmid, and digested with the same restriction endonucleases to obtain mutant *gyrB* expression plasmid (Figure 2). The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit and an ABI Prism 3130xI genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase subunits

Recombinant DNA gyrase subunits were expressed and purified as previously described [13,14,16,20]. Briefly, expression plasmids carrying the *gyrA* and *gyrB* of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21 (DE3) pLysS, respectively. The transformants were grown in Luria-Bertani (LB) medium in the presence of 100 μg/mL Ampicillin to the log phase and the expression of DNA gyrase was induced with the addition of 1 mM isopropyl-beta-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Osaka, Japan), followed by further incubation at 14°C for 16 h. The harvested *E. coli* were lysed by sonication (Sonifier 250; Branson, Danbury, CT) and the recombinant DNA gyrase subunits in supernatants after centrifugation (10,000 × g for 30 min) were purified by Ni-NTA Agarose resin (Life Technologies Corp.) column chromatography and dialyzed against DNA gyrase dilution buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA). The purified protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling assay and inhibition by FQs

ATP-dependent and FQ-inhibited DNA supercoiling assays were performed according to previous reports [13,14,16,20]. DNA supercoiling activity was examined with reaction mixture consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (0.3 μg), and GyrA and GyrB subunits (50 ng each) in a total volume of 30 μl. Reactions were run at 30°C for 1.5 h followed by stopping with the addition of 30 μl chloroform/iso-amyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading solution. The total reaction mixtures were subjected to electrophoresis on 1% agarose gels in 1× Tris-borate-EDTA (TBE) buffer and stained by ethidium bromide (0.7 μg/ml). The extent of supercoiled DNA was quantified with ImageJ (http://rsbweb.nih.gov/ij) and the inhibitory effects of FQs on DNA gyrase were assessed by determining the drug concentration required to inhibit the supercoiling activity of the DNA gyrase by 50% (IC_{50}s) in the

Table 1. Nucleotide sequences of primers used in this study.

Primer name	Primer sequence (Nucleotide Position)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGATGGACATCCAGGAAACGAACATCC-3′ (2013–2037)
D464N_Fw	5′-A GTG GAA GGT **AAT** TCG GCT GGT G
D464N_Rv	5′-C ACC AGC CGA **ATT** ACC TTC CAC T
N502D_Fw	5′-A GTG CTA AAG **GAC** ACC GAA GTT C
N502D_Rv	5′-G AAC TTC GGT **GTC** CTT TAG CAC T
E504V_Fw	5′-A AAG AAC ACC **GTA** GTT CAA GCA A
E504V_Rv	5′-T TGC TTG AAC **TAC** GGT GTT CTT T

Mutated codons are indicated in bold face.
doi:10.1371/journal.pntd.0001838.t001

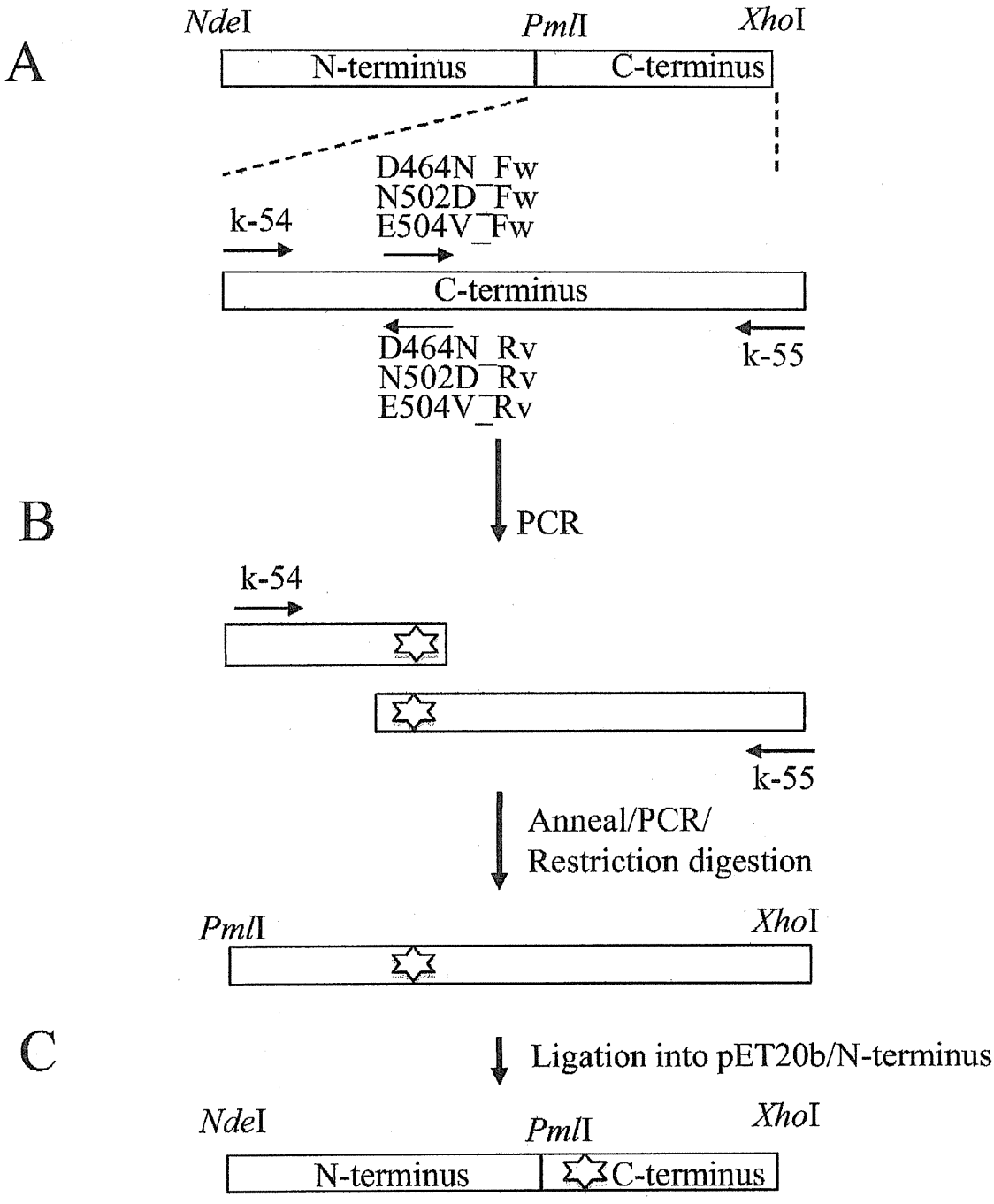

Figure 2. Construction of WT and mutant DNA gyrase expression plasmid. (A) Primer pairs k-54+D464N_Rv, N502D_Rvor E504V_Rv (Table 1) were used for amplifying the DNA fragment encoding N-terminus half (amino acid 424 to 467, 505 or 507, respectively) of C-terminus region of GyrB carrying Asp464Asn, Asn502Asp and Glu504Val, respectively. Primer pairs k-55+D464N_Fw, N502D_Fw or E504V_Fw (Table 1) were used for amplifying the DNA fragment encoding the C-terminus half (amino acid 461, 499 or 501 to 678, respectively) of the C-terminus region of GyrB carrying Asp464Asn, Asn502Asp and Glu504Val, respectively. (B) To complete the C-terminus region encoding cassette, DNA fragments encoding the N-terminus half and C-terminus half of the C-terminus region of GyrB were annealed and reamplified by PCR using the primer pair of k-54 and k-55. (C) The mutated *gyrB*-C cassettes were digested with *PmlI* and *XhoI* restriction endonucleases and ligated into the expression plasmid containing the WT*gyrB*N-terminus region DNA fragment digested by the same enzymes.
doi:10.1371/journal.pntd.0001838.g002

presence or absence of serial two-fold increases in the concentrations of OFX, MXF, SIT, CIP and LVX. Enzymatic assays were performed at least three times to confirm the reproducibility.

FQ-mediated DNA cleavage assay

DNA cleavage assays were also carried out as described in previous reports [13,14,16,20,21]. Briefly, the reaction mixture

(total volume 30 µl) contained DNA gyrase assay buffer, purified DNA gyrase subunits, supercoiled pBR322 DNA (0.3 µg) and increasing concentrations of OFX, MXF, SIT, CIP and LVX. After incubation for 2 h at 30°C, cleavage reactions were stopped by adding 3 µl of 2% SDS and 3 µl proteinase K (1 mg/ml). After subsequent incubation for 30 min at 30°C, proteinase K reactions were stopped by the addition of 3 µl of 0.5 mM EDTA, 30 µl chloroform/iso-amyl alcohol (24:1 mixture) and 3 µl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ (http://rsbweb.nih.gov/ij) and the FQ concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Results

Construction and purification of recombinant WT and mutant DNA gyrase subunits

The WT GyrA and GyrB expression plasmids constructed in our previous work [14] were used. DNA fragments with mutations causing amino acid substitutions at position 464, 502 and 504 in GyrB were amplified from WT GyrB expression plasmid [14] and introduced into expression vector pET-20b (+). Recombinant GyrA and GyrB were expressed as C-terminus hexa-histidine tagged protein for ease of purification, as the His-tag has been shown not to interfere with the catalytic functions of GyrA and GyrB [13–16,20,22]. Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.4 to 1.7 mg soluble His-tagged protein with molecular weights of 80 kDa and 75 kDa for GyrA and GyrB, respectively, from 500 ml cultures. The purity of recombinant proteins was confirmed by SDS-PAGE (Figure S1). All of the recombinant proteins were obtained with high purity (>90–95%).

ATP-dependent DNA supercoiling activities of WT and mutant DNA gyrases

Combinations of WT GyrA and WT or mutant GyrBs (GyrB-Asp464Asn, GyrB-Asn502Asp or GyrB-Glu504Val) were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Figure S2). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Figure S2 A–D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Figure S2 A–D, lane 4, 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction condition (Figure S2 A–D, lane 6). Consequently, ATP-dependent DNA supercoiling activities were confirmed with WT and three mutant DNA gyrases.

IC_{50}s of five FQs for WT and mutant DNA gyrases

FQs-inhibited DNA supercoiling activities were assessed for the determination of IC_{50}s. Figure 3 shows a representative result of the inhibitory effect of OFX and the results for the other FQs are presented in Figure S3. Results show the dose-dependent inhibition of five FQs against WT and mutant DNA gyrases, as summarized in Table 2. The five FQs inhibited the DNA supercoiling activities of WT DNA gyrase at low concentration (Table 2).

CC_{25}s of five FQs for WT and mutant DNA gyrases

DNA cleavage assay was performed in the presence of increasing concentrations of FQs to estimate CC_{25}s. Figure 4 presents the results of a representative DNA cleavage assay using

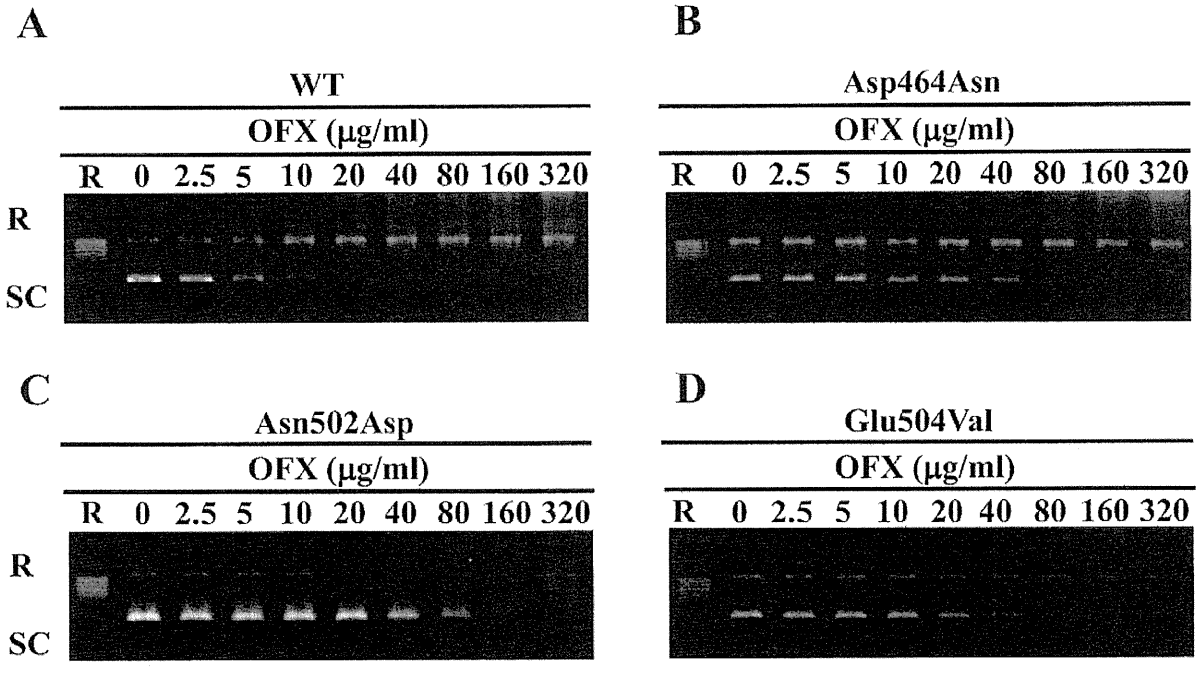

Figure 3. OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 mg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. FQ-inhibited supercoiling activity assay was performed in combination of WTGyrA+WTGyrB (A), GyrB-Asp464Asn (B), GyrB-Asn502Asp (C) and GyrB-Glu504Val (D). R and SC denote relaxed and supercoiled pBR322 DNA, respectively.
doi:10.1371/journal.pntd.0001838.g003

Table 2. IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases.

Drug	IC_{50} (μg/ml)				CC_{25} (μg/ml)			
	WT	Asp464Asn	Asn502Asp	Glu504Val	WT	Asp464Asn	Asn502Asp	Glu504Val
OFX	5.7±0.8	53.9±9.0	106.6±25.1	34.6±4.3	2.4±0.2	32.7±6.3	78.2±12.6	30.0±7.9
MXF	1.7±0.3	4.1±0.4	17.8±2.6	13.9±0.6	0.6±0.0	3.3±0.9	15.3±2.6	9.6±1.7
SIT	0.5±0.1	1.8±0.3	1.6±0.6	1.7±0.2	0.2±0.0	0.9±0.0	1.0±0.2	0.7±0.1
CIP	2.3±0.3	11.3±2.7	257.9±46.1	49.3±9.4	0.9±0.2	6.5±0.6	42.5±13.6	24.7±0.5
LVX	4.5±0.3	32.9±3.2	46.8±1.1	19.9±2.9	1.4±0.1	18.6±4.9	51.7±10.6	9.3±0.7

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OFX, and Figure S4 shows those using other FQs. Table 2 shows the CC_{25}s of FQs for WT and mutant DNA gyrases. Highest CC_{25}s of FQs were observed for GyrB-Asn502Asp DNA gyrase.

Discussion

We focused on amino acid substitutions at position 464, 502 and 504 in GyrB in *M. leprae* equivalent to 461, 499 and 501, respectively, in *M. tuberculosis*, as amino acid substitutions at these positions in *M. tuberculosis* are known to contribute to FQ resistance [13–16,20,22,23]. We carried out a FQ-mediated supercoiling activity inhibition assay and a DNA cleavage assay using recombinant WT and mutant DNA gyrases at 30°C, the optimal temperature of *M. leprae* growth [24], and calculated IC_{50}s and CC_{25}s of five FQs, including OFX, MXF, SIT, CIP and LVX. All FQs inhibited DNA supercoiling activities of WT DNA gyrase at low concentration (Table 2). In strong contrast, three mutant DNA gyrases showed reduced sensitivity to all five FQs. GyrB-Asn502Asp DNA gyrase exhibited the lowest FQ sensitivity among the three mutant DNA gyrases. IC_{50}s of OFX, MXF, SIT, CIP and LVX for GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrases were 2.4- to 9.5-fold, 3.2- to 112.1-fold and 3.4- to 21.4-fold higher than those for WT DNA gyrase (Figure 3, 5, S3 and Table 2). A similar tendency was observed in the DNA cleavage assay. Namely, CC_{25}s of OFX, MFX, SIT, CIP and LVX for GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrases were 4.5- to 13.6-fold, 5.0- to 47.2-fold and 3.5- to 27.4-fold higher than for WT DNA gyrase (Figure 4, 5, S4, Table 2). These results suggested the contribution of these amino acid substitutions in GyrB to reduced FQ sensitivity and the possible emergence of *M. leprae* with mutant GyrB, although previously identified Asp to Asn amino acid substitution in GyrB at position 205 [8] was revealed not to have an effect on FQ susceptibility [13]. It is noteworthy that mutant DNA gyrases exhibited a similar

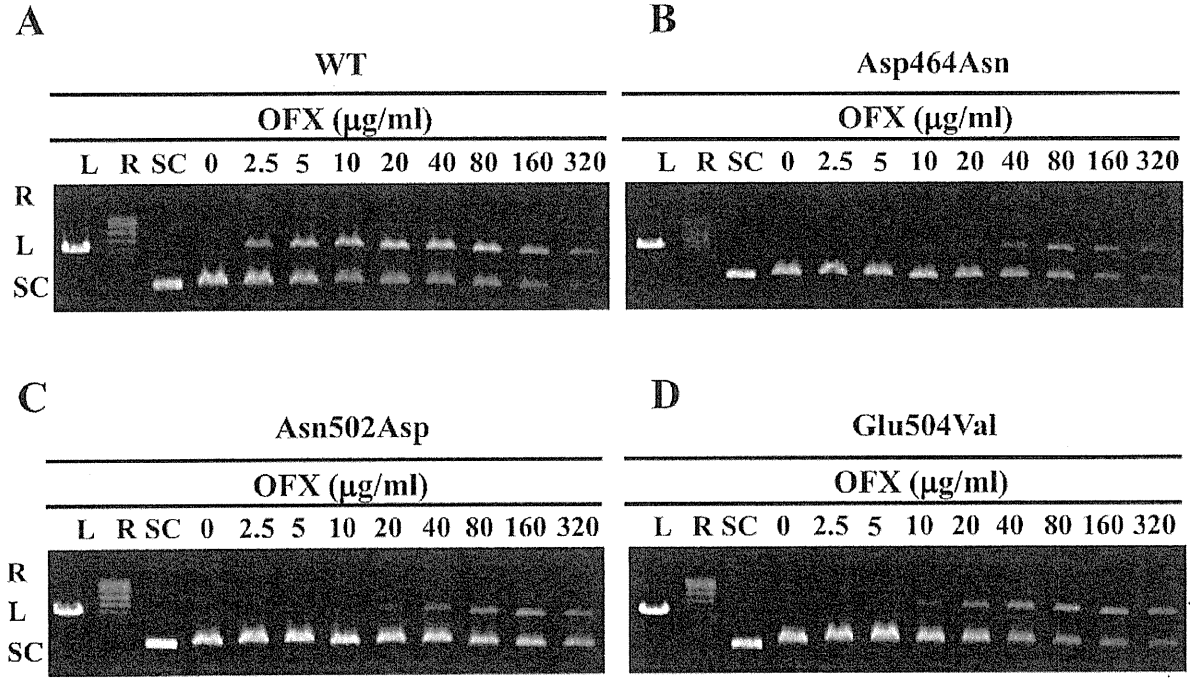

Figure 4. OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 mg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed in combination of WT GyrA+WT GyrB (A), GyrB-Asp464Asn (B), GyrB-Asn502Asp (C) and GyrB-Glu504Val (D). R, L and SC denote relaxed, linear and supercoiled pBR322 DNA, respectively.
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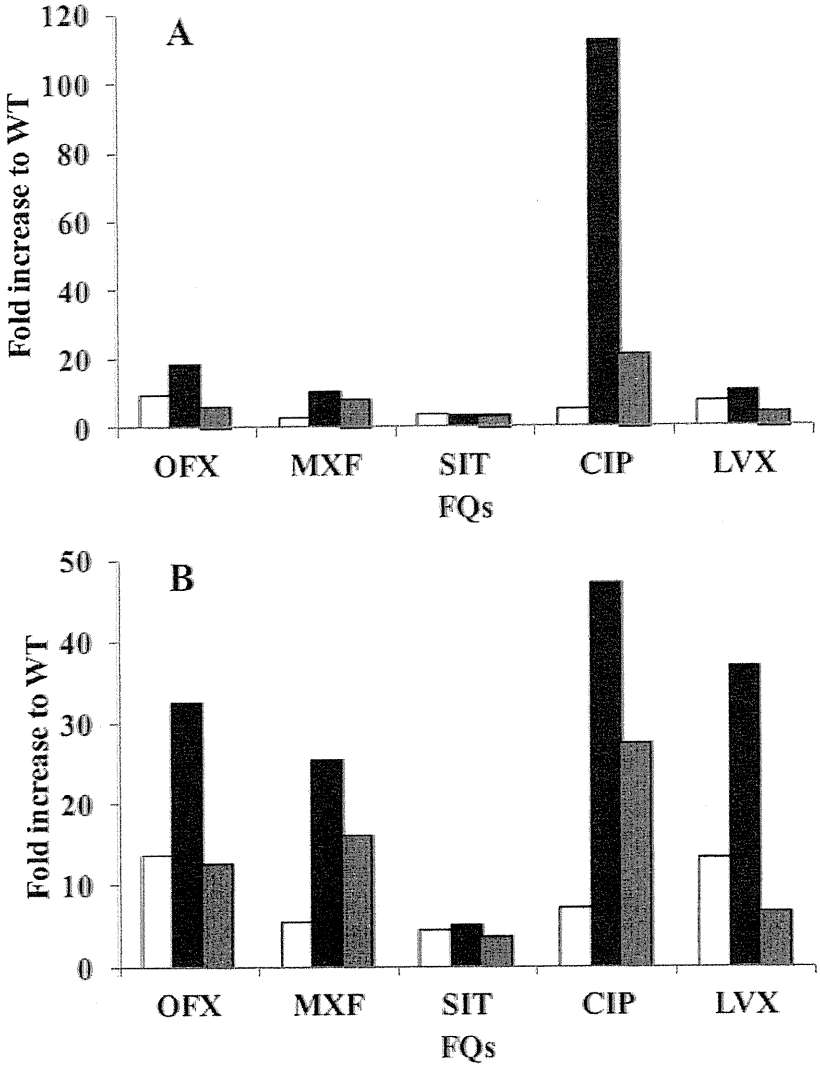

Figure 5. Increased IC_{50}s and CC_{25}s of FQs for mutant DNA gyrases. IC_{50}s and CC_{25}s were calculated by the quinolone-inhibited supercoiling assay and FQ-mediated cleavage assay, respectively. Fold increase of each FQ for mutant DNA gyrases was plotted. (A) IC_{50}s, (B) CC_{25}s. Open, closed and hatched bar denotes the value for GyrB- GyrB-Asp464Asn, GyrB-Asn502Asp and GyrB-Glu504Val DNA gyrase, respectively.
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sensitivity pattern to those reported for mutant GyrB in *M. tuberculosis*. *M. leprae* GyrB-Asn502Asp DNA gyrase had lower FQ sensitivity than GyrB-Asp426Asn and GyrB-Glu504Val DNA gyrase, as has been shown in *M. tuberculosis* [15–18]. The high homology of the entire GyrB and full sequence match in QRDR between *M. leprae* and *M. tuberculosis* might lead to a similar tendency of FQ sensitivity. It is interesting that the Asp to Asn amino acid substitution in *E. coli* at position equivalent to 464 in *M. leprae* showed enhancing effect on CIP resistance [25] where Glu to Asp or Ala amino acid substitution in *Streptococcus pneumonia* at position equivalent to 504 in *M. leprae* showed little or reducing effect on CIP resistance, respectively [26]. Overall or QRDR structure of GyrB might affect the acquisition of FQ resistance.

IC_{50}s of FQs were 8 to 40 times higher than the minimum inhibitory concentrations (MICs) in *M. tuberculosis* [17,18,22]. This non-proportionality presumably reflects basic differences in the cell-permeating properties and the accumulation of different FQs [22]. We investigated the inhibitory effects of OFX, GAT, MXF, LVX and SIT against WT and mutant DNA gyrases. IC_{50}s of OFX for WT DNA gyrase was 5.7 μg/ml (Table 2) and it seemed reasonable that OFX has been used by a single application of 400 to 600 mg for leprosy patients with a single lesion and two or three doses of 400 to 600 mg in combination with first-line drugs, DDS and RIF [27] for the treatment of patients with MDR leprosy. On the contrary, IC_{50}s of OFX for GyrB-Asp464Asn, Asn502Asp and Glu504Val showed 9.5, 18.7 and 6.1 fold higher concentration comparing to WT DNA gyrase, respectively, and OFX seems not to have the ability to inhibit *M. leprae* with DNA gyrase with these mutations. On the other hand, the order of inhibitory activity was SIT>MXF>CIP>LVX>OFX. Namely, SIT most effectively inhibited WT and mutant DNA gyrases among five FQs. IC_{50}s of SIT for WT was 0.5 μg/ml and the increase was 3.6-, 3.2- and 3.4-fold for GyrB-Asp464Asn, GyrB-Asn502Asp and GyrB-Glu504Val DNA gyrases, respectively. In addition, the maximum

serum concentration (Cmax) of OFX, SIT, CIP and LVX in 100 mg dosage was determined in clinical trials to be 0.95, 1.00, 1.33 and 1.22 μg/ml, respectively [28–31], and that of MFX in 400 mg dose to be 4.13 [32]. SIT might strongly inhibit *M. leprae* carrying GyrB-Asp464Asn, Asn502Asp and Glu504Val DNA gyrase as well as that carrying GyrA-Ala90Val, Asp95Gly, and Asp95Asn [14,16,20]. Thus, SIT is a promising candidate for the treatment of leprosy caused by OFX-resistant *M. leprae* with these problematic gyrases. Although SIT is now only approved in Japan and mild gastrointestinal disorders as adverse reactions have been reported, our data in this study might encourage the use of SIT for OFX-resistant leprosy.

In conclusion, we revealed the contribution of Asp464Asn, Asn502Asp and Glu504Val amino acid substitution to reduced sensitivity to FQ in *M. leprae* by an *in vitro* assay. This suggested the possible emergence of FQ-resistant *M. leprae* carrying GyrB with these amino acid substitutions in the future. Hence we would like to propose the analysis of these amino acid substitutions in GyrB to detect FQ-resistant leprosy. Additionally, effectiveness of sitafloxacin to the mutant DNA gyrases suggested the potential use of this FQ for the treatment of ofloxacin resistant cases.

Supporting Information

Figure S1 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were over expressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M: Protein marker (NEB), 1: WTGyrA, 2: WTGyrB, 3: GyrB-Asp464Asn, 4: GyrB-Asn502Asp, 5: GyrB-Glu504Val. 300 ng of each protein was loaded on 5–20% gradient polyacrylamide gel.
(TIF)

Figure S2 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A), DNA gyrases bearing GyrB-Asp464Asn (B), Asn502Asp (C) and Glu504Val (D) were analyzed. Relaxed pBR322 (0.3 mg) was incubated with GyrA (50 ng) or GyrB (50 ng) or both. Lanes: 1: relaxed pBR322 alone, 2: relaxed pBR322 and ATP, 3: relaxed pBR322, ATP, GyrA and GyrB, 4: relaxed pBR322, ATP and GyrA, 5: relaxed pBR322, ATP and GyrB, 6: relaxed pBR322, GyrA and GyrB.
(TIF)

Figure S3 Inhibitory activities of (A) MXF, (B) SIT, (C) CIP and (D) LVX on supercoiling activities against *M. leprae* WT and mutant DNA gyrases. Relaxed pBR322 DNA (0.3 mg) was incubated with 50 ng each of GyrA and GyrB in the absence or presence of the indicated concentration (in mg/ml) of three FQs. The reactions were stopped, and the DNA products were analyzed by electrophoresis on 1% agarose gel. R and SC denote relaxed and supercoiled pBR322 DNA, respectively.
(TIF)

Figure S4 DNA cleavage activity of (A) MXF, (B) SIT, (C) CIP and (D) LVX against *M. leprae*WT and mutant DNA gyrases. Supercoiled pBR322 DNA (0.3 mg) was incubated with 50 ng each of GyrA and GyrB in the absence or presence of the indicated concentration (in mg/ml) of three FQs. The reactions were stopped, and the processed DNA products were analyzed by electrophoresis on 1% agarose gel. R, L and SC denote relaxed, linear and supercoiled pBR322 DNA, respectively.
(TIF)

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Author Contributions

Conceived and designed the experiments: TM MM CN YS. Performed the experiments: KY HK CN. Analyzed the data: KY CN YS. Contributed reagents/materials/analysis tools: KY TM MM. Wrote the paper: KY TM MM CN YS.

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Amino Acid Substitutions at Position 95 in GyrA Can Add Fluoroquinolone Resistance to *Mycobacterium leprae*

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Amino acid substitutions at position 89 or 91 in GyrA of fluoroquinolone-resistant *Mycobacterium leprae* clinical isolates have been reported. In contrast, those at position 94 in *M. tuberculosis*, equivalent to position 95 in *M. leprae*, have been identified most frequently. To verify the possible contribution of amino acid substitutions at position 95 in *M. leprae* to fluoroquinolone resistance, we conducted an *in vitro* assay using wild-type and mutant recombinant DNA gyrases. Fluoroquinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay revealed the potent contribution of an amino acid substitution of Asp to Gly or Asn at position 95 to fluoroquinolone resistance. These results suggested the possible future emergence of quinolone-resistant *M. leprae* isolates with these amino acid substitutions and the usefulness of detecting these mutations for the rapid identification of fluoroquinolone resistance in leprosy.

Leprosy is a chronic human infectious disease caused by *Mycobacterium leprae* which may cause severe disabilities due to damage to the peripheral nerves (33). The World Health Organization (WHO) reported the global number of registered new cases in 2010 to be 228,474, while during 2009 it was 244,796 (37). Although the number of new cases detected globally fell by 16,322 (6.7%) during this period, new leprosy cases are still detected every year, mainly in Asia, Latin America, and Africa (21, 37). In the 1980s, the WHO introduced multidrug therapy (MDT), composed of dapsone (DDS), rifampin (RIF), and clofazimine (36). Recently, fluoroquinolones (FQs), especially ofloxacin (OFX), have been recommended for the treatment of leprosy with a single lesion. The emergence of multidrug-resistant (MDR) leprosy, resistant to both DDS and RIF owing to therapeutic failure or low compliance, has been reported (17, 29), and FQs are thought to be important. For appropriate treatment, early assessment of drug susceptibility is essential; however, *M. leprae* cannot be cultivated on artificial media and a drug susceptibility test depending on *in vitro* growth is not available. Consequently, antibiotic susceptibility tests have relied on the mouse footpad leprosy model, requiring 8 to 12 months because of the slow growth of *M. leprae* (18). Recently, genetic analysis of drug-resistant *M. leprae* substantiated the correlation of DDS, RIF, and OFX resistance with mutations in *folP1*, encoding dihydropteroate synthetase (5, 15, 19, 23–25, 35); *rpoB* (4, 6, 12, 19, 23–25, 33), encoding the beta subunit of RNA polymerase; and *gyrA*, encoding the A subunit of DNA gyrase (4, 19, 24, 26, 40), respectively. Among these, data for *folP1* in *M. tuberculosis* are not available as DDS is not used for the treatment of tuberculosis. Mutations in *rpoB* observed in *M. leprae* showed good agreement with those obtained from RIF-resistant *M. tuberculosis*. In contrast, the distribution of mutations in *gyrA* of FQ-resistant *M. tuberculosis* was distinct from that in *gyrA* of OFX-resistant *M. leprae* (Fig. 1). Namely, amino acid substitutions at position 94 in GyrA were found in approximately half of FQ-resistant *M. tuberculosis* isolates, whereas no amino acid substitutions at position 95, equivalent to position 94 in *M. tuberculosis*, have been reported in *M. leprae*, and 11 cases with amino acid substitutions at position 91, equivalent to position 94 in *M. tuberculosis*, were reported from a total of six countries (4, 19, 24, 26, 40). Thus, elucidation of the contribution of amino acid substitutions at position 95 of GyrA in *M. leprae* to FQ resistance is important for the gene-based detection of fluoroquinolone resistance.

FQs inhibit type II DNA topoisomerases, DNA gyrase, and topoisomerase IV, which play crucial roles in DNA replication during cell division (8). As *M. leprae* has only DNA gyrase, this is the sole target of FQs. DNA gyrase, consisting of two GyrA and two GyrB subunits, catalyzes the negative supercoiling of the circular bacterial chromosome by cleaving double strands and passing the enwrapped DNA, followed by resealing the double strands (8, 13). To reveal the significance of amino acid substitution at position 95 to FQ resistance, we conducted the FQ-mediated supercoiling activity inhibition assay and DNA cleavage assay using recombinant DNA gyrases having an amino acid substitution in GyrA at position 95, Asp to Gly (GyrA-Asp95Gly) or Asp to Asn (GyrA-Asp95Asn). These mutations are frequently found in FQ-resistant *M. tuberculosis* strains (1, 7, 9, 10, 32, 34, 39) but not in FQ-resistant *M. leprae* strains.

MATERIALS AND METHODS

Materials. The Thai-53 strain of *M. leprae* (22), maintained at the Leprosy Research Center, National Institute of Infectious Diseases (Tokyo, Japan), was used to prepare *M. leprae* DNA. *Escherichia coli* strains TOP-10 (Life Technologies Corp., Carlsbad, CA), Rosetta-gami 2, and BL21(DE3)(pLysS) (Merck KGaA, Darmstadt, Germany) were used for cloning and protein expression. GyrA and GyrB expression plasmids were constructed on the basis of pET-20b(+) (Merck KGaA). OFX and gatifloxacin (GAT) were purchased from LKT Laboratories, Inc. (St. Paul, MN); moxifloxacin (MXF) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Sitafloxacin (SIT) was a gift from Daiichisankyo Pharmaceutical, Co., Ltd. (Tokyo, Japan). Ampicillin was purchased from Meiji Seika Pharma, Ltd. (Tokyo, Ja-

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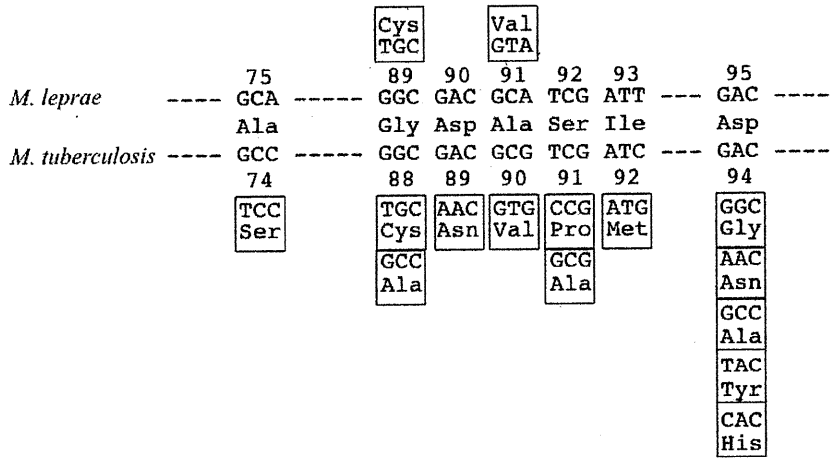

FIG 1 Nucleotide substitutions encoding the quinolone resistance-determining region in *gyrA* of WT and FQ-resistant *M. leprae* and *M. tuberculosis*. Nucleotide sequences encoding the quinolone resistance-determining region of WT *M. leprae* and *M. tuberculosis* GyrA were aligned with the amino acid sequence at the corresponding positions indicated by the numbers. Altered amino acids and the corresponding nucleotide substitutions of *M. leprae* and *M. tuberculosis* are placed above and below WT sequences, respectively.

pan). Oligonucleotide primers were synthesized by Life Technologies Corp. Restriction enzymes were obtained from New England BioLabs, Inc. (Ipswich, MA). The supercoiling assay kit and supercoiled and relaxed pBR322 DNA were purchased from John Innes Enterprises Ltd. (Norwich, United Kingdom).

Construction of recombinant wild-type (WT) and mutant DNA gyrase expression plasmids. DNA gyrase expression vectors were constructed basically as previously described (16), and Fig. 2 presents an overview of the procedure. The sequences of the primers used in the study

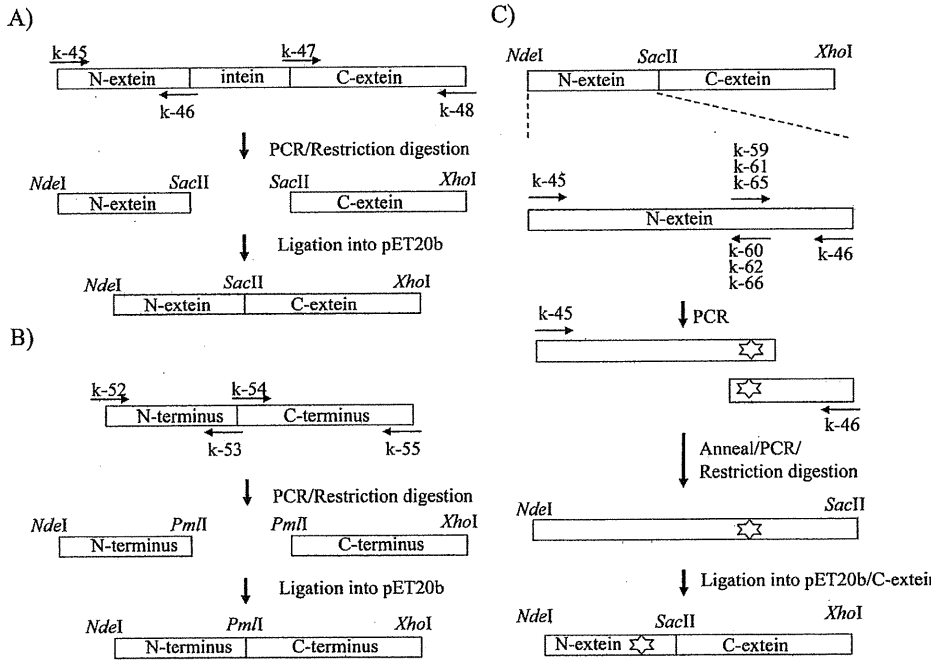

FIG 2 Construction of WT and mutant DNA gyrase expression plasmid. (A) DNA fragments encoding N-extein (amino acids 1 to 130) and C-extein of GyrA (amino acids 125 to 830) were amplified by PCR with primer pairs k-45/k-46 and k-47/k-48 (Table 1), respectively. Similarly, those encoding the N-terminal (amino acids 1 to 428) and C-terminal (amino acids 424 to 679) regions of GyrB were amplified with primer pairs k-52/k-53 and k-54/k-55 (Table 1), respectively. PCR products encoding N-extein and C-extein of GyrA were digested by NdeI-SacII and SacII-XhoI, respectively, and introduced simultaneously into NdeI-XhoI-digested plasmid pET-20b (+). (B) DNA fragments encoding the N-terminal and C-terminal regions of GyrB were digested by NdeI-PmaCI and PmaCI-XhoI, respectively, and introduced into pET20b as described above. (C) Primer pairs consisting of primer k-45 and primer k-60, k-62, or k-66 (Table 1) were used for amplifying the DNA fragment encoding the N-terminal portion (amino acids 1 to 94) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. Primer pairs consisting of primer k-46 and primer k-59, k-61, or k-65 (Table 1) were used for amplifying the DNA fragment encoding the C-terminal portion (amino acids 88 to 130) of N-extein carrying Ala91Val, Asp95Gly, and Asp95Asn, respectively. To complete the N-extein-encoding cassette, DNA fragments encoding the N-terminal and C-terminal regions of N-extein of GyrA were annealed and reamplified by PCR using the primer pair k-45/k-46. The mutated *gyrA*-N cassettes were digested with NdeI and SacII restriction endonucleases and ligated into the expression plasmid containing WT *gyrA* C-extein DNA fragment digested by the same enzymes.

TABLE 1 Nucleotide sequences of primers used in PCR

Primer name	Primer sequence (nucleotide positions)[a]
k-45	5′-GGCATATGACTGATATCACGCTGCCACCAG-3′ (1–25)
k-46	5′-ATAACGCATCGCCGCGGGTGGGTCATTACC-3′ (361–390)
k-47	5′-CACCCGCGGCGATGCGTTATACCGAGGCTCGGCTTACTCC-3′ (371–410)
k-48	5′-GGCTCGAGTTAATGATGATGATGATGATGACCGACACCGCCGTCGG-3′ (2471–2490)
k-52	5′-GGCATATGGCTGCCCAGAGGAAG-3′ (1–18)
k-53	5′-CTAACTCACGTGCTTTACGTGCAGCTATTC-3′ (1259–1288)
k-54	5′-CGTAAAGCACGTGAGTTAGTGCGTCGAAAAAGTGCC-3′ (1270–1305)
k-55	5′-GGCTCGAGCTAATGATGATGATGATGATGGACATCCAGGAAACGAACATCC-3′ (2013–2037)
k-59	5′-GCACGGCGACGTGTCGATTTATG-3′ (261–283)
k-60	5′-CATAAATCGACACGTCGCCGTGC-3′ (261–283)
k-61	5′-CATCGATTTATGGCACGTTAGTGC-3′ (272–295)
k-62	5′-GCACTAACGTGCCATAAATCGATG-3′ (272–295)
k-65	5′-CATCGATTTATAACACGTTAGTGC-3′ (272–295)
k-66	5′-GCACTAACGTGTTATAAATCGATG-3′ (272–295)

[a] Six-histidine tag codons are underlined, and mutated codons are shown in bold type.

are shown in Table 1. All PCRs were carried out in a thermal cycler (Applied Biosystems) under the following conditions: predenaturation at 98°C for 2 min; 35 cycles of denaturation at 98°C for 10 s, annealing at 50 to 60°C for 15 s, and extension at 68°C for 1 to 2.5 min; and then a final extension at 68°C for 2 min. The nucleotide sequences of the DNA gyrase genes in the plasmids were confirmed using a BigDye Terminator (version 3.1) cycle sequencing kit (Life Technologies Corp.) and an ABI Prism 3130*xl* genetic analyzer (Life Technologies Corp.) according to the manufacturer's protocol.

Expression and purification of recombinant DNA gyrase. DNA gyrase subunits were purified as previously described (2, 3, 16, 20, 21, 31). Expression plasmids carrying the *gyrA* (WT and mutants) and WT *gyrB* genes of *M. leprae* were transformed into *E. coli* Rosetta-gami 2 and BL21(DE3)(pLysS), respectively. Expression of GyrA and GyrB was induced with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (Wako Pure Chemical Industries Ltd., Tokyo, Japan), followed by further incubation at 14°C for 16 h. The recombinant DNA gyrase subunit in the supernatant of the sonication lysate (by Sonifier 250; Branson, Danbury, CT) was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Life Technologies Corp.) column chromatography. The protein fractions were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

DNA supercoiling activities and inhibition by FQs. ATP-dependent and quinolone-inhibited DNA supercoiling assays were carried out as previously described (2, 3, 16, 20, 21, 31) with the following modifications. DNA supercoiling activity was examined with a reaction mixture (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and purified GyrA and GyrB (50 ng each) subunits. Reactions were performed at 30°C for 1.5 h and stopped by adding an equal volume of chloroform-isoamyl alcohol (24:1 mixture) and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer, followed by ethidium bromide (0.7 μg/ml) staining. Supercoiling activity was evaluated by tracing the brightness of the bands with the software ImageJ (http://rsbweb.nih.gov/ij). Gyrase bearing an Ala91Val amino acid substitution in GyrA was used as a positive control for all assays (20). The inhibitory effect of FQs on DNA gyrases was assessed by determining the drug concentrations required to inhibit the supercoiling activity of the enzyme by 50% (IC_{50}s). All enzyme assays were performed at least three times to confirm reproducibility.

Quinolone-mediated DNA cleavage assay. DNA cleavage assays were carried out as previously described (16, 20, 21, 31). The reaction mixture (total volume, 30 μl) contained DNA gyrase reaction buffer, recombinant DNA gyrase subunits (50 ng), supercoiled pBR322 DNA (300 ng), and 2-fold serially increasing concentrations of FQs. After incubation for 2 h at 30°C, 3 μl of 2% SDS and 3 μl proteinase K (1 mg/ml) were added to the reaction mixture. After subsequent incubation for 30 min at 30°C, reactions were stopped by the addition of 3 μl of 0.5 mM EDTA, 30 μl chloroform-isoamyl alcohol (24:1 mixture), and 3 μl of 10× DNA loading dye. The total reaction mixtures were subjected to electrophoresis in 0.8% agarose gels in 1× TBE buffer, followed by ethidium bromide staining. The extent of DNA cleavage was quantified with ImageJ, and the quinolone concentrations required to induce 25% of the maximum DNA cleavage (CC_{25}s) were determined.

Temperature sensitivity of *M. leprae* DNA gyrase. The reactions with mixtures (total volume, 30 μl) consisting of DNA gyrase reaction buffer, relaxed pBR322 DNA (300 ng), and recombinant DNA gyrase subunits

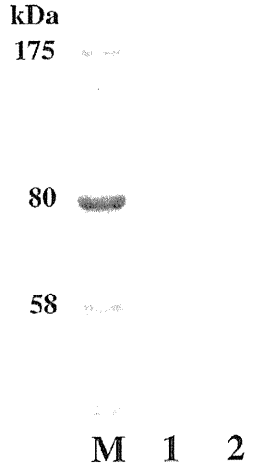

FIG 3 SDS-PAGE analysis of purified *M. leprae* DNA gyrases. The His-tagged recombinant DNA gyrases were overexpressed in *E. coli* and purified by Ni-NTA affinity resin chromatography. Lanes: M, protein marker (NEB); 1, WT GyrA; 2, GyrA-Ala91Val; 3, GyrA-Asp95Gly; 4, GyrA-Asp95Asn; 5, WT GyrB. Three hundred nanograms of each protein was loaded onto a 5 to 20% gradient polyacrylamide gel.

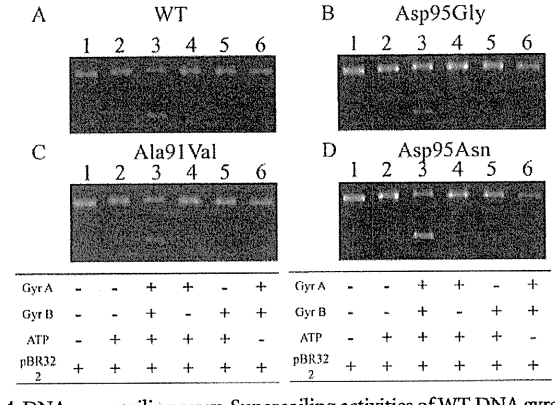

FIG 4 DNA supercoiling assay. Supercoiling activities of WT DNA gyrase (A) and DNA gyrases bearing GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) were analyzed. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) or GyrB (50 ng), or both. Lanes: 1, relaxed pBR322 alone; 2, relaxed pBR322 and ATP; 3, relaxed pBR322, ATP, GyrA, and GyrB; 4, relaxed pBR322, ATP, and GyrA; 5, relaxed pBR322, ATP, and GyrB; 6, relaxed pBR322, GyrA, and GyrB.

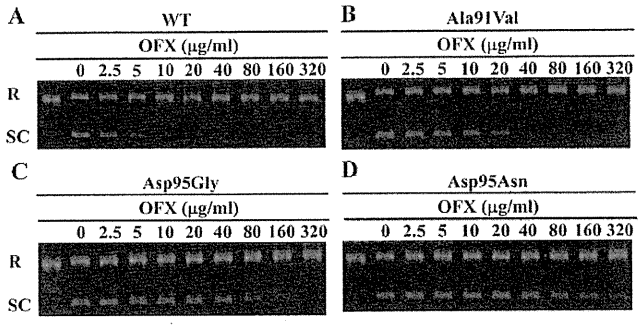

FIG 5 OFX-inhibited DNA supercoiling assay. Relaxed pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. Quinolone-inhibited supercoiling activity assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R and SC, relaxed and supercoiled pBR322 DNA, respectively.

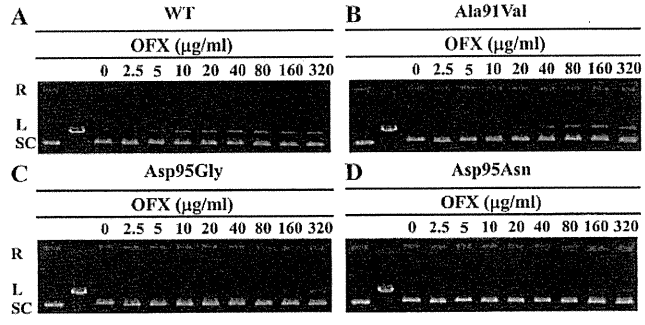

FIG 6 OFX-mediated DNA cleavage assay. Supercoiled pBR322 (0.3 μg) was incubated with GyrA (50 ng) and GyrB (50 ng) in the presence of the indicated concentration of OFX. DNA cleavage assay was performed with combinations consisting of WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D). R, L, and SC, relaxed, linear, and supercoiled pBR322 DNA, respectively.

(50 ng) were run at 25, 30, 33, 37, and 42°C for 1.5 h. Supercoiling activities of recombinant DNA gyrases were evaluated at each reaction temperature as described above.

RESULTS

Construction and purification of recombinant His-tagged GyrA and GyrB proteins. DNA fragments, including the *gyrA* and *gyrB* genes, were successfully amplified from *M. leprae* Thai-53 strain DNA and inserted in frame downstream of a T7 promoter in pET-20b (+). GyrA and GyrB were expressed as C-terminal hexahistidine-tagged proteins for ease of purification, as the His tag has been shown not to interfere with the catalytic functions of GyrA and GyrB (2, 3, 16, 20, 21, 31). Expressed recombinant WT and mutant DNA gyrase subunits were purified as 0.3 to 1.5 mg soluble His-tagged 80-kDa protein of GyrA and 75-kDa protein of GyrB from 500-ml cultures. The purity of the recombinant proteins was confirmed by SDS-PAGE (Fig. 3). All of the recombinant proteins were obtained with high purity (>95%).

DNA supercoiling activities. Combinations of GyrA WT, Ala91Val, Asp95Gly, or Asp95Asn and WT GyrB subunits were examined for DNA supercoiling activities using relaxed pBR322 DNA as a substrate in the presence or absence of ATP (Fig. 4). DNA supercoiling activities were observed in the presence of ATP and recombinant DNA gyrase subunits (Fig. 4A to D, lane 3), while neither subunit alone exhibited DNA supercoiling activity (Fig. 4A to D, lanes 4 and 5). In addition, no supercoiling activity was observed when ATP was omitted from the reaction mixture (Fig. 4A to D, lane 6).

Inhibition of DNA gyrase activities by FQs. The IC_{50}s of FQs were determined using the quinolone-inhibited DNA supercoiling assay (Fig. 5). Representative data showing the inhibitory effects of OFX against DNA gyrase are shown in Fig. 5, and data for other FQs are presented in Fig. S1 in the supplemental material. IC_{50}s of each FQ against WT and mutant DNA gyrases are summarized in Table 2. Each FQ showed dose-dependent inhibition, with IC_{50}s ranging from 0.4 to 262.3 μg/ml. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher IC_{50}s to quinolones (Table 2; Fig. 5; see Fig. S1 in the supplemental material) than WT gyrase (Table 2). These DNA gyrases also showed higher resistance than DNA gyrase bearing GyrA-Ala91Val, which was simultaneously analyzed as a positive control for resistance to FQs. Inhibitory effects of FQs were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

FQ-mediated DNA-cleavable complex formation. The CC_{25}s of FQs were determined. Figure 6 shows the result of a DNA cleavage assay using OFX, and Fig. S2 in the supplemental material presents the results using GAT, MXF, and SIT. Table 2 summarizes the CC_{25}s of each DNA gyrase. DNA gyrases bearing GyrA-Asp95Gly and -Asp95Asn showed significantly higher CC_{25}s to quinolones than WT gyrase (Table 2). These DNA gyrases also showed higher CC_{25}s than gyrase bearing GyrA-Ala91Val (Table 2). Effects on cleavable complex formation were ranked SIT > GAT > MXF > OFX in all DNA gyrases.

Temperature sensitivity of *M. leprae* DNA gyrase. Figure 7 shows the effects of temperature on DNA gyrase activities. The highest DNA supercoiling activities were observed at 33°C in all DNA gyrases. WT and GyrA-A91V DNA gyrases showed reduced DNA supercoiling activities at 37°C, whereas Gyr-Asp95Gly and Asp95Asn DNA gyrases maintained activities comparable to those at 33°C. No supercoiling activities were observed in any of the DNA gyrases at 42°C.

TABLE 2 IC_{50}s and CC_{25}s of FQs against WT and mutant DNA gyrases[a]

Drug	IC_{50}				CC_{25}			
	WT	Ala91Val	Asp95Gly	Asp95Asn	WT	Ala91Val	Asp95Gly	Asp95Asn
OFX	6.8 ± 0.8	39.4 ± 15.5 (5.8)	161.2 ± 44.2 (23.7)	262.3 ± 105.8 (38.6)	7.3 ± 0.5	75.5 ± 16.8 (10.1)	240.5 ± 30.7 (32.1)	269.5 ± 76.5 (35.9)
GAT	1.0 ± 0.1	3.1 ± 0.7 (3.1)	7.5 ± 1.6 (7.5)	13.8 ± 1.6 (13.8)	1.1 ± 0.2	4.3 ± 0.2 (3.9)	15.6 ± 3.6 (14.2)	13.5 ± 3.1 (12.3)
MXF	1.5 ± 0.3	5.2 ± 1.0 (3.5)	21.5 ± 4.7 (14.3)	34.7 ± 3.1 (23.1)	1.0 ± 0.1	4.5 ± 1.0 (4.5)	25.5 ± 3.7 (25.5)	20.8 ± 5.0 (20.8)
SIT	0.4 ± 0.0	1.0 ± 0.2 (2.5)	2.2 ± 0.5 (5.5)	3.9 ± 0.6 (9.8)	0.3 ± 0.0	0.9 ± 0.0 (3.0)	2.2 ± 0.6 (7.3)	2.3 ± 0.4 (7.7)

[a] IC_{50}s and CC_{25}s are in μg/ml, and data in parentheses represent the fold increase compared to WT.

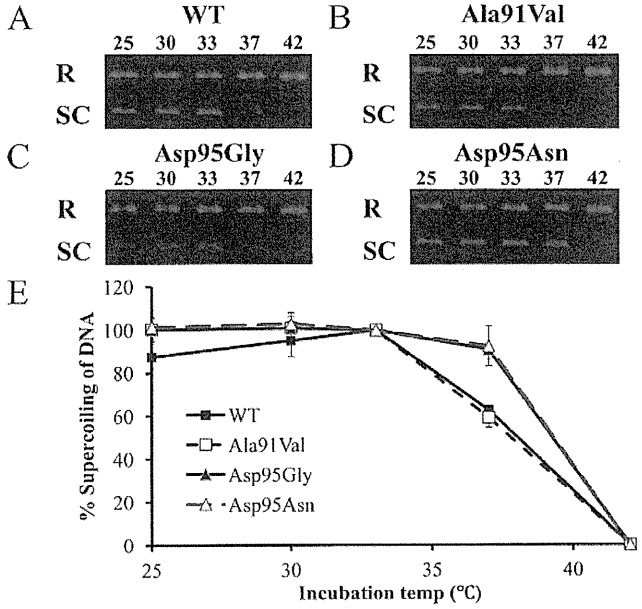

FIG 7 Temperature-dependent DNA supercoiling activity of DNA gyrases. Relaxed pBR322 (0.3 μg) was incubated with WT GyrB-WT GyrA (A), GyrA-Ala91Val (B), GyrA-Asp95Gly (C), and GyrA-Asp95Asn (D) at the temperatures (in °C) indicated above the lanes. The proportion of supercoiled DNA compared to that of WT DNA gyrase at 33°C is plotted for each incubation temperature.

DISCUSSION

Mutations in the *gyrA* gene of quinolone-resistant *M. leprae* clinical isolates have predominantly been reported at codon 91, and a smaller number have been reported at codon 89 (4, 19, 24, 26, 40). Amino acid substitutions at other positions have not been reported, in strong contrast to the substitutions reported in *M. tuberculosis*, with predominant mutations in codon 94 (1, 7, 9, 10, 32, 34, 39), equivalent to codon 95 in *M. leprae* (Fig. 1). This study aimed to obtain basic data for the rapid detection of FQ-resistant leprosy by elucidating the correlation between mutations at codon 95 and quinolone resistance.

To explain the discrepancy described above, we first hypothesized that amino acid substitution at position 95 in GyrA of *M. leprae* has less of an influence on FQ resistance. Hence, we carried out a quinolone-mediated supercoiling activity inhibition assay and DNA cleavage assay at 30°C, the optimal temperature of *M. leprae* growth, using recombinant DNA gyrases and calculated IC_{50}s and CC_{25}s of four FQs, OFX, MXF, GAT, and SIT. The DNA gyrase bearing GyrA-Ala91Val, used as a control, exhibited resistance, having approximately 2- to 10-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase, as has been reported previously (20, 21). Interestingly, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn showed resistance, having approximately 5- to 40-fold higher IC_{50}s and CC_{25}s of FQs than WT DNA gyrase (Table 2). Namely, amino acid substitution from Asp to Gly or Asn at position 95 added higher resistance to DNA gyrase than that from Ala to Val at position 91. This was similar to the observation in *M. tuberculosis* (2, 3). These results suggested that a possible property of Asp95Gly and Asp95Asn amino acid substitutions in GyrA is to give higher FQ resistance to DNA gyrase in *M. leprae*.

We then hypothesized that amino acid substitutions at position 95 place a disadvantage on the enzymatic property of DNA gyrases, especially lower or abolished activity at higher temperatures, and thus, we conducted a DNA supercoiling assay at various temperatures: 25, 30, 33, 37, and 42°C. DNA supercoiling activities of WT and GyrA-Ala91Val DNA gyrase showed a similar temperature dependence, with the highest activity being at 25 to 33°C, reduced activity occurring at 37°C, and activity being completely abolished at 42°C. In contrast, DNA gyrases bearing GyrA-Asp95Gly or -Asp95Asn maintained their activities even at 37°C. Our hypothesis was rejected by these data.

The influence of the clear usage of FQs for the treatment of leprosy and tuberculosis might solve this question. For leprosy patients with a single lesion, a single application of 400 to 600 mg of OFX is used. For the treatment of MDR leprosy, two or three doses of 400 to 600 mg in combination with first-line drugs DDS and RIF (11) are applied. In contrast, for tuberculosis, OFX is taken twice daily at 400 mg each time with first-line drugs such as isoniazid and rifampin for several months (11, 36). The maximum serum concentration (C_{max}) of OFX has been reported to show a dose-dependent increase. The C_{max}s achieved with administration of 100 mg, 300 mg, and 600 mg of OFX in humans were 1.00, 2.81, and 6.81 μg/ml, respectively (14). The blood concentration of OFX is low in leprosy patients and is maintained at a high level in tuberculosis patients because of the treatment regimen. Thus, *M. leprae* carrying DNA gyrase with lower resistance, such as GyrA-Ala91Val, might be predominantly selected for various reasons in leprosy patients, whereas GyrA-Asp94Gly or -Asp94Asn is predominantly found in *M. tuberculosis*-infected patients (1, 7, 9, 10, 32, 34, 39); however, the possible emergence in the future of highly FQ-resistant *M. leprae* having an amino acid substitution at position 95 cannot be rejected, especially when MDR leprosy is treated by repeated administration of FQs.

We investigated the inhibitory effects of OFX, GAT, MXF, and SIT against WT and mutant DNA gyrases. IC_{50}s of OFX for WT and GyrA-Ala91Val, -Asp95Gly, and -Asp95Asn DNA gyrases were 6.8, 39.4, 161.2, and 262.3 μg/ml, respectively (Table 2). The order of FQ inhibitory activity was SIT > GAT > MXF > OFX. OFX does not have the ability to inhibit *M. leprae* with DNA gyrase carrying GyrA-Asp95Gly or -Asp95Asn. The IC_{50} of SIT was the lowest of the four quinolones, with IC_{50}s of 0.4, 1.0, 2.2, and 3.9 μg/ml for WT, A91V, D95G, and D95N gyrases, respectively. As the C_{max}s of OFX, GAT, MXF, and SIT at the 100-mg dosage were determined in clinical trials to be 1.00, 0.87 to 5.41, 4, and 0.3 to 1.9 μg/ml, respectively (14, 27, 28, 30), SIT might strongly inhibit *M. leprae* carrying GyrA-Ala91Val DNA gyrase and be a promising candidate for the treatment of the majority of cases of FQ-resistant leprosy.

In conclusion, we revealed the contribution of the GyrA-Asp95Gly and -Asp95Asn amino acid substitutions to FQ resistance in *M. leprae* by an *in vitro* assay. This suggested the possible emergence in the future of FQ-resistant *M. leprae* carrying GyrA with these amino acid substitutions, although further analysis is needed to clarify a direct relationship to *in vivo* resistance. Hence, we would like to propose analysis for these amino acid substitutions to detect FQ-resistant leprosy.

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