38) Honore N, Roche PW, Grosset JH, Cole ST. A method for rapid detection of rifampicin-resistant isolates of *Mycobacterium leprae*. Lepr Rev 72: 441-448, 2001.
39) 儀同政一、斉藤肇. 治らい薬研究の現状. Jpn J Leprosy 64: 174-187, 1995.
40) Shepard CC. A kinetic method for the study of activity of drugs against *Mycobacterium leprae* in mice. Int J Lepr 35: 429-435, 1967.
41) Buddemeyer E, Hutchinson R, Cooper M. Automatic Quantitative Radiometric assay of bacterial metabolism. Clin Chem 22: 1459-1464, 1976.
42) Franzblau SG. Drug susceptibility testing of Mycobacterium leprae in the BACTEC 460 system. Antimicrob Agents Chemother 33: 2115-2117, 1989.
43) 山崎利雄. 生物発光を用いた結核菌の迅速薬剤感受性測定. 臨床検査 47：197-199, 2003.
44) Gidoh M, Tsutsumi S. Activity of sparfloxacin against *Mycobacterium leprae* inoculated into footpads of nude mice. Lepr Rev 63: 108-116, 1992.
45) Rosalind M, Van Landingham, Walker LL, O'Sullivan JF, Shinnick TM. Activity of phenazine analogs against *Mycobacterium leprae*. Int J Lepr 61: 406-414, 1993.
46) Gidoh M, Tsutsumi S, Yamane T, Yamashita K, Hosoe K, Hidaka T. Bactercidal action at low doses of a new rifamycin derivative, 3'-hydroxy-5'- (4-isobutyl-1-piperazinyl) benzoxazinorifamycin(KRM-1648) on *Mycobacterium leprae* inoculated into footpads of nude mice. Lepr Rev 63: 319-328, 1992.
47) Yoder LJ, Jacobson RR, Hastings RC. The activity of rifabutin against *Mycobacterium leprae*. Lepr Rev 62: 280-287, 1991.
48) Bryskier A. Ketolides-telithromycin, an example of a new class of antibacterial agents. Clin Infect 6: 661-669, 2000.
49) Williams DL, Gillis TP, Portaels F. Geographically distinct isolates of *Mycobacterium leprae* exhibit no genotypic diversity by restriction fragment length polymorphism analysis. Mol Microbiol 4: 1653-1659, 1990.
50) Matsuoka M, Maeda S, Kai M, Nakata N, Chae GT, Gillis TP, Kobayashi K, Izumi S, Kashiwabara Y. *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. Int J Lepr 68: 121-128, 2000.
51) Shin YC, Lee H, Lee H, Walsh GP, Kim JD, Cho SN. Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in train differentiation. J Clin Microbiol 38: 4543-4538, 2000.
52) Sonoda S, Li HC, Cartier L, Nunez L, Tajima K. Ancient HTLV type 1 provirus DNA of Andean mummy. AIDS Res Hum Retroviruses 16: 1753-1756, 2000.
53) Sugimoto C, Kitamura T, Guo J, Al-Ahdal MN, Shchelkunov SN, Otova B, Ondrejka P, Chollet JY, El-Safi S, Ettayebi M, Gresenguet G, Kocagoz T, Chaiyarasamee S, Thant KZ, Thein S, Moe K, Kobayashi N, Taguchi F, Yogo Y. Typing of urinary JC virus DNA offers a novel means of tracing human migrations. Proc Natl Acad Sci USA 94: 9191-9196, 1997.
54) Covacci A, Telford JL, Giudice GD, Parsonnet J, Rappuoli R. *Helicobacter pylori* virulence and genetic geography. Science 284: 1328-1333, 1999.
55) Hanihara K. Dual structure model for the population history of the Japanese. Japan Review 2: 1-33, 1991.
56) Greenberg JH, Turner II CG, Zegura SL. The settlement of the Americas: A comparison of the linguistic, dental, and genetic evidence. Curr Anthropol 27: 477-497, 1986.
57) Matsuoka M, Zhang L, Budiawan T, Saeki K, Izumi S. Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. J Clin Microbiol 42: 741-745, 2004.
58) van Beers SM, Izumi S, Madjid B, Maeda Y,

Day R, Klatser PR. An epidemiological study of leprosy infection by serology and polymerase chain reaction. Int J Lepr 62: 1-9, 1994.

59) Abe M, Ozawa T, Minagawa F, Yoshino Y. Immunoepidemiological studies on studies on substantial infection in leprosy II. Geographical distribution of seropositive responders with special reference to their possible source of infection. Int J Lepr 59: 162-168, 1990.

60) Groathouse NA, Rivoire B, Kim H, Lee H, Cho SN, Brennan PJ, Vissa VD. Multiple polymorphic loci for molecular typing of strains of *Mycobacterium leprae*. J Clin Microbiol 42: 1666-1672, 2004.

61) Truman RW, Fontes AB, de Miranda AB, Suffys P, Gillis TP. Genotypic variation and stability of four variable-number tandem repeats and their suitability for discriminating strains of *Mycobacterium leprae*. J Clin Microbiol 42: 2558-2562, 2004.

Current advances in the leprosy research activities

Masahiko Makino[1)]*, Koichi Suzuki[1)], Yasuo Fukutomi[1)],Yasuko Yamashita[1)], Yumi Maeda[1)], Yuji Miyamoto[1)], Tetsu Mukai[1)], Noboru Nakata[1)], Masanori Kai[1)], Toshio Ymazaki[1)], Msaichi Gidoh[2)], Masanori Matsuoka[2)]

1)Department of Microbiology,

2)Department of Bioregulation, Leprosy Research Center, National Institute of Infectious Diseases, Tokyo.

[Received: 12 Nov. 2004]

Key words : Leprosy, *Mycobacterium leprae*, Topics

Due to the advent of multi-drug therapy (MDT) recommended by the WHO, for the treatment of leprosy, presently, leprosy is regarded as a "curable disease". The number of new cases in Japan is relatively very low, due to which the disease is likely to be neglected, but on scientific grounds, there is a necessity to perform in depth studies. Leprosy caused by *M. leprae* is still unclear on various aspects including transmission, immunology, nerve damage etc. Here we introduce the recent advances in the field of basic leprosy research.

*Corresponding author :
Department of Microbiology, Leprosy Research Center,
National Institute of Infectious Diseases, Tokyo, 189-0002,
Japan.
TEL: 042-391-8211 FAX: 042-391-8212
E-mail: mmaki@nih.go.jp

Jpn.J.Leprosy 74, 43-48(2005)

新規フルオロキノロンWQ-3345およびWQ-3402の抗らい菌活性

儀同　政一*
国立感染症研究所ハンセン病研究センター

〔受付：2004年10月5日、掲載決定：2004年11月15日〕

キーワード：フルオロキノロン、多剤併用療法、Buddemeyer法、マウス足蹠法

新規フルオロキノロンWQ-3345およびWQ-3402の抗らい菌活性をBuddemeyer法とヌードマウス足蹠法で検討した。Buddemeyer法では、RFP＞WQ-3402＞sparfloxacin（SPFX）＞gatifloxacin（GFLX）＞WQ-3345＞levofloxacinの順で、WQ-3402の抗らい菌活性はSPFXを凌ぎフルオロキノロン中最強であったが、WQ-3345はGFLXより弱かった。他方、ヌードマウス足蹠法ではWQ-3345とWQ-3402は何れも20mg/kg投与でらい菌増殖の不完全抑制を示すに留まった。さらに、Buddemeyer法で抗らい菌活性の強かったWQ-3402を50mg/kgに増量してヌードマウス足蹠法で検討したところ、20mg/kg投与の場合と同様に不完全抑制を認めたに過ぎなかった。以上の成績よりWQ-3402は、Buddemeyer法では強い*in vitro*抗らい菌活性を示すものの、ヌードマウス足蹠法での*in vivo*抗らい菌活性はSPFXのそれに比べては著しく劣ることが明らかになった。

はじめに

ハンセン病の治療は、多剤併用療法（multidrug therapy, MDT）の普及により有病率は低下したが、今なお新患は60万人を超えている[1]。ハンセン病の治療は、ハンセン病治療指針[2]でも少菌型で6ヶ月、多菌型で1年以上と長い治療期間を要すること、また薬剤耐性菌の増加などの問題が生じている。rifampicin（RFP）耐性に対しRFPと同様な殺菌的薬剤はフルオロキノロンのみで、保険適用薬であるofloxacin（OFLX）の、*in vitro, in vivo*活性は弱く[3]、最も強い抗らい菌活性を持つsparfloxacin（SPFX）[4, 5]は、光毒性[6]に問題がある。また、優れた抗らい菌活性を保持しつつ光毒性を弱めた6-fluoro-8-methoxy quinolone, gatifloxacin[7, 8]（GFLX）は、低血糖、高血糖[9, 10]の発症の克服が今後解決すべき課題として残されている。

WQ-3345[11, 12]は7位にアミノ基を、WQ-3402[11]は、7位にメチルアミノ基、1位に5-アミノ-2，4-ジフルオロフェニル基を導入した新規フルオロキノロンでグラム陽性菌及びグラム陰性菌に優れた抗菌活性を有するとともに、ciprofloxacin耐性methicillin耐性黄色ブドウ球菌にも優れた抗菌活性を示す薬剤として湧永製薬で開発された[11, 12]。フルオロキノロンの光毒性は、SPFX＞CPFX＞LVFX＞GFLX＞WQ-3402,WQ-3345の順で、WQ-3402とWQ-3345はキノリン骨格の8位にCH_3基を導入することで光毒性を軽減し

*Corresponding author :
国立感染症研究所ハンセン病研究センター
〒189-0002　東村山市青葉町4-2-1
Tel：042-391-8211　Fax：042-394-9092
E-mail：m-gidoh@nih.go.jp

た抗菌薬である[11, 12]。薬剤耐性化の防止または遅延、副作用軽減、治療期間の短縮に対応するため、光毒性が弱く既存の同系薬より強い抗菌活性を持つ新規フルオロキノロンWQ-3402とWQ-3345の抗らい菌活性をBuddemeyer法[13, 14]とヌードマウス足蹠法で検討した。

材料と方法

1）抗菌薬

WQ-3402：1-（5-amino-2, 4-difluorophenyl）-6-fluoro-8-methyl-7-methylamino-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid ethanolamine salt（湧永製薬）、WQ-3345：7-amino-1-（5-amino-2, 4-difluorophenyl）-6-fluoro-8-methyl-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid ethanolamine salt（湧永製薬）、SPFX（大日本製薬）、GFLX（杏林製薬）、levofloxacin（LVFX, 第一製薬）は、各製薬会社から原末の提供を受けた。rifampicin（RFP）は、市販品（和光純薬）を用いた。

Buddemeyer法[13, 14]に用いた抗菌薬WQ-3402, WQ-3345, GFLX, LVFX, SPFXは0.2N-NaOHで、RFPはdimethyl sulfoxideで溶解後、phosphate buffered saline（PBS）（pH 7.0）で最終濃度が8.0, 2.0, 0.5, 0.125 μg/mlになるよう調整した。

ヌードマウス足蹠法に用いた薬剤は、実験1ではSPFXは10 mg/kg, WQ-3345は10, 20mg/kg, WQ-3402は10, 20mg /kg、実験2ではSPFXは10 mg/kg, WQ-3402は30, 40, 50 mg/kgになるよう0.001% Tween80含有PBS（pH7.0）で調整した。

2）接種菌の精製

Mycobacterium leprae（Thai 53株）を接種後11ヶ月目のヌードマウス（BALB/c-*nu/nu*、雌、5週令、日本クレア）の両後肢足蹠を切除後、3%ヨード液、70%エタノールで消毒しPBS（pH 7.0）で洗浄後、足蹠乳剤を遠心操作により集菌後、0.05%トリプシン（最終濃度）で処理、1%水酸化ナトリウム液（最終濃度）で処理後集菌精製[15]した。Shepard法[16]で菌数計算後、PBS（pH 7.0）で$2x10^8$/mlに希釈した。

3）動物

ヌードマウス（BALB/c-*nu/nu*、雌、5週令）は、日本クレアから購入し、ビニールアイソレータ（三基科学工芸株式会社）中で滅菌したマウス用耐圧固形飼料MB-6E（船橋農場）で飼育した。

4）抗らい菌活性の測定

Buddemeyer法：4 mlガラスバイアル中に7H12培地 500 μl、らい菌（$2x10^8$/ml）100 μl、薬剤（最終濃度8.0, 2.0, 0.5, 0.125 μg /ml）300 μlを加えよく混合する。このガラスバイアルのキャップを緩く締め、32℃の炭酸ガス培養器中で4日間培養後、^{14}C-パルミチン酸（57mCi/mmol）100 μl（1 μCi）を加え混合後、再びキャップを緩く締めたガラスバイアルを、NaOHとシンチレータで処理後乾燥したろ紙片を入れたプラスチックバイアルに入れキャップを強く締める。更に32℃の培

Table 1. *In vitro* anti-*M. leprae* activities of various fluoroquinolones measured by Buddemeyer method

Drugs	μg/ml	$\chi \pm \sigma$ (cpm)	Inhibition(%)[b]
Control		40,193±748[a]	·····
WQ-3345	8.0	7,599±633	81.1
	2.0	18,582±983	53.8
	0.5	27,243±254	32.2
	0.125	32,355±634	19.5
WQ-3402	8.0	5,443±57	86.5
	2.0	11,474±772	71.5
	0.5	23,041±211	42.7
	0.125	26,942±705	33.0
LVFX	8.0	14,050±633	65.0
	2.0	21,031±406	47.7
	0.5	30,637±804	23.8
	0.125	33,176±1,494	17.5
SPFX	8.0	8,750±158	78.2
	2.0	12,403±327	69.1
	0.5	22,175±438	44.8
	0.125	30,580±440	23.9
GFLX	8.0	11,614±1,383	71.1
	2.0	15,295±519	61.9
	0.5	25,888±797	35.6
	0.125	29,979±640	25.4
RFP	8.0	9,055±231	77.5
	2.0	10,585±562	73.7
	0.5	11,699±647	70.9
	0.125	21,861±508	45.6

a. Date are given as the mean±standard deviation of triplicate samples.

b. [(specimen of control group(c)-that of a drug group)/c] x 100 (%)

養器で7日間培養を継続し、発生した$^{14}CO_2$量を液体シンチレーションカウンターで測定し、WQ-3345, WQ-3402の抗らい菌活性をLVFX, GFLX, SPFX, RFPと比較検討した。

マウス足蹠法：1群10匹のヌードマウス（BALB/c-*nu/nu*, 雌, 5週令, 日本クレア）の両後肢足蹠にらい菌（$2x10^8$/ml）の0.05mlを接種した。菌接種後60日から152日の93日間、ステンレスカテーテルで薬剤を週5日毎日経口投与した。接種後8ヶ月から11ヶ月まで4回、2匹4足蹠内のらい菌数を計数し、1足蹠当たりの平均菌数を求め、各薬剤の抗らい菌活性を求めた。

結　果

Buddemeyer法の結果を表1に示した。

各抗菌薬2μg/mlでの抗らい菌活性はRFP＞WQ-3402＞SPFX＞GFLX＞WQ-3345＞LVFXで、WQ-3402にフルオロキノロン中最も強いSPFXを凌ぐ強い抗らい菌活性を認めたが、WQ-3345はGFLXより弱かった。

ヌードマウス足蹠法の結果を図1・2に示した。対照薬のSPFXは10 mg/kgでヌードマウス足蹠内のらい菌の増殖を完全抑制したが、WQ-3345とWQ-3402は20mg/kgでも不完全抑制を示すに留まった（実験1）。

Buddemeyer法で抗らい菌活性の強いWQ-3402は、30, 40, 50mg/kgまで増量したが不完全抑制であった（実験2）。

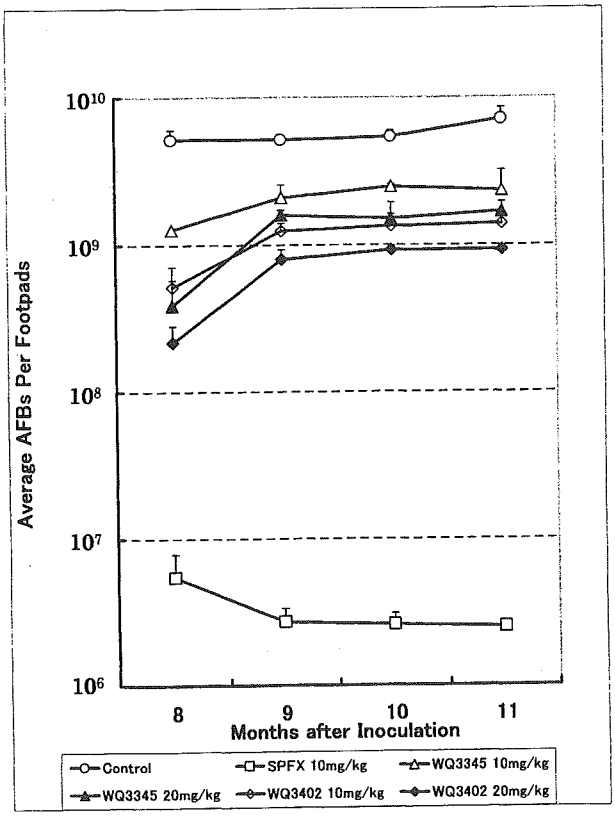

Fig. 1. Nude mice were infected with *M. leprae*, strain Thai-53, by inoculating $1x10^7$ bacilli into each of hind footpads, followed by oral treatment with WQ-3402, WQ-3345 or SPFX, given once a day, 5 times weekly, between days 60 to 152 postinfection at a daily dose of 10 or 20 mg/kg. At 8,9,10 and 11 months after inoculation, the mice were killed and the number of AFBs in the 4 hind footpads of 2 mice was counted according to the method of Shepard.

考　察

MDT（DDS,B663,RFP）に用いられている薬剤を除き、優れた抗らい菌活性を持つ薬剤は、マクロライド系ではclarithromycin, テトラサイクリン系ではminocycline、フルオロキノロン系ではSPFX、GFLX、LVFX、OFLXなどで、臨床で使用できる薬剤は少ない。抗らい菌活性の弱い

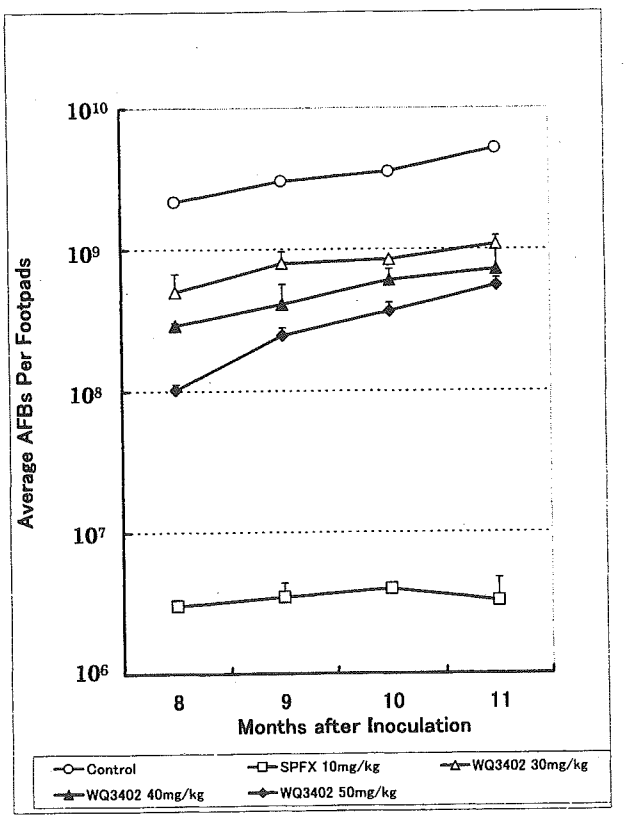

Fig. 2. Antibacterial activity at high doses of WQ-3402 against *M. leprae* inoculated into footpads of nude mice. Inoculation of *M. leprae* and counting of AFBs were performed according to the methods shown in the legend to Figure 1.

OFLXの低用量投与が行われた結果、OFLX耐性菌が増加してきている。また、ハンセン病の治療に使用されている抗らい菌薬の中で殺菌作用を持つのは、RFPとフルオロキノロンのみである。臨床からは抗らい菌活性が強く、光毒性などの副作用の少ないフルオロキノロンの開発が求められている。

新規フルオロキノロンWQ-3345の抗らい菌活性は、Buddemeyer法とヌードマウス足蹠法ともSPFX及びGFLXより弱かった、WQ3402は、Buddemeyer法では、フルオロキノロン中最強のSPFXより強い抗らい菌活性を持っていたが、ヌードマウス足蹠法は50 mg/kgでも不完全抑制で、SPFXやGFLXより弱いという結果になった。

ハンセン病は、らい菌により末梢神経や皮膚などが主に侵される慢性感染症であることから、血中濃度が持続的[17]で良好な組織移行性、特に皮膚内移行率の高い[18] SPFXは、*in vivo*法で強い抗らい菌活性を示すと考えられる。さらにWQ-3402（$T_{1/2}$=2.7h）[11]は、SPFX（Dogs, per os, $T_{1/2}$=8.0h）[19]やGFLX（$T_{1/2}$=6.2h）[20]と比べ血中半減期が短く、組織移行性や蛋白結合率など体内動態が劣ることから、*in vivo*で強い抗らい菌活性は得られなかったものと考えられる[11, 12]。

謝　辞

WQ-3345,WQ-3402をご提供いただいた湧永製薬株式会社と本研究にご協力いただきました湧永製薬創薬研究所の大下嘉弘先生に深謝致します。

文　献

1）World Health Organization Leprosy Elimination Project, Status Report 2003. WHO, Geneva 2004.

2）後藤正道、石田　裕、儀同政一、長尾榮治、並里まさ子、石井則久、尾崎元昭：ハンセン病治療指針．日本ハンセン病学会雑誌、69: 157-177,2000.

3）Gelber, R, H., Iranmanesh, A., Murray, L., Siu, P. and Tsang,M.: Activities of various quinolone antibiotics against *Mycobacterium leprae* in infected mice. Antimicrob. Agent Chemother. 36: 2544-2547,1992.

4）Gidoh, M. and Tsutsumi, S. : Activity of sparfloxacin against *Mycobacterium leprae* inoculated into footpads of nude mice. Lepr. Rev. 63: 108-116. 1992.

5）Gertrude P. C., Bernadette Y. G.,Virginia E. C. Jocelyn B. L., Claribel L. J. Manuela Luisa R. P., Jocelyn B. L. and Franzblau S. G.: Clinical trial of sparfloxacin for lepromatous leprosy. Antimicrob. Agents Chemother. 38: 61-65. 1994.

6）Engler, M., Rusing, G., Sorgel, F. and Holzgrabe, U.: Defluorinated sparfloxacin as a new photoproduct identified by liquid chromatography coupled with UV detection and tandem mass spectrometry. Antimicrob. Agents Chemother. 42: 1151-1159. 1998.

7）Hosaka, M., Yasue, T., Fukuda, H., Tomizawa, H., Aoyama, H. and Hirai, K: *In vitro* and *in vivo* antibacterial activities of AM-1155, a new 6-fluoro-8-methoxy quinolone. Antimicrob. Agents Chemother. 36: 2108-2117.1992.

8）Kusajima, H., Ishida, R. and Uchida, H. : Phototoxicity of gatifloxacin, a new quinolone, and its related drugs in guinea pigs. Jpn. Pharmacol. Ther. 26: 1655-1660, 1998.

9）Baker S. E. and Hangii, M. C.: Possible gatifloxacin-induced hypoglycemia. Ann. Pharmacother. 36: 1722-1726. 2002.

10）Biggs. W. S.: Hypoglycemia and hyperglycemia associated with gatifloxacin use in elderly patients. J. Am. Board. Fam. Pract. 16: 455-457, 2003.

11）Kuramoto Y., Ohshita Y., Amano H., Hirano Y., Hayashi N., Aoki S., Niino Y. and Yazaki A.: Structure-activity relationships of novel acid, 7-amino or 7-alkylamino-1-（5-amino-2,4-difluorophenyl）-8-methylquinolones. 40th Interscience Conference on Antimicrobial Agents and Chemotherapy. 2000（Toront,Canada）.

12) Hayashi N., Ohshita Y., Amano H., Hirao Y., Niino Y. and Yazaki A.: WQ-3330 and WQ-2942; Structure-activity relationships of novel 8-methl quinolones containing an aminophenyl group at the 1-position. 39th Interscience Conference on Antimicrobial Agents and Chemotherapy. 1999 (San Francisco,USA).

13) Buddemeyer, E., Hutchinson, R. and Cooper, M.: Automatic quantitative radiometric assay of bacterial metabolism. Clin. Chem. 22: 1459-1464, 1976.

14) Franzblau, S. G.: Oxidation of palmitic acid by *Mycobacterium leprae* in an axenic medium. J. Clin. Microbiol. 26: 18-21, 1988.

15) 中村昌弘：らい菌接種ヌードマウス足蹠乳剤内迷入雑菌の除去. 日本ハンセン病学会雑誌, 64: 47-50, 1994.

16) Shepard, C. C. and D. H. McRae.: A method for counting acid-fast bacteria. Int. J. Lepr. 36: 78-82. 1968.

17) 中島光好、金丸光隆、植松俊彦、滝口祥令：ピリドンカルボン酸系抗菌剤sparfloxacinの臨床第Ⅰ相試験. 臨床医薬, 7: 1639-1684, 1991.

18) 秋山尚範、鳥越利加子、山田　琢、阿部能子、下江敬生、神崎寛子、荒田次郎、赤木理、山本康生、池田政身：ラットにおける各種抗生物質の皮膚内移行について. 日皮会誌 101: 943-950, 1991.

19) 中村信一、黒部暢之、大植富夫、橋本昌久、清水當尚：Sparfloxacinの各種動物における吸収、分布および排泄.　日本化学療法学会雑誌、39, 123-130, 1991.

20) 大塚　毅、石川紅美、町田正明、草嶋久生、石田了三、内田　広：新キノロン系抗菌薬gatifloxacin及びその光学異性体の体内動態. Ⅰ. 各種実験動物における体内動態. 日本化学療法学会雑誌、47: 112-123, 1999.

In vitro and *in vivo* activities of newly synthesized fluoroquinolnes WQ-3345 and WQ-3402 against *Mycobacterium leprae*

Masaichi Gidoh*

Leprosy Research Center, National Institute of Infectious Diseases

[Received: 5 Oct. 2004 / Accepted: 15 Nov. 2004]

Key words : fluoroquinolone, multidrug therapy, Buddemeyer method, mouse footpad method

Activities of newly synthesized fluoroquinolnes WQ-3345 and WQ-3402 against *M.leprae* were measured by using the Buddemeyer method. The % inhibition of the examined drugs for *M. leprae* was in the order of RFP＞WQ-3402＞SPFX＞GFLX＞WQ-3345＞LVFX. The anti-*M. leprae* activity of WQ-3402 was found to be strongest in these five fluoroquinolones when examined by this method, and the activity of WQ-3345 was weaker than that of GFLX.

The anti-*M.leprae* activities of WQ-3345 and WQ-3402 were measured by a mouse footpad method using nude mice. The inhibitory effects on the growth of *M. leprae* inoculated into the footpads were found to be incomplete after orally administered with WQ-3345 or WQ-3402 respectively at dosages of 10 and 20 mg/kg, and the incomplete inhibition was again found even at a dosage of 30, 40 or 50 mg/kg in the latter.

*Corresponding author :
Leprosy Research Center, National institute of Infectious Diseases4-2-1, Aoba-cho, Higashimurayama-shi, Tokyo, 189-0002, Japan
Tel:+81-42-391-8211 Fax: +81-42-394-9092
E-mail: m-gidoh@nih.go.jp

ELSEVIER

FEMS Microbiology Letters 243 (2005) 311–315

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Polymorphism in the *rpoT* gene in *Mycobacterium leprae* isolates obtained from Latin American countries and its possible correlation with the spread of leprosy

Masanori Matsuoka [a,*], Liangfen Zhang [a], Mary Fafutis Morris [b], Pedro Legua [c], Carlos Wiens [d]

[a] *Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002 Tokyo, Japan*
[b] *CIINDE, Department of Physiology, CUCS, University of Guadalajara, Federalismo, Norte, 3102 Guadalajara, Jalisco 44220, Mexico*
[c] *Alexander Von Humboldt Tropical Disease Institute, Peruana Cayetano Heredia University, A.P. 4314 Lima, 100, Peru*
[d] *Mennonita K81 Hospital D.d.C. 166, Asuncion, Paraguay*

Received 6 September 2004; received in revised form 25 November 2004; accepted 12 December 2004

First published online 7 January 2005

Edited by M. Mitsuyama

Abstract

The genotypes of *Mycobacterium leprae* isolates originating from Mexico, Peru and Paraguay were analysed for the polymorphism of short tandem repeats in the *rpoT* gene. The genotype with four copies of the six-base tandem repeats in the *rpoT* gene was prominently predominant in Mexico, but the genotype of all isolates from Peru and Paraguay contained three copies of the six-base tandem repeats. These obvious different distributions might reflect the spread of leprosy by the different strains of *M. leprae* harboured by the various human races that moved to the American continent, as has been demonstrated in other infectious diseases.

Keywords: Leprosy; *Mycobacterium leprae*; *rpoT* gene; Genotyping; Latin American countries

1. Introduction

Genotyping of causative agents of infectious diseases is essential for epidemiological analysis of transmission. Until recently, it was believed that there is no genomic diversity among *Mycobacterium leprae* isolates, a useful feature for the analysis of leprosy transmission. However, in 2000, two independent studies reported some genomic polymorphisms suitable for genotyping of *M. leprae* [1,2]. Since then other polymorphic genomic regions which might be applicable for genotyping have been also revealed [3]. One of them is the different copy number of six-base tandem repeats in the *rpoT* gene of *M. leprae* [1]. Specifically, *M. leprae* isolates can be divided into two sub-genotypes based on the polymorphism in the *rpoT* gene, which contains either four or three copies of a six-base tandem repeat. Our previous study showed the prominent distribution of the genotype with four copies of the six-base tandem repeats in the *rpoT* gene in the East Asia, Korea and the main island of Japan [1]. For some infectious diseases, the distribution of microorganisms with specific genotype in specific geographical areas has been noticed to correspond with the worldwide movement of human races

* Corresponding author. Tel.: +81 42 391 8211; fax: +81 42 394 9092.
E-mail address: matsuoka@nih.go.jp (M. Matsuoka).

0378-1097/$22.00
doi:10.1016/j.femsle.2004.12.031

[4–8]. Studies of the genomic similarity of microorganisms, other than *M. leprae*, suggest that disease strains were distributed in Korea and the main island of Japan by one of the Mongoloid groups that migrated into these areas [6,9–11]. Our data with *M. leprae* [1] substantiated this observation.

Intrigued with this, it was of interest to determine the genotypes of *M. leprae rpoT* gene that exist in the American continent. Because of the complicated history of invasion, conquest and migration, the American continent consists of multiple races. The genotypes of *M. leprae rpoT* gene distributed in Latin American countries are investigated in this study.

2. Materials and methods

2.1. Source of M. leprae isolates and preparation of genomic DNA

A total of 72 *M. leprae* isolates were used in the present study. Twenty-seven samples from Mexico, 25 samples from Peru and 20 samples from Paraguay were examined. In Mexico, 2 samples from Sinaloa State, 15 samples from Jalisco State and 10 samples from Guanajuato State were collected. Samples from Peru and Paraguay were collected randomly from patients throughout the countries. Almost all of the patients were Mestizo. Twenty-four samples originated from Philippines were also included in the analysis (kindly supplied by Dr. A. Abraham, Dr. Jose N. Rodriguez Memorial Hospital, Philippines). Samples were collected from multibacillary cases [12], since paucibacillary specimens gave poor PCR products in the preliminary study, as reported by others [13]. Furthermore, leprosy disease types are defined by host immune response rather than causative strains. Slit-skin smear specimens were collected from the skin lesion of patient in the same manner as the routine slit-skin smear test for Bacterial Index examination. The sample on the disposable surgical blade was soaked in 70% ethanol and kept at a room temperature until use. The bacilli were removed from the blade and collected as a pellet by centrifugation at 10,000*g* for 20 min and then washed with phosphate-buffered saline. Template was prepared by treatment with lysis buffer as mentioned elsewhere [13].

2.2. rpoT genotyping by PCR and electrophoresis

PCR was carried out using a G mixture of FailSafe PCR System (EPICENTRE, Madison, WI, USA) in a 50 μl volume of reaction mixture. Primers A (5′-ATGCCGAACCGGACCTCGACGTTGA-3′) and B (5′-TCGTCTTCGAGGTCGTCGAGA-3′) (GenBank Accession No. AB019194) were used for amplification to span the 91 or 97-bp fragment containing the target region with three copies of the six-base tandem repeats or four copies of the six-base tandem repeats in the *rpoT* gene [1]. For comparing the differences of the repeats in the *rpoT* gene, 91- or 97-bp products were separated by electrophoresis in a 4% Meta Phore™ agarose gel (FMC Bioproducts, Rockland, ME, USA) using TBE (Tris/Borate/EDTA, pH 8.0) buffer at 50 V.

2.3. Sequencing

The numbers of six-base repeats were confirmed by direct sequencing. DNA samples for sequencing were recovered by MinElute Gel Extraction Kit (QIAGEN, GmbH, Germany) after electrophoresis of PCR products in 1.2% Seakem GTG agarose gel (Cambrex Bio Science Rockland Inc., Rockland, ME, USA). Samples were sequenced as described previously [14].

2.4. Genotyping of TTC repeats

Templates prepared for the *rpoT* genotyping were also analysed TTC repeats [2]. The target region was amplified and sequenced as described previously [14].

2.5. Confirmation of M. leprae

To identify of *M. leprae*, the *groEL* gene was amplified by PCR from the template samples using the method of Plikaytis et al. [15].

2.6. Ethical approval

Informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee of National Institute of Infectious Diseases, Japan. Bacillary samples of slit-skin smears were collected when informed consent was obtained.

3. Results

3.1. Geographic distribution of M. leprae with different rpoT genotypes

PCR products of different sizes, 91- or 97-bp, were obtained according to the number of six-base tandem repeats in the *rpoT* gene (Fig. 1). Sequencing revealed that there were three copies of the six-base tandem repeats in the 91-bp PCR products and four copies in the 97-bp PCR products, respectively. No other *rpoT* genotype was detected. Of 27 samples from Mexico, only two samples from Jalisco state harboured three copies of the six-base tandem repeats. Both samples from Sinaloa state, 13 samples from Jalisco state and 10 samples from Guanajuato state revealed a genotype with four copies of the six-base tandem repeats. In contrast, all samples

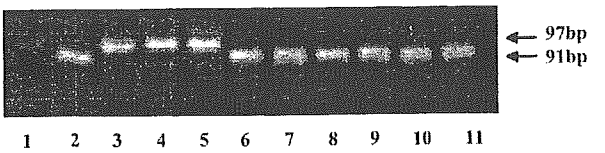

Fig. 1. The *rpoT* gene polymorphism in *M. leprae* obtained from Mexico, Peru and Paraguay. PCR products were resolved on a 4% Meta Phore agarose gel. Samples were: lane 1, the DNA size marker of 100 bp; lane 2, *M. leprae* with 3-copy of 6-bp tandem repeats; lane 3, *M. leprae* with four copies of the 6-bp tandem repeats; lane 4, isolate from Sinaloa state in Mexico; lane 5, isolate from Guanajuato state in Mexico; lane 6, isolate from Jalisco state in Mexico; lane 7, isolate from Peru; lane 8, isolate from Peru; lane 9, isolate from Peru; lane 10, isolate from Paraguay; lane 11, isolate from Paraguay.

Table 2
Frequency (%) of TTC genotypes in each country

No. of repeats	Country		
	Mexico	Peru	Paraguay
9	5		10
10	57	35	36
11	24	22	24
12	3	35	20
13	3	8	5
14	3		5
18	5		
Total	100% (27 cases)	100% (25 cases)	100% (20 cases)

from Peru, Paraguay and the Phillipines showed three copies of the six-base tandem repeats (Table 1).

3.2. Frequency of each TTC genotype

The copy number of TTC repeats varied from 9 to 18 copies (Table 2). No biased distribution of some specific TTC repeat genotypes was recognized in these three countries. The 10-copy TTC genotype was the most frequent in all three countries.

3.3. Verification of bacterial materials

All samples examined showed the 364-bp PCR products of the *groEL* gene and indicated that samples contained *M. leprae.*

4. Discussion

The first stage of our study showed a prominent biased distribution of *M. leprae* with four copies of the six-base tandem repeats in the *rpoT* gene in the main island of Japan and Korea. The predominance of three copies of the same repeats in Okinawa, the island off the southern end of Japan, was associated with human migration [1]. It has been a very influential hypothesis that modern Japanese are derived from a base of Jomon ancestry compounded with later Yayoi immigrants who had migrated to Japan through the Korean Peninsula [16]. Additional data to support the hypothesis that *M. leprae* was spread by one of the Mongoloid lineage which migrated to Japan through the Korean Peninsula include the similarity of genetic marker of Korean and Japanese [17]; low seroprevalent frequency of HTLV-1 carriers in Koreans and Japanese [18]; and the resemblance between isolates from Korea and Japan on genotypes of JC virus, *Mycobacterium tuberculosis*, *Helicobacter pylori* and Hepatitis B virus [6,7,9–11].

In this study, the geographical distribution of *M. leprae* discriminated by *rpoT* gene polymorphism was compared in some Latin American countries. The most noticeable finding of this study was that even although all of the isolates from Peru and Paraguay harboured three copies of the six-base tandem repeats of *M. leprae rpoT* gene, in Mexico, was a predominance of bacilli with four copies of the repeats.

Genotyping of microorganisms distributed throughout the American continent and Asia has proved the concordant spread of some infectious diseases with the migration of human races [4–8]. It must be of interest to pursue whether there were any correlations between the genotypes of *M. leprae rpoT* gene distribution in the American continent and the spread of leprosy with the intercontinental movement of human races in the past because there have been reports that the movement of leprosy patients initiated the transmission of *M. leprae* [19]. Notably, the genotype of four copies of the six-base tandem repeats of the *M. leprae rpoT* gene was typically dominant in Mexico whilst in all isolates from Peru and Paraguay there were only three copies. According to a random analysis of *M. leprae rpoT* gene on an isolate from armadillo in the USA, three isolates from Brazil and an isolate from Haiti, it was supposed that *M. leprae* with three copies of the six-base tandem repeats existed on the American

Table 1
Number of each *rpoT* genotype isolated in the three Latin American countries

rpoT Genotype	Mexico			Peru	Paraguay
	Sinaloa state	Guanajuato state	Jalisco state		
4-copy	2	10	13		
3-copy			2	25	20

continent [1]. For this reason, the predominance of four copies of the six-base tandem repeats in Mexico was unexpected. These limited data might indicate that leprosy was introduced into Mexico and other countries in different ways, for example, from different human races such as from Africa by slaves, from Asia by different groups of Mongoloid people and from Europe countries by Caucasians. Such ideas have been explored in the context of other infectious diseases [4,5,8,20]. However, more samples from more other related countries would help to develop a more comprehensive correlation between the genotype of *M. leprae* and the history of introduction of leprosy to Latin American countries. This would with no doubt contribute to the study of anthropology and archaeology.

It is generally said that leprosy was introduced to Mexico from the Philippines during the Spanish colonial era. Until now, the preliminary study of all 24 isolates from the Philippines has shown three copies of the six-base tandem repeats, which does not support this view. More isolates of *M. leprae* from Mexico and other countries around Mexico for genotyping are required to resolve this point.

Polymorphism of TTC repeats exhibited a wide range of variation; however, the variation of samples from Peru was relatively limited, and there was no biased distribution of some specific genotypes in any countries. The bacilli with 10 copies of TTC repeats were detected most frequently and this was same with our previous results obtained in Indonesia [14].

Genotypes of *M. leprae* that are particularly distributed in some specific areas, other than three or four copies of six base tandem repeats in the *rpoT* gene, might facilitate the epidemiological and anthropological analysis of the origin and spread of leprosy. Unfortunately, even though microsatellite and minisatellite nucleotide sequences with polymorphism have been used for genotyping [3], none of them showed such biased geographical distribution as *rpoT* genotyping in our preliminary study (data ware not shown). Other short tandem repeats with polymorphism that are valuable for genotyping are under investigation for epidemiological analysis of leprosy transmission and the worldwide spread in the past.

Acknowledgements

This study was supported by the grant of a Health Research Grant of Emerging and Re-emerging Infectious Diseases, Ministry of Health, Labour and Welfare, Government of Japan, the grant of the Postdoctoral Fellowship Program, Japan Society for the Promotion of Science, the grant of Foundation for Hansen's diseases in Asia and the grant of US–Japan Cooperative Medical Science Program.

References

[1] Matsuoka, M., Maeda, S., Kai, M., Nakata, N., Chae, G.-T., Gillis, T.P., Kobayashi, K., Izumi, S. and Kashiwabara, Y. (2000) *Mycobacterium leprae* typing by genomic diversity and global distribution of genotypes. Int. J. Lepr. 68, 121–128.

[2] Shin, Y.-C., Lee, H., Lee, H., Walsh, G.P., Kim, J.-D. and Cho, S.-N. (2000) Variable numbers of TTC repeats in *Mycobacterium leprae* DNA from leprosy patients and use in strain differentiation. J. Clin. Microbiol. 38, 4535–4538.

[3] Groathouse, N.A., Rivoire, B., Kim, H., Lee, H., Cho, S.-N., Brennan, P.J. and Vissa, V.D. (2004) Multiple polymorphic loci for molecular typing of strains of *Mycobacterium leprae*. J. Clin. Microbiol. 42, 1666–1672.

[4] Falush, D., Wirth, T., Linz, B., Pritchard, J.K., Stephens, M., Kidd, M., Blaser, M.J., Graham, D.Y., Vacher, S., Perez-Perez, G.I., Yamaoka, Y., Mégraud, F., Otto, K., Reichard, U., Katzowitsch, E., Wang, X., Achtman, M. and Suerbaum, S. (2003) Traces of human migrations in *Helicobacter pylori* populations. Science 299, 1582–1585.

[5] Sonoda, S., Li, H.-C., Cartier, L., Nunez, L. and Tajima, K. (2000) Ancient HTLV type I provirus DNA of Andean mummy. AIDS Res. Hum. Retroviruses 16, 1753–1756.

[6] Sugimoto, C., Kitamura, T., Guo, J., Al-Ahdal, M.N., Shchelkunov, S.N., Otova, B., Ondrejka, P., Chollet, J.-Y., El-Safi, S., Ettayebi, M., Grésenguet, G., Kocagöz, T., Chaiyarasamee, S., Thant, K.Z., Thein, S., Moe, K., Kobayashi, N., Taguchi, F. and Yogo, Y. (1997) Typing of urinary JC virus DNA offers a novel means of tracing human migrations. Proc. Natl. Acad. Sci. USA 94, 9191–9196.

[7] van Soolingen, D., Qian, L., de Haas, P.E.W., Douglas, J.T., Traore, H., Portaels, F., Qing, H.Z., Enkhsaikan, D., Nymadawa, P. and van Embden, J.D.A. (1995) Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. J. Clin. Microbiol. 33, 3234–3238.

[8] Yamaoka, Y., Orito, E., Mizokami, M., Gutierrez, O., Saitou, N., Kodama, T., Osato, M.S., Kim, J.G., Ramirez, F.C., Mahachai, V. and Graham, D.Y. (2002) *Helicobacter pylori* in North and South America before Columbus. FEBS Lett. 517, 180–184.

[9] Covacci, A., Censini, S., Bugnoli, M., Petracca, R., Burroni, D., Macchia, G., Massone, A., Papini, E., Xiang, Z., Figura, N. and Rappuoli, R. (1993) Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. Proc. Natl. Acad. Sci. USA 90, 5791–5795.

[10] Covacci, A., Telford, J.L., Giudice, G.D., Parsonnet, J. and Rappuoli, R. (1999) *Helicobacter pylori* virulence and genetic geography. Science 284, 1328–1333.

[11] Huy, T.T.-T., Ushijima, H., Quang, V.X., Win, M.K., Luengrojanakul, P., Kikuchi, K., Sata, T. and Abe, K. (2004) Genotype C of hepatitis B virus can be classified into at least two subgroups. J. Gen. Virol. 85, 283–292.

[12] WHO Expert Committee on Leprosy. (1998) Seventh Report. Technical Report Series 874, WHO, Geneva.

[13] de Wit, M.Y.L., Faber, W.R., Krieg, S.R., Douglas, J.T., Lucas, S.B., Montreewasuwat, N., Pattyn, S.R.N., Hussain, R., Ponnighaus, J.M., Hartskeerl, R.A. and Klatser, P.R. (1991) Application of polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissue. J. Clin. Microbiol. 29, 906–910.

[14] Matsuoka, M., Zhang, L., Budiawan, T., Saeki, K. and Izumi, S. (2004) Genotyping of *Mycobacterium leprae* on the basis of polymorphism of TTC repeats for analysis of leprosy transmission. J. Clin. Microbiol. 42, 741–745.

[15] Plikaytis, B.B., Gelber, R.H. and Shinnick, T. (1990) Rapid and sensitive detection of *Mycobacterium leprae* using nested-primer gene amplification assay. J. Clin. Microbiol. 28, 1913–1917.
[16] Hanihara, K. (1991) Dual structure model for the population history of the Japanese. Jpn. Rev. 2, 1–33.
[17] Park, M.H., Hwang, Y.S., Park, K.S., Tokunaga, K., Akaza, T., Juji, T. and Kim, S.I. (1998) HLA haplotypes in Koreans based on 107 families. Tissue Antigens 51, 347–355.
[18] Ishida, T. and Hinuma, Y. (1986) The origin of Japanese HTLV-I. Nature 322, 504.
[19] Trautman, J.R. (1994) The history of leprosy In: (Hastings, R.C., Ed.), Leprosy, Vol. 2, pp. 11–25. Churchill Livingstone, Edinburgh.
[20] Miura, T., Fukunaga, T., Igarashi, T., Yamashita, M., Ido, E., Funahashi, S., Ishida, T., Washio, K., Ueda, S., Hashimoto, K., Yoshida, M., Osame, M., Singhal, B.S., Zaninovic, V., Cartier, L., Sonoda, S., Tajima, K., Ina, Y., Gojobori, T. and Hayami, M. (1994) Phylogenic subtype of human T-lymphotropic virus type I and their relations to the anthropological background. Proc. Natl. Acad. Sci. USA 91, 1124–1127.

JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2005, p. 5221–5229
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.10.5221–5229.2005

Vol. 43, No. 10

Diversity of Potential Short Tandem Repeats in *Mycobacterium leprae* and Application for Molecular Typing

Liangfen Zhang,[1] Teky Budiawan,[2] and Masanori Matsuoka[3]*

Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002, Tokyo, Japan[1]; Leprosy-TB Program, Provincial Health Service, Jl 17, Agustus, Manado, North Sulawesi, 95117, Indonesia[2]; and Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, 189-0002, Tokyo, Japan[3]

Received 18 January 2005/Returned for modification 15 April 2005/Accepted 24 July 2005

A recent advance in molecular typing for tracing the transmission of leprosy is the discovery of short tandem repeats (STRs) in *Mycobacterium leprae*. To substantiate polymorphic loci from STR as promising candidates for molecular typing tools in leprosy epidemiology, 44 STR loci including 33 microsatellites and 11 minisatellites were investigated among 27 laboratory strains by sequencing PCR products. Not all STRs were necessarily polymorphic. Thirty-two out of the 44 loci were polymorphic. Nine polymorphic loci were suitable for identifying genotypes according to the discriminatory capacity, stability, and reproducibility. All the strains were classified into independent genotypes by the selected nine loci. Three multicase households were subjected to molecular typing. *M. leprae* obtained from household cases showed identical copy numbers by TTC triplet alone, but the isolates from one family contact case were divided into different genotypes by adding eight other polymorphic loci. The combination of information from multiple loci allows increasing levels of discrimination and it is likely that the generation and documentation of data will result in the choice of a potential molecular typing tool for leprosy epidemiology.

Mycobacterium leprae is an obligate intracellular parasite with tropism for macrophages and Schwann cells and the only species of mycobacteria to infect peripheral nerves (19). It causes leprosy, a chronic granulomatous infection of the skin and peripheral nerves with characteristic deformities and disability (1). It is generally accepted today that the worldwide implementation of standardized multidrug therapy for leprosy has decreased the number of registered leprosy cases from a peak of 10 to 15 million to a current total of less than 1 million. However, the annual confirmed new cases remain at 500,000 to 700,000. This continuing number suggests that effective multidrug therapy fails to disrupt the chain of leprosy transmission (26).

Even though leprosy is one of the oldest recorded diseases, the source for *M. leprae*, the portal of its exit/entry, and the mode of transmission are still under investigation. Some regard human beings as a host for the bacteria, while others are still considering more possibilities. It was proposed that the nasal mucosa are the exit/entry pathway of *M. leprae* (9, 17, 18). In light of the transmission mode, human-to-human direct contact was first generally accepted (6, 16), with time as airborne (23), as vector-borne (14, 25) and as vehicle-borne (3, 12) routes from evidence that has been obtained. All the progress in leprosy epidemiology was helpful in understanding the chain of transmission, yet at the same time was overwhelming.

In the past few years, studies focusing on leprosy transmission by molecular genotyping have shed new light on it (8, 10, 11, 22, 24, 27, 28). The most recent one was the report from Groathouse et al. (8). By in silico analysis, 44 promising polymorphic short tandem repeat (STR) sites, including both 33 microsatellite loci (repeat units of 1 to 5 bp) and 11 minisatellite (repeat units of >5 bp) were selected from the *M. leprae* TN genome sequence. Variable-number tandem repeats (VNTRs) at 9 of 11 STR loci from four clinical isolates of *M. leprae* were found. Clearly, it is urgent to accumulate extensive data for developing powerful typing tools to tracking the transmission of leprosy to finally reach a world free of leprosy.

In this study, we identified 32 polymorphic loci throughout the 44 STRs in a batch of 27 laboratory strains by sequencing and demonstrating the applicability and feasibility of nine potential loci acting as genetic markers to discriminate different *M. leprae* strains.

MATERIALS AND METHODS

***M. leprae* strains.** A panel of 27 laboratory strains of *M. leprae* was subjected for genetic analysis. Strains were maintained by inoculation into nude mice footpads annually in our laboratory. All the strains used in this study were recovered from multibacillary cases. Excluding Thai-53, Thai-311 and Thai-237 were from Thailand, Indonesia-1 was from Indonesia, and Korea 3-2 was from Korea, and the others were from Japan.

All patients were from geographically distinct regions. Four strains, Thai-53, Kyoto-1, Zensho-4, and Korea3-2, and 17 samples, namely, the fourth generation of Thai-53 (Thai-53 4th), Thai-53 7th, Thai-53 11th, Kyoto-1 3rd, Kyoto-1 5th, Kyoto-1 7th, Kyoto-1 8th, Zensho-4 (biopsy specimen), Zensho-4 1st, Zensho-4 2nd, Zensho-4 3rd, Zensho-4 4th, Korea3-2 (biopsy specimen), Korea3-2 1st, Korea3-2 2nd, Korea3-2 3rd, and Korea3-2 4th (Table 6) were employed for the stability testing of loci. Partially purified bacterial materials were prepared from the inoculated footpads by deferential centrifuging and suspension in a phosphate buffered saline at concentration of 10^{5-6}/ml.

Primer selection. Primer sets for the amplification regions of DNA containing the STR sites were referred to the study of Groathouse et al. (8). The sequences of primer pairs were listed (Table 1).

* Corresponding author. Mailing address: Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1, Aobacho, Higashimurayama-shi, Tokyo 189-0002, Japan. Phone: 81-42-391-8211. Fax: 81-42-394-9092. E-mail: matsuoka@nih.go.jp.

TABLE 1. Primers used in this study[a]

Primer	Sequence (5′ to 3′)	Repeat unit (bp)	Locus	Amplicon size (bp)	Accession no.[b]
RP1-1	ATA ACC AAC CGT TTC GCT GC GCA GAT TGC ACC CTG CAG TA	1	(T)8(A)6	108	*ML0258-ML0259*
RP1-2	ATG ATC CCG GTG TGA GCT CG TCT CTG CAG CCC ACA CCC TG	1	(T)6(N)7(T)8	149	ML0415/−
RP1-3	AAG AAC GTA CAA TCA CC AGC GAC CAC CCA CTA GCA TC	1	(A)9	145	*ML1196-ML1197*
RP1-4	TTG TGC TGG TCT CGT AGG CG TGC CGA CCT CGG TCT GCT TC	1	(G)9	150	ML0823/+
RP1-5	CGG ATA TAA TTA ACC TTC GG GTT GCG GGT CTA CTT GGT TG	1	(C)9	131	ML2238/−
RP1-6	GGG CGA TGC CGC CTT CGA AC CAG ACC CGG AAA CAG GAT AC	1	(G)10a	128	ML0266/+
RP1-7	AAT CCA AGC TGA TCG GCC AG TGC CCT GCA ATG TGC CGA TG	1	(G)10b	137	ML0350/−
RP1-8	GCG CAC TCT GTT AAT GAT GC ACA CCG ACA ACC GGT TAC CG	1	(G)11	136	ML1126/−
RP1-9	TGG CCA TCG TGG TGC TGT TC CAA CCT CGG CGG ACG CAT AC	1	(G)12	119	**ML0946/+**
RP1-10	TCG AGT TTT GGA CGG CAC CG AGT GCT TGT GCG GTC CAC AC	1	(C)16(G)8	152	*ML1647-ML1648*
RP1-11	ACT TCG GCG ACT GCG GTA AC GGT CAC TGG GCG TTG TGG TC	1	(C)20	159	*ML0237-ML0238*
RP1-12	GAA CTG GCC GGG TTA AAA GG TGC GCC TTC ACT GTG AAA TG	1	(G)22	176	ML0164/+
RP2-1	CAG GTC TTC GCG CCA ATT CT CCC GCG ACT CCC TGG CTT GC	2	(CG)6	140	**ML2472/−**
RP2-2	GTG TTA CGC GGA ACC AGG CA CCA TCT GTT GGT ACT ACT GA	2	(AC)8a	124	ML1285/−
RP2-3	GAT GCG ACT ATC ACT CGC AC GCT GGT TTC CTT CTA GTC CC	2	(AC)8b	140	*ML1824-ML1825*
RP2-4	GCC TGG TGC CCG GAC AAT GC ACT GAT CTC GCC GGC GCT GT	2	(AC)9	140	*ML1227-ML1228*
RP2-5	CAA CAG ATC GGT CGG AGA GG CTG GGT AGC CAT CTG GCT GC	2	(CA)6	160	*ML2106-ML2107*
RP2-6	GAC CCG GTT GCT TCA TGT AT GTA AGT TAC AAA CAA GCT GT	2	(TA)8	143	*ML2676-ML2677*
RP2-7	CGG AAA GTG TTG TGG TCG AC CCA AAC ACT TAG TCC ACT AA	2	(TA)9	189	*ML2375-ML2376*
RP2-8	TAA TTT TCT TAA AGA TAG AG TGT GAT CTT TGC ACT GTC GT	2	(TA)10	139	*ML1450-ML1451*
RP2-9	ATG AGC CGT AAG GAA TTA AC ATG CTG CCA CTC ATT AGA GG	2	(TA)13	158	*ML0235-ML0236*
RP2-10	CTG AGT AGA CCA GTC GAC AG ATC GCG GAG CAA CAA TTT CT	2	(AT)10	150	*ML2476-ML2477*
RP2-11	TTG GCG CTT CTG ATA TGG CT GGC GAG CCC GTC TGG CTC GA	2	(AT15)	164	*ML0798-ML0799*
RP2-12	TTA GCA GGA CGA TTG TAC AG ACC CGG AAT TCC TCC AAG	2	(AT)17	160	ML2183/−
RP2-13	CCG ACT CGC CGA AGC GAA AC CCG CGT TAG CAT AGG CAA T	2	(TA)18	119	ML0830/−
RP3-1	GTC ACG GCG CCG GGA ACG CA CCG ATT CTG CTG GGC TGC TG	3	(ACC)5	150	ML1645/−
RP3-2	TCA CCA TCG ACG CTC CGG GT TCG GCC TGG TTG TCT GCC TT	3	(GGT)5	161	*ML2159-ML2160*
RP3-3	CGT GAC AAT TCC TGG CAC AT CGA GAA GTG TAC CAC CAT CC	3	(AGT)5a	143	ML1073/−
RP3-4	GGC CAA ACG GTC AGG TTG TT ACA TCG AAT CGC TGT TGT AC	3	(AGT)5b	149	ML1118/−
RP3-5	GTA CGC CAA GGA CCG TGA CT GGT TGT TGA TGT TGG TGG TG	3	(ACT)5	120	ML2236/+
RP3-6	ACG CTG CGG TTT CGC AGC CT AAT ATG CAT GCC GGT GGT	3	(GTA)9	148	*ML2172-ML2173*
RP3-7	GGA CCT AAA CCA TCC CGT TT CTA CAG GGG GCA CTT AGC	3	(AGA)20	168	*ML2344-ML2345*
RP5-1	GAA GTT GAA TTC TTA TTG CC GCG GAG GGC ATC ACA CAG AA	5	(CACCG)3	130	*ML2158-ML2159*

Continued on facing page

TABLE 1—*Continued*

Primer	Sequence (5′ to 3′)	Repeat unit (bp)	Locus	Amplicon size (bp)	Accession no.[b]
RP6-1	AAG CGT CGA TAC AAA GGC ACC GT AGT AGC TTC GCCATC CTC GGT TT	6	6-3a	91	**ML1022/−**
RP6-2	GTT TGT CAA CAT TGG CAG GT CTG GAA CCG CGT GGC CCA CT	6	6-3b	148	**ML1918/−**
RP6-3	CTA CTT GCG CGC CAC CGC CA CCG TCG CCA GGT TTT GCA GA	6	6-7	191	**ML1505/+**
RP7-1	CTG GGG CGC GCT CAA TCG CT CGG GTT CGG GTG TAA CGA CA	7	7-3	160	**ML0213/+**
RP10-1	GTA CGC CAC CAG GAC AAC TC TGG CGG GCG TGC AGC CAA AC	10	10-4	203	ML0970/+
RP12-1	AGT AGC TTC CAT CCC CTC AT GCG ACG AAA GCA TTT ACG GC	12	12-5	180	**ML1182**
RP15-1	GCT GAG GTT AGG CGC CGA TC TCG GCA GAG TCC TGG CCA TC	15	15-3	195	*ML2454-ML2455*
RP18-1	GCT ATG GGC AGC CTG GGT AT AGC CGG TTA CCA AGA TGG CA	18	18-8	330	**ML1334/+**
RP21-1	TGT TGA AAT TTG GCG GCC AT TGC AAG GAG TGC TCA GCT AT	21	21-3	179	ML0058/−
RP23-1	CAG TCG CCC GGA TAC TGT TA TAA ATC CGC TCC CAA ATC TT	23	23-3	190	*ML2469-ML2470*
RP27-1	GTG CTG TGC CTG CCG TT TCC CCA AAG CCG CCG AAT CC	27	27-5	270	**ML0568/+**

[a] Primers were designed to amplify STRs reported by Groathouse et al. (8).
[b] Protein coding genes are in bold type. Intergenic regions are in italic type. Pseudogenes are underlined.

Slit-skin smears from multicase household. Eleven slit skin smears were collected as the same manner as that for Bacterial Index examination from leprosy patients in a total of three households. Among them, five smears from five patients in household I; four smears from two patients in household II, sample 6 from the left earlobe and sample 7 from the right earlobe within a single individual; sample 8 from the earlobe and sample 9 from the back in another individual; and two smears from two patients in household III (Table 7).

Preparation of *M. leprae* DNA from strains and slit skin smears and sequencing analysis. *M. leprae* templates from both strains and slit-skin smears were prepared by treatment with lysis buffer at 60°C overnight as described previously (13). PCR amplification of STR sites as well as sequencing analysis was performed under the same condition as described elsewhere using the listed primer pairs (10, 11, 12, 13). Briefly, target loci were amplified using a G mixture and a FailSafe PCR system (EPICENTRE, Madison, Wis.). DNA samples for sequencing were recovered with a MinElute gel extraction kit (QIAGEN GmbH, Hilden, Germany) after electrophoresis of PCR products. Samples were sequenced with a BigDye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, Norwalk, Conn.) and an ABI Prism 310 genetic analyzer (Perkin-Elmer). The nucleotide sequences obtained were analyzed using DNASIS software (Hitachi Software Engineering, Yokohama, Japan).

Multiplication of *M. leprae* strains in nude mice footpad. Inoculums of each strain were prepared from BALB/c-*nu/nu* nude mice which were inoculated with isolates of the third to fifth generations about 8 to 10 months before by Nakamura's method as described previously (15). Five-week-old male nude mice were injected with the inoculums containing 10^4 bacilli/0.05 ml of Hanks' balanced salt solution. Bacillary number in the footpads of six nude mice at 10, 20, 30, 40, and 50 weeks growth were examined by Shepard's method (21).

Ethical approval. Informed consent was obtained from all subjects, and the study was approved by the institutional ethics committee of National Institute of Infectious Diseases, Tokyo, Japan. Bacillary samples of nasal mucus and slit skin smears were collected when informed consent was obtained. Animal experiments were conducted under the approval of the institutional animal experiment committee.

RESULTS

Allelic diversity of 33 microsatellite loci in *M. leprae* strains. In the 27 *M. leprae* strains, 8 out of 33 selected microsatellite loci showed the same copy number of allele whereas the other 25 loci, (A)9, (G)9, (C)9, (G)10a, (G)10b, (G)11, (G)12, (C)16(G)8, (C)20, (G)22, (AC)8a, (AC)8b, (AC)9, (CA)6, (TA)8, (TA)9, (TA)10, (TA)13, (AT)10, (AT)15, (AT)17, (TA)18, (GGT)5, (GTA)9, and (AGA)20, presented at least two types of allele. All locus identifications are from Groathouse et al. (8) to maintain integrity. This result validated the comments from Groathouse group on the polymorphic (C)20, (AT)17, (TA)18, (GTA)9, and (AGA)20 and no diversity of (CG)6, albeit (AC)9 was also examined for considerable polymorphism here which was predicted as nonpolymorphic loci by them. However, the last repeat unit of some results was not easily defined for unknown reasons (Table 2).

Allelic diversity of eleven minisatellite loci in *M. leprae* strains. Throughout the 27 *M. leprae* strains, four out of 11 selected minisatellite loci showed no diversity while the other seven loci, 6-3a, 6-7, 12-5, 18-8, 21-3, 23-3, and 27-5, exhibited variable characteristics, which also verified Groathouse et al. (8) reports on the polymorphism of loci 6-7, 12-5, 18-8, 21-3, and 27-5 (Table 3).

Variations of allelic diversity at STR loci in *M. leprae* strains. Forty-four selected STR loci consisted of nine protein coding genes, 19 intergenic regions, and 16 pseudogenes. Polymorphism was revealed in six among nine (67%) protein coding genes, 16 out of 19 (84%) intergenic genes, and 10 of 16 (63%) pseudogenes. Almost all of the STR loci located in intergenic regions or in pseudogenes and were unlikely involved in biological functions. Overall, thirty-two out of 44 STR loci were polymorphic and 12 loci were invariable that might be of limited value as epidemiological markers. The variations were between 2 and 11 alleles (Table 4). Notably, at locus (CA)6, isolate Thai-237 differed from the other 26 strains by one repeat unit (Table 2). Similarly, at locus 23-3, isolate Hoshizuka-1 alone, and at locus 27-5, isolate Airaku-2 alone

TABLE 2. Allelic diversity of microsatellite loci in *M. leprae* strains

Strain	Copy no. of repeat element(s) at locus[a]:																	
	(T)8(A)6	(T)6(N)7(T)8	(A)9	(G)9	(C)9	(G)10a	(G)10b	(G)11	(G)12	(C)16(G)8	(C)20	(G)22	(CG)6	(AC)8a	(AC)8b	(AC)9	(CA)6
Zensho-2	8 & 6	6 & 7 & 8	8	9	10	10	10	13†	9	16† & 7	16†	17†	6	7	8	7	6
Airaku-2	8 & 6	6 & 7 & 8	8	9	10	9	9	13	14†	12† & 8	9	14†	6	9	6	8	6
Airaku-3	8 & 6	6 & 7 & 8	8	9	10	9	11	11	10	16† & 8	13†	18†	6	10	6	8	6
Ryukyu-2	8 & 6	6 & 7 & 8	8	9	12	7	9	14	11	10 & 8	8	16†	6	12	7	8	6
Zensho-9	8 & 6	6 & 7 & 8	8	9	9	9	11	13	10	16† & 8	8	19†	6	9	7	8	6
Gushiken	8 & 6	6 & 7 & 8	9	9	10	12	11	10	9	14† & 8	9	16†	6	11	7	7	6
Thai-53	8 & 6	6 & 7 & 8	9	9	10	12	11	10	9	14† & 8	9	17†	6	11	7	7	6
Thai-311	8 & 6	6 & 7 & 8	8	10	10	9	11	10	9	16† & 8	13†	16†	6	9	6	8	6
Amami-1	8 & 6	6 & 7 & 8	8	9	9	8	9	11	9	17† & 8	8	12†	6	8	7	8	6
Indonesia-1	8 & 6	6 & 7 & 8	9	9	13†	11	9	14	9	18† & 8	12†	9	6	11	7	9	6
Thai-237	8 & 6	6 & 7 & 8	9	9	9	9	11	9	9	14† & 8	9	13†	6	9	7	7	7
Zensho-12	8 & 6	6 & 7 & 8	9	9	9	10	9	11	9	12 & 7	10	14†	6	10	7	10	6
Hoshizuka-1	8 & 6	6 & 7 & 8	8	9	12†	9	10	13	9	10 & 7	10	14†	6	8	8	7	6
Hoshizuka-5	8 & 6	6 & 7 & 8	8	10	12†	9	10	13	11	14† & 7	17†	13†	6	10	8	7	6
Izumi	8 & 6	6 & 7 & 8	8	9	10	13†	11	11	9	17† & 7	9	17†	6	11	8	7	6
Kanazawa	8 & 6	6 & 7 & 8	8	9	10	9	13†	14†	9	16† & 7	11	13†	6	6	8	7	6
Keifu-4	8 & 6	6 & 7 & 8	8	9	11	9	10	11	9	14† & 7	16†	12†	6	8	10	7	6
Kitazato	8 & 6	6 & 7 & 8	8	9	9	13†	12	13†	10	15† & 7	15†	14†	6	9	7	7	6
Zensho-4	8 & 6	6 & 7 & 8	8	9	10	11	12	11	9	17† & 7	14†	14†	6	8	8	7	6
Kusatsu-3	8 & 6	6 & 7 & 8	8	9	10	12	10	10	12†	18† & 7	13†	12†	6	9	8	8	6
Kyoto-2	8 & 6	6 & 7 & 8	8	9	11	11	9	13	10	19† & 7	10	15†	6	6	8	7	6
Oku-4	8 & 6	6 & 7 & 8	8	9	9	10	13†	14	9	10 & 8	15†	14†	6	9	8	7	6
Zensho-5	8 & 6	6 & 7 & 8	8	9	12	11	11	13	11	16† & 7	13†	14†	6	10	8	7	6
Kusatsu-6	8 & 6	6 & 7 & 8	8	9	10	10	12	10	10	17† & 7	14†	16†	6	9	8	7	6
Korea3-2	8 & 6	6 & 7 & 8	8	9	11	11	13†	12	9	12† & 7	9	18†	6	7	8	7	6
Hoshizuka-4	8 & 6	6 & 7 & 8	8	9	10	10	11	13	9	19† & 7	10	10	6	8	8	7	6
Kyoto-1	8 & 6	6 & 7 & 8	8	9	11	10	12	13	9	13† & 8	11	18†	6	7	8	7	6

[a] †, difficult to read the sequence across the junction of stretch of repeat.

had one copy difference allele (Table 3). Additionally, at locus 12-5, a five-copy-repeat unit was obtained from *M. leprae* TN, but all of the strains in this study showed three- and four-copy-repeat units instead of the five-copy-repeat unit (Table 3).

Comparison among the loci fall within protein coding genes with two variations. Locus 6-3a is in the *rpoT* gene coding RNA polymerase sigma factor in *M. leprae* contained two variations, three- and four-copy alleles (2, 13). Twelve strains, Zensho-2 to Zensho-12, harbored the three-copy allele whereas 15 strains, Hoshizuka-1 to Kyoto-1, had a four-copy allele in the *rpoT* gene (Table 3). Geographic distribution of each genotype of *M. leprae rpoT* revealed the distinguished distribution in several countries in the world (13, 10). Intriguingly, we compared the copy numbers of the other two protein coding genes 12-5 and 18-8 having two alleles with the strains carrying three-copy and four-copy *rpoT* that were closely associated with geographic distributions and no correlation was exploited.

Discriminatory capacity, stability, and reproducibility of nine potential polymorphic loci detected among serial passage strains by nude mice. Nine polymorphic microsatellite loci, (AC)9, (AC)8b, (AC)8a, (TA)10, (AT)17, (AT)15 (GTA)9, (TA)18, and (AGA)20 (also named TTC) were selected for discriminatory capacity analysis. All 27 strains were divided into two groups, three-copy *rpoT* and four-copy *rpoT*, based on the *rpoT* polymorphism. By adding the conjunction of 9 loci, they were distinguished from each other (Table 5). Then stability and reproducibility testing was carried out among them through serial passage strains by nude mice owing to the nature of susceptibility of replication slippage of microsatellite (28). One generation designated approximately 1 year, these strains have been in passage for 4 to 11 years. Not only the identical profile of allele was shared among the different generations of the strains at each locus, but also the copy number of repeats was in agreement with that in the repeated experiment of the same strains, which ensured these polymorphic microsatellite loci were highly stable and reproducible (Tables 1 and 6). The stability of TTCwas done previously (11)]. It was the rationale for these nine polymorphic microsatellite loci to be a significant source of informative markers for the identification and discrimination of *M. leprae* strains.

Application of polymorphic microsatellite loci for multicase households. Based on the condition of leprosy patients living in same household and possessing the identical TTC pattern, eleven smears from three household were chosen (Table 7). Five bacterial materials from household I shared identical 13-copy of TTC repeat were also subjected to loci (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15, (AT)17, (TA)18, and (GTA)9 and presented the identical copy number of 12, 7, 10, 10, 13, 15, 14, and 9, respectively. In household II which had 12 copies of the TTC repeat, four smears at the above loci showed equal repeats of 10, 7, 9, 10, 13, 13, 11, and 11. There was an exact match by copy number at each locus between samples 6 and 7 as well as samples 8 and 9 within one individual. In household III, two smears harboring eight copies of the TTC repeat, the copy number of loci (AC)8a, (AC)8b, (AC)9, (TA)10, (AT)15 and (TA)18 was 10, 7, 9, 8, 16, and 15. respectively. Strikingly, at locus (AT)17, a difference of 9 and 10 copies and a mismatch of 11 and 12 copies at locus (GTA)9 were found in these two smears, respectively.

TABLE 2—*Continued*

Copy no. of repeat element(s) at locus[a]:														
(TA)9	(TA)10	(TA)13†	(AT)10	(AT15)	(AT)17	(TA)18	(ACC)5	(GGT)5	(AGT)5a	(AGT)5b	(ACT)5	(GTA)9	(AGA)20	(CACCG)3
8	14	23	9	14	15	15	5	4	5	5	5	14	9	3
12	7	19	7	15	14	17	5	4	5	5	5	10	13	3
10	7	21	7	18	13	17	5	4	5	5	5	11	13	3
6	10	22	7	19†	13	13	5	4	5	5	5	8	14	3
7	13	15	7	21	16	12	5	4	5	5	5	11	14	3
8	11	23	8	13	13	20	5	5	5	5	5	11	14	3
9	11	23	8	13	13	21	5	5	5	5	5	11	14	3
12	7	19	7	14	11	19	5	4	5	5	5	9	25	3
6	10	24	8	17	10	15	5	4	5	5	5	9	15	3
8	10	19	9	14	13	10	5	5	5	5	5	9	15	3
9	11	18	9	11	15	10	5	5	5	5	5	9	16	3
10†	12	22	9	16	18	16	5	6	5	5	5	9	11	3
9	15	15	8	21†	14	13	5	4	5	5	5	15	9	9
14	13	19	8	15	14	12	5	4	5	5	5	15	9	3
8	12†	16	8	13	13	16†	5	4	5	5	5	11	9	3
9	14	19	8	17	13	11	5	4	5	5	5	12	10	3
7	11	19	9	21†	15	19	5	4	5	5	5	13	10	3
14†	13	18	8	12	12	14	5	4	5	5	5	16	10	3
8	10	17	9	20	13	15	5	4	5	5	5	13	10	3
10	9	24	8	13	12	17	5	4	5	5	5	16	11	3
14	16	18	9	18	15	14	5	4	5	5	5	16	11	3
10	13	19	9	14	15	12	5	4	5	5	5	13	11	3
8	12†	18	8	14	14	14	5	4	5	5	5	18	11	3
12	16	19	8	18	12	17	5	4	5	5	5	16	16	3
11	15	20	8	17	14	15	5	4	5	5	5	14	13	3
10	12†	20	8	20†	14	17	5	4	5	5	5	13	12	3
12	12	23	9	15	12	16	5	4	5	5	5	15	9	3

TABLE 3. Allelic diversity of minisatellite loci in *M. leprae* strains

Strain	No. of copies at locus:										
	6-3a	6-3b	6-7	7-3	10-4	12-5	15-3	18-8	21-3	23-3	27-5
Zensho-2	3	3	7	3	4	3	3	7	2	2	5
Airaku-2	3	3	6	3	4	4	3	7	2	2	3
Airaku-3	3	3	6	3	4	4	3	8	1	2	5
Ryukyu-2	3	3	6	3	4	4	3	8	2	2	5
Zensho-9	3	3	6	3	4	4	3	8	2	2	5
Gushiken	3	3	6	3	4	4	3	7	3	2	5
Thai-53	3	3	6	3	4	4	3	8	3	2	5
Thai-311	3	3	6	3	4	4	3	8	2	2	5
Amami-1	3	3	6	3	4	3	3	8	2	2	5
Indonesia-1	3	3	6	3	4	4	3	8	3	2	5
Thai-237	3	3	6	3	4	4	3	8	3	2	5
Zensho-12	3	3	7	3	4	4	3	8	3	2	5
Hoshizuka-1	4	3	6	3	4	3	3	7	2	3	5
Hoshizuka-5	4	3	6	3	4	3	3	7	1	2	5
Izumi	4	3	6	3	4	3	3	7	1	2	5
Kanazawa	4	3	10	3	4	3	3	7	2	2	5
Keifu-4	4	3	6	3	4	3	3	7	2	2	5
Kitazato	4	3	6	3	4	3	3	7	1	2	5
Zensho-4	4	3	9	3	4	3	3	7	3	2	5
Kusatsu-3	4	3	6	3	4	3	3	7	2	2	5
Kyoto-2	4	3	8	3	4	3	3	7	3	2	5
Oku-4	4	3	5	3	4	3	3	7	2	2	5
Zensho-5	4	3	6	3	4	3	3	7	1	2	5
Kusatsu-6	4	3	6	3	4	3	3	7	2	2	5
Korea3-2	4	3	7	3	4	3	3	7	1	2	5
Hoshizuka-4	4	3	9	3	4	3	3	7	2	2	5
Kyoto-1	4	3	6	3	4	3	3	7	3	2	5

Linkage between the growth rate of *M. leprae* in nude mice footpad and allelic diversity. Shepard et al. (20) reported the hereditary fast-slow growth difference among *M. leprae* strains in conventional mouse footpads, which was also observed in nude mice footpads in our laboratory. However, in this study, the growth curve of *M. leprae* in nude mice footpads gave no difference between strains with three-copy *rpoT* (Zensho-2, Airaku-3, and Thai-53) and those with four-copy *rpoT* (Izumi and Zensho-4) except Kyoto-2, which revealed the copy number of *rpoT* was irrelevant to the fastidious growth. In addition, no correlation was found between the VNTR of the other protein coding genes and *M. leprae* growth.

DISCUSSION

Poor understanding of leprosy transmission has hindered us from eradicating the disease even though *M. leprae* was identified as the pathogen as early as 1873 by Hansen (19). The unusual biological traits of being especially slow growing as well as the inability to culture *M. leprae* in vitro have partially accounted for the setbacks of detailed studies on leprosy. However, the determination of the complete *M. leprae* TN genome sequence (4) is a breakthrough for leprosy research, which has served as a public domain used by the Groathouse study group for the screening of potential VNTR for molecular typing (8). Tandem repeats are usually classified among satellites (spanning megabases of DNA, associated with heterochromatin), minisatellites (repeat units in the range from 6 to 100 bp, spanning hundreds of base pairs) and microsatellites (repeat units in the range from 1 to 5 bp, spanning a few tens of nucleotides) (5, 7). Both microsatellite and minisatellite loci

TABLE 4. Variation of allelic diversity at STR loci in *M. leprae* strains[a]

No.	Locus	No. of repeats and range	No.	Locus	No. of repeats and range	No.	Locus	No. of repeats and range
1	(T)8(A)6	No variation	16	(AC)**9***	7, 8, 9, 10	31	(GTA)**9***	8, 9, 10, 11, 12, 13, 14, 15, 16, 18
2	(T)6(N)7(T)8	No variation	17	(CA)6	6, 7	32	(AGA)20*	9, 10, 11, 12, 13, 14, 15, 16, 25
3	(A)9	8, 9	18	(TA)8	6, 7, 8, 9, 10	33	(CACCG)3	No variation
4	(G)9	9, 10	19	(TA)9	6, 7, 8, 9, 10, 11, 12, 14	**34**	**6-3a**	3, 4
5	(C)9	9, 10, 11, 12	20	(TA)10*	7, 9, 10, 11, 12, 13, 14, 15, 16	**35**	**6-3b**	No variation
6	(G)10a	7, 8, 9, 10, 12, 13	21	(TA)13	15, 16, 17, 18, 19, 20, 21, 22, 23, 24	**36**	**6-7**	5, 6, 7, 8, 9, 10
7	(G)10b	9, 10, 11, 12, 13,	22	(AT)10	7, 8, 9, 10, 12, 14	**37**	**7-3**	No variation
8	(G)11	10, 11, 12, 13, 14	23	(AT15)*	13, 14, 15, 16, 17, 18, 19, 20, 21	38	10-4	No variation
9	**(G)12**	9, 10, 11, 12, 14	24	(AT)17*	10, 11, 12, 13, 14, 15, 16, 18	**39**	**12-5**	3, 4
10	(C)16(G)8	7, 8 & 10, 12, 13, 14, 15, 16, 17, 18, 19	25	(TA)18*	11, 12, 13, 14, 15, 16, 17, 19	40	15-3	No variation
11	(C)20	8, 9, 10, 11, 12, 13, 14, 15, 14, 15, 16	26	(ACC)5	No variation	**41**	**18-8**	7, 8
12	(G)22	9, 10, 12, 13, 14, 15, 16, 17, 18, 19	27	(GGT)5	4, 5, 6	42	21-3	1, 2, 3
13	**(CG)6**	No variation	28	(AGT)5a	No variation	43	23-3	2, 3
14	(AC)8a*	6, 7, 8, 9, 10, 11, 12	29	(AGT)5b	No variation	**44**	**27-5**	3, 5
15	(AC)8b*	6, 7, 8, 10,	30	(ACT)5	No variation			

[a] Protein coding genes are in bold type. Pseudogenes are underlined. Intragenic genes are in standard type. *, selected loci as the combined one for genotyping.

have been selected for this study to substantiate the polymorphic loci as promising candidates used as the molecular typing tools for leprosy epidemiology.

In order to generate comprehensive and reliable data, a battery of 27 laboratory strains was used to develop PCR systems to amplify 44 STR target loci and the PCR products were sequenced. Unexpectedly, the results disclosed that not all the STR loci were polymorphic. Some of the loci were with

TABLE 5. Discriminatory capacity of polymorphic microsatellite loci[a]

Strain	No. of repeats									
	6-3a	(AC)9	(AC)8b	(AC)8a	(TA)10	(AT)17	(AT)15	(GTA)9	(TA)18	(AGA)20 or TTC
Gushiken	3	7	7	11	11	13	13	11	20	14
Thai-53	3	7	7	11	11	13	13	11	21	14
Thai-237	3	7	7	9	11	15	11	9	10	16
Zensho-2	3	7	8	7	14	15	14	14	15	9
Thai-311	3	8	6	9	7	11	14	9	19	25
Airaku-2	3	8	6	9	7	14	15	10	17	13
Airaku-3	3	8	6	10	7	13	18	11	17	13
Amami-1	3	8	7	8	10	10	17	9	15	15
Zensho-9	3	8	7	9	13	16	21	11	12	14
Ryukyu-2	3	8	7	12	10	13	19	8	13	14
Indonesia-1	3	9	7	11	10	13	14	9	10	15
Zensho-12	3	10	7	10	12	18	16	9	16	11
Kitazato	4	7	7	9	13	12	12	16	14	10
Kanazawa	4	7	8	6	14	13	17	12	11	10
Kyoto-2	4	7	8	6	16	15	18	16	14	11
Kyoto-1	4	7	8	7	12	12	15	15	16	9
Korea3-2	4	7	8	7	15	14	17	14	15	13
Zensho-4	4	7	8	8	10	13	20	13	15	10
Hoshizuka-4	4	7	8	8	12	14	20	13	17	12
Hoshizuka-1	4	7	8	8	15	14	21	15	13	9
Oku-4	4	7	8	9	13	15	14	13	12	11
Kusatsu-6	4	7	8	9	16	12	18	16	17	16
Zensho-5	4	7	8	10	12	14	14	18	14	11
Hoshizuka-5	4	7	8	10	13	14	15	15	12	9
Izumi	4	7	8	11	12	13	13	11	16	9
Keifu-4	4	7	10	8	11	15	21	13	19	10
Kusatsu-3	4	8	8	9	9	12	13	16	17	11

[a] Strains with identical *rpoT* genotypes were discriminated by the combination of STRs with polymorphism. All isolates were divided into independent genotypes.