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Simultaneous Determination of *Mycobacterium leprae* Drug Resistance and Single-Nucleotide Polymorphism Genotype by Use of Nested Multiplex PCR with Amplicon Sequencing

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ABSTRACT *Mycobacterium leprae* is the predominant cause of leprosy worldwide, and its genotypes can be classified into four single-nucleotide polymorphism (SNP) types and 16 subtypes. Determining *M. leprae* drug resistance and genotype is typically done by PCR and Sanger DNA sequencing, which require substantial effort. Here, we describe a rapid method involving multiplex PCR in combination with nested amplification and next-generation sequence analysis that allows simultaneous determination of *M. leprae* drug resistance and SNP genotype directly from clinical specimens. We used this method to analyze clinical samples from two paucibacillary, nine multibacillary, and six type-undetermined leprosy patients. Regions in *folP1*, *rpoB*, *gyrA*, and *gyrB* that determine drug resistance and those for 84 SNP-InDels in the *M. leprae* genome were amplified from clinical samples and their sequences determined. The results showed that seven samples were subtype 1A, three were 1D, and seven were 3K. Three samples of the subtype 3K had *folp1* mutation. The method may allow more rapid genetic analyses of *M. leprae* in clinical samples.

KEYWORDS *Mycobacterium leprae*, single-nucleotide polymorphism, nested multiplex PCR

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that mainly affects the skin and peripheral nerves (1). In 2019, 202,185 new cases of leprosy were reported worldwide (2). Leprosy is also caused by *Mycobacterium lepromatosis* closely related to *M. leprae* (3, 4). The first case of drug-resistant *M. leprae* was reported in 1964 for dapsone (DDS) and in 1976 for rifampin (RIF) (5, 6). In 1981, multidrug therapy (MDT) against leprosy comprising three drugs, DDS, RIF, and clofazimine, was recommended by the World Health Organization (WHO). The MDT strategy has significantly reduced the number of leprosy patients, but several multidrug-resistant *M. leprae* strains, such as 92008 and Zensho-4, have been reported (7, 8).

Since *M. leprae* still cannot be cultivated on artificial media, drug susceptibility testing of *M. leprae* is performed via a time-consuming mouse footpad assay (9). DNA diagnosis to detect missense mutations in the drug resistance-determining regions (DRDRs) of *M. leprae folP1*, *rpoB*, and *gyrA* is used to determine the potential for resistance to DDS, RIF, and fluoroquinolones, respectively (10–14). WHO Guidelines for Global Surveillance of Drug Resistance in Leprosy recommend that mutation detection of DRDRs in the three drug resistance genes be performed using PCR and DNA sequencing (15).

Several complete genome sequences of *M. leprae*, including strain TN isolated from a patient in Tamil Nadu, India, and strain Br4923 isolated from a patient in Brazil, have been reported (16, 17). Based on screening of >600 *M. leprae* isolates collected from different regions around the world, *M. leprae* can be classified into four single-nucleotide polymorphism (SNP) types (1 to 4) and 16 subtypes (A to P) (18, 19). The SNP genotyping

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TABLE 1 Clinical information for leprosy patients in this study[a]

National origin	Age (yr)	Sex	WHO classification	Clinical specimen	Type of case	yr sample was obtained	Sample name
Brazil	30–39	F	Unknown	Skin biopsy	New case	2014	ML015
Bangladesh	50–59	M	PB	Skin biopsy	New case	2015	ML007
East Timor	30–39	M	Unknown	Skin biopsy	New case	2014	ML026
Indonesia	20–29	M	MB	Skin biopsy	New case	2014	ML025
Japan	80–89	F	MB	Skin biopsy	New case	2016	ML003
	70–79	F	MB	Skin-slit smear	Relapse	2014	ML009
	60–69	M	Unknown	Skin-slit smear	Relapse	2013	ML010
	80–89	F	Unknown	Skin biopsy	Relapse	2014	ML024
	70–79	M	MB	Skin biopsy	New case	2015	ML028
Nepal	20–29	M	MB	Skin biopsy	New case	2018	ML001
	20–29	F	Unknown	Skin biopsy	New case	2018	ML002
	30–39	M	MB	Skin biopsy	New case	2016	ML004
	30–39	M	PB	Skin biopsy	New case	2016	ML012
Philippines	50–59	F	MB	Skin biopsy	New case	2015	ML005
	40–49	F	MB	Skin biopsy	New case	2015	ML008
	50–59	M	Unknown	Skin biopsy	New case	2019	ML013
	50–59	M	MB	Skin biopsy	New case	2014	ML023

[a]MB, multibacillary leprosy; PB, paucibacillary leprosy; unknown, not classified. F, female; M, male.

was defined by surveying 78 informative SNPs and six single-base insertion/deletions (InDels) (18) and is useful for identifying sources of *M. leprae* and tracking clonal transmission patterns.

DNA diagnosis of *M. leprae* drug resistance and SNP genotyping are usually performed by PCR and Sanger sequencing or whole-genome sequence (WGS) analysis (10–14, 17–19). Several computer programs to predict drug resistance of *Mycobacterium tuberculosis* strains based on WGS data have been developed (20), but obtaining WGS data for *M. leprae* from clinical specimens is challenging due to the presence of contaminating human DNA that is often present at much greater abundance relative to the amount of bacterial DNA, and that can preclude amplification of target *M. leprae* DNA by standard PCR (21, 22). Nested PCR, a modification of PCR that can be used to reduce nonspecific binding in products, addresses this challenge and is useful for rare templates or PCR with high background, but this method requires larger numbers of total cycles (23).

To monitor drug resistance trends and understand transmission patterns and genetic diversity in leprosy, we developed a rapid method that uses multiplex PCR in combination with nested amplification (nested multiplex PCR, or nmPCR) and amplicon sequencing to facilitate rapid and straightforward prediction of *M. leprae* drug resistance and determination of SNP genotype in clinical specimens.

MATERIALS AND METHODS

Ethics statement. This study was approved by the medical research ethics committee of the National Institute of Infectious Diseases for the use of human subjects (approval number 1172). All procedures in the study, including biological sample collection and testing involving human subjects, were performed in accordance with the Helsinki Declaration, ethical guidelines for medical research involving human subjects.

Clinical specimens. Seventeen clinical samples were randomly selected from our collections of skin biopsy specimens and slit-skin smears obtained from multibacillary (MB), paucibacillary (PB), and type-undetermined leprosy patients between 2013 and 2019. Patient characteristics are listed in Table 1.

DNA preparations from clinical specimens. DNA was purified from clinical specimens using a QIAamp DNA micro kit (Qiagen, Valencia, CA). Approximately 10 mg skin tissue was used as starting material and the extraction was carried out according to the manufacturer's protocol. The DNA was then eluted with 50 μl AE buffer.

Primer design. The primers used in this study are shown in Tables S1 and S2 in the supplemental material. Primers for DRDRs of the drug resistance genes and SNP genotyping in nmPCR were designed based on the genome sequence of *M. leprae* strain TN (GenBank accession number AL450380.1). The length of the outer and inner primers ranged from 20 to 25 bases. The guanine-cytosine contents of the primers were between 50 and 60%, except for several primers targeting the GC-rich regions. Amplicons ranging from 230 bp to 260 bp were expected in the first round of PCR, whereas the second round of PCR was expected to generate amplicons ranging from 130 to 150 bp.

nmPCR. The scheme for nmPCR is shown in Fig. 1. Two nmPCR procedures to amplify target regions were designed. One procedure amplified all 89 target regions in one tube (nmPCR-All). The other divided

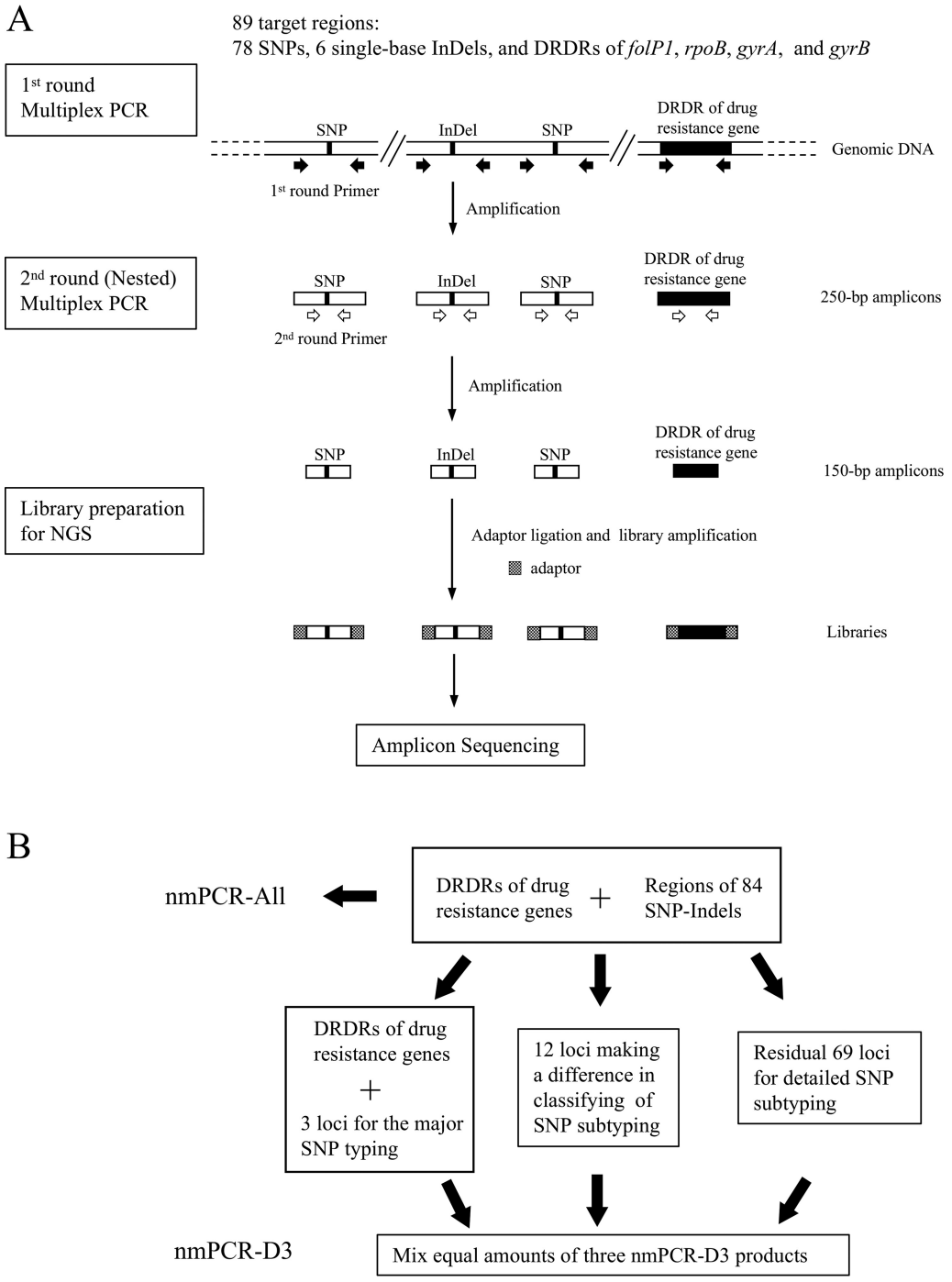

FIG 1 Scheme for nmPCR amplification of 89 target regions (A) and two nmPCR procedures (nmPCR-All and nmPCR-D3) to amplify target regions (B).

the target regions into three groups that were amplified in three separate tubes (nmPCR-D3). The three groups included the following: group 1, three loci for the major SNP typing and five loci for drug resistance determination; group 2, 12 loci that allowed classification of SNP subtype; and group 3, 69 residual loci for detailed SNP subtyping.

The first-round nmPCR-All was performed with PrimeSTAR HS DNA polymerase and GC buffer (TaKaRa Bio Inc., Shiga, Japan) in a 50-μl reaction volume, comprising 25 μl 2× GC buffer, 4 μl deoxynucleoside triphosphate (dNTP) mix (2.5 mM each dNTP), 0.25 μl PrimeSTAR HS DNA polymerase (2.5 U/μl), 8 μl of equimolar primer mixture containing 92 outer primer pairs (16 μM in total), and 100 ng DNA extracted from clinical specimens. For SNP14676, SNP1642875, and DRDR of *folp1*, two sets of primer pairs were used in the first-round PCR to overcome difficulties in amplification. The cycling conditions were 98°C for 5 min in the initial

denaturing step, followed by 40 cycles of 98°C for 10 s, 55°C for 5 s, 72°C for 15 s, and finally one cycle of extension at 72°C for 5 min. The reactions were carried out in a TaKaRa PCR Thermal Cycler Dice Touch (TaKaRa Bio Inc., Shiga, Japan). The second-round multiplex PCR was conducted with the same polymerase as that used in the first-round mPCR in a 50-μl reaction volume, using the equimolar primer mixture containing 89 internal primer pairs (16 μM total) and 1 μl of the first-round PCR mixture as the template. The temperature and cycling conditions were the same as those for the first round of PCR.

For nmPCR-D3, the same conditions as those for nmPCR-All were used, except for the primer mixture. In multiplex PCR for major SNP typing and drug resistance determination, 11 primer pairs for the first-round PCR and 8 primer pairs for the second-round PCR were used (Tables S1 and S2). In the remaining nmPCRs for 12 loci used for subtyping and 69 loci used for detailed subtyping, 12-12 and 69-69 sets of outer-inner primer pairs were used, respectively.

The amplified products were electrophoresed on 2% agarose gels containing 0.5 μg/ml ethidium bromide and were visualized with a UV transilluminator.

Preparation of amplicon libraries. After electrophoresis, DNA bands of around 150 bp were excised from the agarose gels. Purification of the amplified products was performed using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad Laboratories, Hercules, CA, USA) with the following modification: DNA was eluted in 200 μl Tris-EDTA buffer (pH 8.0). The extracted DNA was treated with phenol-chloroform (1:1) and precipitated with ethanol. The products of nmPCR-All and mixtures of the same amounts of the three nmPCR-D3 products were subjected to next-generation sequencing (NGS) analysis. For library preparation, an NEB Next Ultra II DNA library preparation kit for Illumina with NEBNext Singleplex Oligos for Illumina (New England Biolabs Inc., Ipswich, MA, USA) was used with the manufacturer's protocol.

Sequencing and data analysis for drug resistance determination and SNP genotyping. HiSeq2500 (Illumina, Inc., San Diego, CA, USA) was used for sequencing of PCR products from ML028 in nmPCR-All. NovaSeq 6000 (Illumina, Inc., San Diego, CA, USA) was used for sequencing of all other PCR products. The workflow of the NGS data analysis is summarized in Fig. S1. The data processing was performed on MacOS (version 10.4). The quality of each read was checked by using FastQC (version 0.11.9) (24). Low-quality reads in the raw reads obtained from amplicon sequencing were trimmed using Sickle (version 1.33) with basically the default setting and threshold for trimming based on average quality in a window modified to 30 (25). The trimmed reads were mapped on the reference genome (*M. leprae* strain TN) using the BWA-MEM algorithm with default parameters (26). The output SAM file was converted to a BAM file, and the file information was corrected using SAMtools with default settings (27). SAMtools mplieup and bcftools call with the default parameters were used to generate the VCF files containing SNP information (27, 28). Target region sequences of the BAM files and VCF files were confirmed with IGV software (29).

RESULTS

The nmPCR was optimized first. A total of 89 equimolar primer mixtures having various concentrations (4 μM, 8 μM, 16 μM, and 32 μM in total) were tested in the first- and second-round PCR using ML005 DNA as a template. The primer concentration that yielded the maximum amplification was 16 μM (data not shown). Thus, all nmPCR experiments were performed using 16 μM total primer mixtures. Representative images of results for nmPCR-All and nmPCR-D3 are shown in Fig. 2. In the first-round PCR, bands corresponding to an expected PCR product of approximately 250 bp were not seen. However, in the second-round PCR, products of around 150 bp were amplified in both nmPCRs.

DNA from 10 clinical samples (ML001 to ML005, ML007 to ML010, and ML028) yielded amplification products having the expected size of approximately 150 bp by nmPCR-All and nmPCR-D3. These PCR products were subjected to amplicon sequencing by NGS, and results for nmPCR-All and nmPCR-D3 were compared (see Table S3 in the supplemental material). The results showed that nmPCR-D3 amplified the target regions more efficiently than nmPCR-All. In 10 clinical specimens, nmPCR-All and nmPCR-D3 yielded sufficient reads ($\geq$26,989,826 raw reads and $\geq$25,916,222 trimmed and mapped reads). However, in seven samples, nmPCR-All obtained no reads for one or two loci in the major SNP typing (Table S3). In four samples (ML001, ML003, ML004, and ML028; Table S4), nmPCR-D3 yielded reads for all 89 loci. In addition, nmPCR-D3 yielded reads for the three loci for SNP typing and for five DRDRs in drug resistance genes in all samples. nmPCR-D3 also obtained reads for the 12 subtyping loci in all the samples, except for SNP313361 in ML015.

Based on these results, we used nmPCR-D3 to test the remaining 7 clinical samples (ML012, ML013, ML015, and ML023 to ML026). Overall, $>$97% of the reads from the amplicons were mapped to the reference genome, with the exception of ML012 and ML015, for which 64.11% and 65.77% of reads, respectively, could be mapped to the reference (Table S3). Positive rates for sequence determination and depths of the 84 SNP-InDels and DRDRs calculated using SAMtools are shown in Table S5. For determination of the major SNP type and drug resistance gene mutations in nmPCR-D3, the positive rates were $>$88.2% in all 17

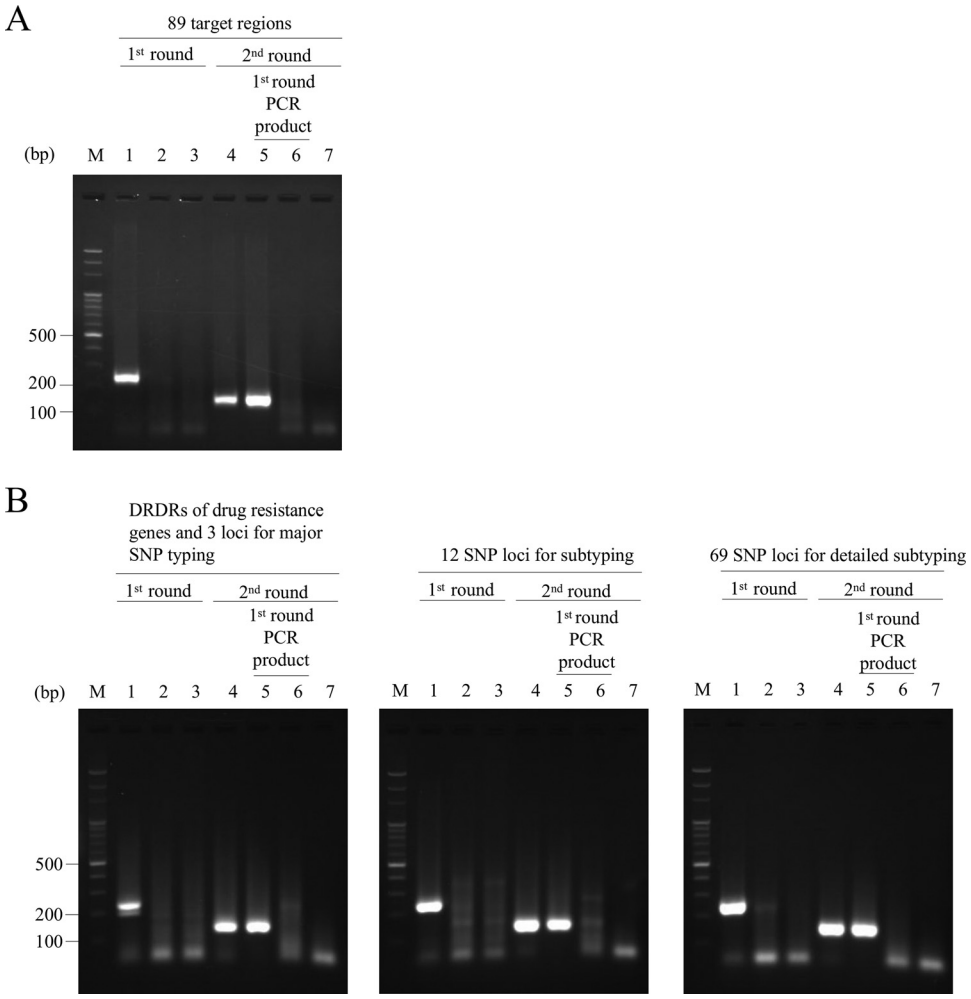

FIG 2 Agarose gel electrophoresis of nmPCR-All (A) and nmPCR-D3 (B). Lane 1, *M. leprae* DNA plus human DNA (100 ng + 100 ng, positive control); lane 2, ML002 clinical sample; lane 3, human DNA (100 ng, negative control); lane 4, *M. leprae* DNA plus human DNA (100 ng + 100 ng); lane 5, first PCR products for ML002; lane 6, first PCR products for human DNA; lane 7, water; M, 100-bp DNA ladder (Watson, Tokyo, Japan). In panel B, the gels show targeted DRDRs of drug resistance genes and 3 loci for major SNP typing (left), 12 SNP loci for subtyping (middle), and 69 SNP loci for detailed subtyping (right).

samples. For the 12 subtyping loci, the positive rates for nmPCR-D3 were also >88%. However, some of the 69 loci (e.g., SNP1324009, SNP298572, and SNP379804) for detailed subtyping had low positive rates for sequence determination.

SNP type, subtype, and drug resistance gene mutations in 17 clinical samples were next investigated by nmPCR and amplicon sequencing (Table 2). Seven samples, including two from PB patients, were subtype 1A, three were subtype 1D, and seven were subtype 3K. Seven samples containing *M. leprae* subtype 1A were from patients from countries in Southeast Asia such as the Philippines and Bangladesh, while one sample, ML015, was from a patient from Brazil. Three samples with *M. leprae* subtype 1D were from patients from Nepal. Among the *M. leprae* subtype 3K samples, 5 of 7 were from patients from Japan, and there was one each from patients living in Indonesia and the Philippines. The results of this study are generally consistent with previous data for the geographical association of *M. leprae* genotypes (Table 3). Of the 5 type 3K samples from Japanese patients, 3 had a mutation in the *folp1* gene. Of these, two (ML003 and ML010) had an A-to-G transition at nucleotide 157 (Thr53Ala) in *folp1,* and one (ML009) had a C-to-T transition at nucleotide 164 (Pro55Leu); these results were consistent with those obtained by standard PCR amplification and Sanger sequencing (data not shown). None of the samples had mutation in the DRDRs of *rpoB*, *gyrA*, and *gyrB*.

TABLE 2 Genotype and DRDR mutation in 17 clinical samples

SNP type	SNP subtype[a]	Origin	DRDR mutation	Sample name
1	A	Brazil	—[b]	ML015
1	A	Bangladesh	—[b]	ML007
1	A	East Timor	—[b]	ML026
1	A	Nepal	—[b]	ML012
1	A	Philippines	—[b]	ML008
1	A	Philippines	—[b]	ML013
1	A	Philippines	—[b]	ML023
1	D	Nepal	—[b]	ML004
1	D	Nepal	—[b]	ML001
1	D	Nepal	—[b]	ML002
3	K	Indonesia	—[b]	ML025
3	K	Japan	*folp1* (T53A)[c]	ML003
3	K	Japan	*folp1* (P55L)[c]	ML009
3	K	Japan	*folp1* (T53A)[c]	ML010
3	K	Japan	—[b]	ML024
3	K	Japan	—[b]	ML028
3	K	Philippines	—[b]	ML005

[a]The genotype of the strain having a sequence of 84 loci for SNP typing was not fully obtained and was determined using the genotyping scheme described by Cole et al. (30, 31, 37).
[b]No mutation.
[c]Deduced amino acid substitution in parentheses.

DISCUSSION

In this study, we developed a method using nmPCR followed by amplicon sequencing to simultaneously determine the presence of mutations in drug resistance genes and the SNP genotype of *M. leprae* in clinical specimens. Using this method, we demonstrated that the 84 informative SNP-InDels and mutations in DRDRs in *folP1*, *rpoB*, *gyrA*, and *gyrB* of *M. leprae* in 17 clinical specimens from MB, PB, and type-undetermined leprosy patients could be detected.

M. leprae SNP genotypes are typically determined by performing separate PCR amplifications for the SNPs, followed by Sanger sequencing or complete genome sequence

TABLE 3 Correlation between SNP genotype and geographical location

SNP type	Countries where the *M. leprae* genotype has been reported[a]	Reference(s)
1A (branch 1)	Bangladesh (B-105), Guyana (I487), India (TN), Indonesia (Indonesia-03), Japan (Japon-35), South Korea (Coree-04), New Caledonia (N-Cal-94003), Nepal (Nepal-02), Philippines (Phi-01), Thailand (Thai53)	18
1B (branch 1)	Antilles (S2_95034), French West Indies (89001), India (Inde-825)	18, 38
1C	Bangladesh (B-107), New Caledonia (N-Cal-97021), Nepal (Nepal-B14230)	18
1D (branch 1)	Bangladesh (B-112), Brazil (Br2016-18), French West Indies (88056), India (S11_Inde2), Japan (Japon-27), Madagascar (Mdg-B107), Malawi (Malawi-02), Nepal (Nepal-21A), Pakistan (Pak), Venezuela (V-17)	18, 19, 38
2E (branch 2E)	Ethiopia (ARLP-08), Malawi (Malawi-01)	18, 19
2F (branch 2F)	Iran (Iran-10), Denmark (Refshale_16),[b] Sweden (3077),[b] Turkey (Turc-1-6), UK (SK8)[b]	18, 38
2G	New Caledonia (N-Cal-82061), Nepal (Nepal-B5008)	18
2H (branch 2H)	Ethiopia (Afr-Eth)	18
3I (branch 3)	Brazil (Brazil-01), Denmark (Jorgen_625),[b] French West Indies (85054), UK (SK2),[b] Morocco (Maroc-01), Mexico (NHDP98), USA (Armadillo-260,[c] NHDP10), Venezuela (V-01)	18, 38
3J	New Caledonia (N-Cal-96008)	18
3K-0 (branch 0)	China (S10_Ch-04), Indonesia (Indonesia-16), Hungary (KD271), [b] Japan (Zensho-4), South Korea (Korea-3-2), New Caledonia (S9_96008), Philippines (CM1)[d]	19, 32, 38, 39
3K-1 (branch 5)	Japan (Zensho-9), South Korea (K02), Marshall Islands (US57), Philippines (PS04), Turkey (Turc2.3)	19
3L (branch 4)	New Caledonia (N-Cal-92041)	18
3M (branch 4)	France (Fr-2310), French West Indies (1261)	18
4N (branch 4)	Benin (Bn7-39), Brazil (Brazil-05), Guinea (Gu4-17), Ivory Coast (C-I-07), French West Indies (81030), Mali (S13_Ml-3-28), Morocco (Maroc-2704), Senegal (Senegal-88063), Venezuela (V-06)	18, 19
4O (branch 4)	Guinea (C30), Ivory Coast (C-I-02), Mali (S14_Ml-2-7), Senegal (Senegal-2662), Venezuela (V-07)	18, 19
4P (branch 4)	Brazil (Br4923), French West Indies (98007), Mali (Mali-C13), Venezuela (V-10)	18

[a]Representative strain is in parentheses.
[b]Ancient DNA extracted from medieval skeletons.
[c]*M. leprae* strains found in wild armadillo.
[d]*M. leprae* strains found in Cynomolgus macaque.

analysis (18, 19, 30, 31). A previous report showed that successful coverage for complete genome analysis of *M. leprae* could be achieved even if the bacillary index (BI) was as low as 1+, but the sequencing success rates were 23% for BI 1+ and 33% for BI 2+ (19). Therefore, PCR amplification would be a good solution to maximize the amount of information that can be obtained for *M. leprae* genomes in clinical specimens.

The results of our study are generally consistent with previous data for the geographical association of *M. leprae* genotypes (Table 3). Recently, phylogenetic analysis of *M. leprae* has defined six branches (branches 0 to 5) (32–34). Branch 1, including SNP subtypes 1A, 1B, and 1D, is mainly distributed in Southeast Asia. Branch 2 has branches 2E, 2F, and 2H, including subtypes 2E, 2F, and 2H, respectively (33). Branches 2E and 2H are mainly distributed in East Africa, and branch 2F is mainly in Europe. Branch 3 (subtype 3I) is distributed mainly in North and South America, while branch 4 (subtypes 3J, 3L, 4M, 4O, and 4P) is distributed in West Africa and South America (33). Branch 0 (subtype 3K-0) is found in Far East Asia and branch 5 (subtype 3K-1) in the Pacific Islands (32–34). In Brazil, subtypes 3I and 4P are predominant, and subtype 1D has been reported (19). In the present study, subtype 1A was detected from a Brazilian patient for the first time.

In two samples (ML012 and ML015), read depths were low in multiple loci, probably because of preferential amplification to the specific loci. The primer sequences may need to be considered. Goulart et al. reported that, compared with larger amplicons, the 130-bp amplicon improved *M. leprae* DNA detection by PCR in samples from skin lesions of tuberculoid (TT) and borderline-tuberculoid (BT) leprosy patients (35). Thus, design of amplicons shorter than 150 bp could improve the positive rates for target amplification. In addition, if the sample preparation for the targeted NGS is made easier, the utility value of our method will increase.

Nested PCR is useful for studies on certain human tissue microbiota, as this approach allows amplification of target DNA, having concentrations that are severalfold lower than that needed for standard PCR (23, 36). Meanwhile, NGS is a powerful tool that enables collection of several gigabases of nucleotide sequence in total. Using the method described in the present study, we could amplify and sequence any *M. leprae* genome region of interest by using appropriate primer pairs. Although NGS analysis currently requires specialized skills, the nmPCR with the sequence determination approach will be valuable for determining the SNP genotype and drug resistance of *M. leprae* in clinical specimens.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 6, XLSX file, 0.02 MB.

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