Table 1 (continued) Primers used for this study

Primer	Sequence	Application
MTRBWTF1	AATTCATGGACCAGAACAACCCGCT	introduction of point mutation at codons 507, 508, 510, 511, 512, and 513 deletion of codons 506–508
MTRBWTF2	CTGTCGGCGCTGGGGCCCGGCGGTC	introduction of point mutation at codons 522, 523, 526, and 531
MTRBWTR1	GGCTCAGCTGGCTGGTGCCGAAGAA	introduction of mutation at codons 514, 516, 518, 519, and 521 deletion of codon 518, and insertion of TTC between codons 514 and 515
MTRBWTR2	TCGGCGCTTGTGGGTCAACCCCGAC	introduction of point mutation TCG531→TTC and TCG531→TTG
MTRB507AGC	GGCTCAGCTGGCTGGTGCTGAAGAA	introduction of point mutation GGC507→AGC
MTRB507GAT	GGCTCAGCTGGCTGGTATCGAAGAA	introduction of point mutation GGC507→GAT
MTRB508CAC	GGCTCAGCTGGCTGTGGCCGAAGAA	introduction of point mutation ACC508→CAC
MTRB508GCC	GGCTCAGCTGGCTGGCGCCGAAGAA	introduction of point mutation ACC508→GCC
MTRB510CAT	GGCTCAGATGGCTGGTGCCGAAGAA	introduction of point mutation CAG510→CAT
MTRB511CCG	GGCTCGGCTGGCTGGTGCCGAAGAA	introduction of point mutation CTG511→CCG
MTRB513AAT1	TGCTCAGCTGGCTGGTGCCGAAGAA	introduction of point mutation CAA513→AAT
MTRB513AAT2	ATTTCATGGACCAGAACAACCCGCT	introduction of point mutation CAA513→AAT
MTRB513GAA	CGCTCAGCTGGCTGGTGCCGAAGAA	introduction of point mutation CAA513→GAA
MTRB516GAG	AATTCATGGAGCAGAACAACCCGCT	introduction of point mutation GAC516→GAG
MTRB516CAC	AATTCATGCACCAGAACAACCCGCT	introduction of point mutation GAC516→CAC
MTRB516GTC	AATTCATGGTCCAGAACAACCCGCT	introduction of point mutation GAC516→GTC
MTRB521ATG	AATTCATGGACCAGAACAACCCGAT	introduction of point mutation CTG521→ATG
MTRB522TTG	TCGGCGCTTGTGGGTCAACCCCAAC	introduction of point mutation TCG522→TTG
MTRB523GCG	TCGGCGCTTGTGGGTCAACGCCGAC	introduction of point mutation GGG523→GCG
MTRB523GGC	TCGGCGCTTGTGGGTCAAGCCCGAC	introduction of point mutation GGG523→GGC
MTRB526CTC	TCGGCGCTTGAGGGTCAACCCCGAC	introduction of point mutation CAC526→CTC
MTRB526TAC	TCGGCGCTTGTAGGTCAACCCCGAC	introduction of point mutation CAC526→TAC
MTRB526GAC	TCGGCGCTTGTCGGTCAACCCCGAC	introduction of point mutation CAC526→GAC
MTRB526TTC	TCGGCGCTTGAAGGTCAACCCCGAC	introduction of point mutation CAC526→TTC
MTRB526AAC	TCGGCGCTTGTTGGTCAACCCCGAC	introduction of point mutation CAC526→AAC
MTRB526CGC	TCGGCGCTTGCGGGTCAACCCCGAC	introduction of point mutation CAC526→CGC
MTRB526CAA	TCGGCGCTTTTGGGTCAACCCCGAC	introduction of point mutation CAC526→CAA
MTRB529AAA	TTTGCGCTTGTGGGTCAACC	introduction of point mutation CGA529→AAA
MTRB531TTC	CTGTTCGCGCTGGGGCCCGGCGGTC	introduction of point mutation TCG531→TTC

359 Table 1 (continued) Primers used for this study

Primer	Sequence	Application
360 MTRB531TTG	CTGTTGGCGCTGGGGCCCGGCGGTC	introduction of point mutation TCG531→TTG
361 MTRB506d	GGCTCAGCTGGCTGAACTCCTTGAT	introduction of mutation 506-508del
362 MTRBin514TTC	AATTCTTCATGGACCAGAACAACCC	introduction of mutation 514insTTC
363 MTRBd518	AATTCATGGACCAGAACCCGCTGTC	introduction of mutation 518del

364

365

366 Table 2 Rifampicin and Rifabutin susceptibilities of the recombinant *M. smegmatis*
367 strains
368

Mutation	Rifampicin		Rifabutin		Reference
	MIC (μg /ml)	Fold increase [a]	MIC (μg /ml)	Fold increase	
M. leprae					
wild type	1		0.25		
GGC507→GGG (silent)	1	1	0.25	1	This study
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	(3)
ACC508→ACA (silent)	1	1	0.25	1	This study
CAG513→GTG (Q513V)	32	32	8	32	(3)
GAT516→AAT (D516N)	32	32	2	8	(14)
CAG517→CAT (Q517H)	1	1	0.25	1	(11)
CAC526→TAC (H526Y)	32	32	8	32	(14)
TCG531→TTG (S531L)	32	32	4	16	(3, 14)
TCG531→TGG (S531W)	32	32	8	32	(14)
GCG532→TCG (A532S)	1	1	0.25	1	(11)
CTG533→CCG (L533P)	32	32	4	16	(14)
GTC547→ATC (V547I)	1	1	0.25	1	This study
M. tuberculosis					
wild type	1	1	0.25		
GGC507→AGC (G507S)	0.5	0.5	0.125	0.5	(1)
GGC507→GAT (G507D)	0.5	0.5	0.125	0.5	(1)
ACC508→CAC (T508H)	0.5	0.5	0.125	0.5	(1)
ACC508→GCC (T508A)	1	1	0.25	1	(1)
CAG510→CAT (Q510H)	1	1	0.25	1	(22)
CTG511→CCG (L511P)	16	16	1	4	(1, 12)
CAA513→AAT (Q513N)	8	8	0.5	2	(1)
CAA513→GAA (Q513E)	32	32	2	8	(1)
GAC516→GAG (D516E)	8	8	0.5	2	(12)
GAC516→CAC (D516H)	1	1	0.25	1	(1)

369
370

371 Table 2 (continued) Rifampicin and Rifabutin susceptibilities of the recombinant *M.*
372 *smegmatis* strains

Mutation	Rifampicin		Rifabutin		Reference
	MIC (μg /ml)	Fold increase	MIC (μg /ml)	Fold increase	
GAC516→GTC (D516V)	32	32	2	8	(12, 21, 22)
CTG521→ATG (L521M)	1	1	0.125	0.5	(21)
TCG522→TTG (S522L)	>32	>32	8	32	(21)
GGG523→GCG (G523A)	1	1	0.125	0.5	(1)
GGG523→GGC (silent)	1	1	0.25	1	(1)
CAC526→CTC (H526L)	32	32	4	16	(12, 22)
CAC526→TAC (H526Y)	>32	>32	8	32	(12, 22)
CAC526→GAC (H526D)	>32	>32	8	32	(12, 22)
CAC526→TTC (H526F)	>32	>32	4	16	(1)
CAC526→AAC (H526N)	32	32	2	8	(8)
CAC526→CGC (H526R)	32	32	8	32	(12, 22)
CAC526→CAA (H526Q)	8	8	0.5	2	(1)
CGA529→AAA (R529K)	32	32	4	16	(22)
TCG531→TTC (S531F)	32	32	4	16	(1)
TCG531→TTG (S531L)	32	32	8	32	(21, 22)
506-508del [b]	16	16	0.5	2	(5)
514insTTC [c]	>32	>32	8	32	(12) (22)
518del [d]	32	32	2	8	(22)

373

374 [a] fold increase in MIC compared to the wild type sequence, [b] deletion of codons 506-508, [c]
375 insertion of TTC between codons 514 and 515, [d] deletion of codon 518

376

377 Figure 1

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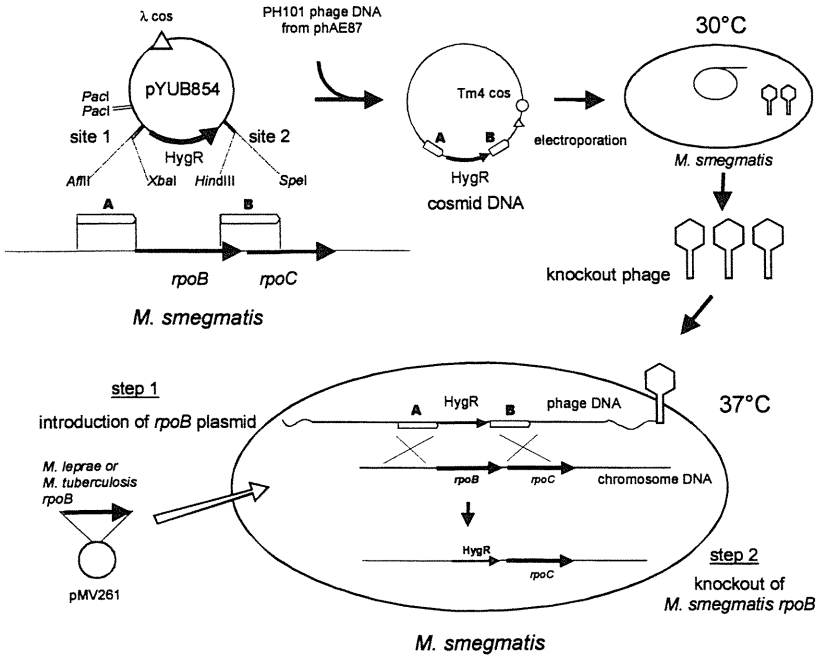

380 Figure 2

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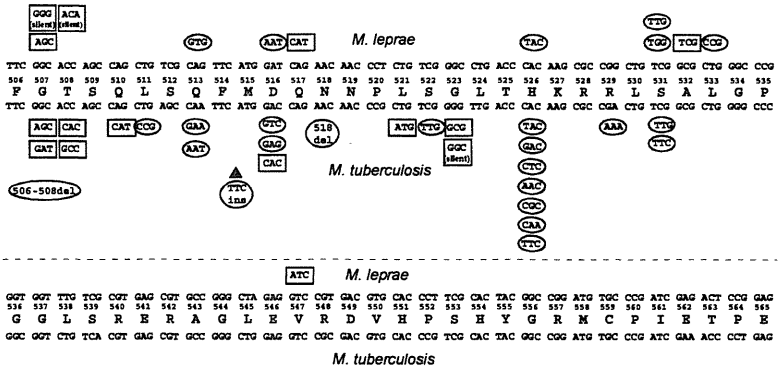

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Short Communication

FTA Card Utility for PCR Detection of *Mycobacterium leprae*

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SUMMARY: The suitability of the FTA® elute card for the collection of slit skin smear (SSS) samples for PCR detection of *Mycobacterium leprae* was evaluated. A total of 192 SSS leprosy samples, of bacillary index (BI) 1 to 5, were collected from patients attending two skin clinics in Myanmar and preserved using both FTA® elute cards and 70% ethanol tubes. To compare the efficacy of PCR detection of DNA from each BI class, PCR was performed to amplify an *M. leprae*-specific repetitive element. Of the 192 samples, 116 FTA® elute card and 112 70% ethanol samples were PCR positive for *M. leprae* DNA. When correlated with BI, area under the curve (AUC) values of the respective receiver-operating characteristic curves were similar for the FTA® elute card and ethanol collection methods (AUC = 0.6). Taken together, our results indicate that the FTA® elute card, which enables the collection, transport, and archiving of clinical samples, is an attractive alternative to ethanol preservation for the detection of *M. leprae* DNA.

In January 2010, the global registered prevalence of leprosy was 211,903 cases (1). To meet future challenges and to sustain the trend of decline in leprosy cases, WHO has developed simple guidelines for annual routine sentinel surveillance programs to monitor drug resistance (2). These programs detect *Mycobacterium leprae* drug resistance mutations using direct PCR sequencing of the drug resistance-determining regions (DRDR) of relevant genes. While most *M. leprae* samples are currently stored in 70% ethanol until laboratory analysis, the FTA® elute card (Cat. no. WB120401; Whatman Inc., Florham Park, N.J., USA) represents an alternative method for the collection and safe transportation of leprosy samples (3).

FTA® elute cards are designed for room temperature collection, shipment, archiving, and purification of nucleic acids from biological samples for PCR analysis. However, no studies analyzing the suitability of the FTA® elute card for preservation of leprosy slit skin smear (SSS) samples to detect *M. leprae* DNA have been reported thus far.

In this study, to compare the efficacy of PCR using DNA samples recovered from FTA® elute cards and from 70% ethanol, we performed nested PCR to amplify the *M. leprae*-specific repetitive element (RLEP). This is a highly sensitive method routinely used in molecular epidemiology for the detection of *M. leprae* DNA. In addition, the suitability of the FTA® elute card for the collection of SSS samples for PCR detection of *M. leprae* was evaluated.

In 2009, 192 multibacillary leprosy patients, with bacterial indices (BI) of 1 to 5, were recruited from the Central Special Skin Center at Yangon General Hospital and from Mandalay General Hospital. After giving informed consent, each patient submitted two SSS samples, which were preserved on an FTA® elute card or in 70% ethanol using separate sterile disposable blades. The first SSS sample was smeared directly onto an FTA® elute card. The second SSS was scraped from the same site and the blade was then immersed in a tube containing 1 ml of 70% ethanol. Samples were stored at room temperature until the tests were performed at the Department of Medical Research, Lower Myanmar (DMR). Forty-four randomly selected DNA samples were sent to the Leprosy Research Center (LRC) at the National Institute of Infectious Diseases (NIID), Tokyo, Japan, for external quality control. The research proposal was approved by the Institutional Ethical Review Committee at DMR. PCR efficacy of the samples harvested using the FTA® elute card or using ethanol was compared for each BI category.

DNA from SSS specimens preserved in 70% ehtanol was extracted according to Klatser's method (4). Briefly, sample tubes were centrifuged at 18,000 × g for 10 min, the supernatant was discarded, and the precipitate was suspended in phosphate buffered saline (PBS) (pH 7.2) and allowed to stand for 30 min. The suspension was then centrifuged at 18,000 × g for 10 min to remove remaining alcohol. The washed precipitate was suspended in 50 μl of lysis buffer containing 10 mg/ml proteinase K in 1 M Tris-HCL, pH 8.5, and 0.5% Tween 20, and incubated at 60°C for 18 h. After boiling at 97°C for 10 min, samples were frozen and thawed

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twice to disrupt bacilli completely. DNA was retrieved from FTA® elute cards according to the manufacturer's directions. Briefly, each was dried in a 60°C incubator for 30 min. The sample area was then cut out using a disposable 4-mm diameter biopsy punch, washed with 500 μl of sterile water, and then suspended in 30 μl of sterile water and heated at 95°C for 25 min. The eluted DNA was stored at −20°C until PCR analysis.

Nested PCR assay was performed to amplify RLEP. The RLEP element was targeted because its copy number in the *M. leprae* genome is known to be more than 35 (5,6). In the first round of PCR, a total reaction volume of 25 μl was used, containing 1 μl of SSS DNA as the template. The second round of PCR used the same reaction volume, and contained 1 μl of the outer PCR product, diluted 1:50 in sterile water, as the template (7). PCR was performed using the following conditions: initial 94°C denaturation for 4 min; 25 cycles of denaturation (95°C for 40 s), annealing (55°C for 1 min), and elongation (72°C for 20 s); and final elongation (72°C for 1 min). Distilled water and DNA from the *M. leprae* Thai-53 strain (8), purified at NIID, served as negative and positive controls, respectively. The PCR products were visulized by gel electrophoresis.

The data were analyzed using a statistical software package (version 9.3.2.0; MedCalc® software [http://www.medcalc.be]) and a receiver operating characteristic (ROC) curve was drawn (9). Additionally, a statistically significant difference between assays was confirmed by the chi-square test (10).

The number of samples in each BI category is shown in Table 1. Of the 192 samples, 116 samples from the FTA® elute cards (60%) and 112 samples from 70% ethanol (58%) were nested PCR positive for RLEP. When correlated with BI classification, 35% of the FTA® elute card and 45% of the 70% ethanol BI-1 samples were positive for *M. leprae*, 56 and 46% in BI-2, 57% each in BI-3, 59 and 62% in BI-4, and 82 and 77% in BI-5 or more, respectively (Table 1). The area under the curve (AUC) values of FTA® elute card and ethanol tubes ROC curves were similar (AUC = 0.6), and each method showed a statistically significant positive correlation between BI value and detection of *M. leprae* DNA by PCR (P = 0.01).

To test the reproducibility of FTA® elute card PCR efficacy, 22 DNA samples were randomly selected from each of the FTA® elute card- and 70% ethanol-harvested groups (approximately 10% of total samples) and sent to the LRC at the NIID for analysis. Twenty of the 22 samples in each group were PCR positive for *M. leprae*.

Table 1. Positive rate of PCR from FTA® elute card sample and 70% ethanol sample

Bacillary index (n)[1)]	FTA elute card (%)	70% ethanol (%)
BI-1 (31)	11 (35)	14 (45)
BI-2 (48)	27 (56)	22 (46)
BI-3 (29)	17 (57)	17 (57)
BI-4 (37)	22 (59)	23 (62)
BI-5 & >(47)	39 (82)	36 (77)
Total (192)	116 (60)	112 (58)

[1)]: Samples for each BI class.

Molecular detection from various samples collected using the FTA® elute card has been reported previously (11–13). In this study, the FTA® elute card was evaluated as a collection medium for SSS leprosy samples, and 60% of the FTA® elute card-harvested samples were found to be PCR positive for *M. leprae* DNA, as compared to 58% of ethanol-harvested samples. Moreover, the AUC values of the ROC curves were similar between FTA® elute card and 70% ethanol samples (AUC = 0.6), when correlated with BI. Taken together, our results indicate that the FTA® elute card provides a fast and reliable method for sample collection and DNA extraction for the detection of *M. leprae* that can replace 70% ethanol collection methods.

Using FTA® elute cards, DNA remains detectable by PCR more than 4 years after specimen collection (14), and the manufacturer claims to have obtained PCR amplifiable DNA after 14 years of storage. DNA elution from FTA® elute cards has several advantages over purification from 70% ethanol, involving a simple water/heat protocol that does not require special reagents or equipment. Direct sequencing of PCR products is widely used for *M. leprae* genotyping and drug resistance identification (15,16). The FTA® elute card provides both a small format and room temperature stability, making it particularly suitable for the collection of samples for *M. leprae* screening.

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Conflict of interest None to declare.

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Real-time PCR and high resolution melt analysis for rapid detection of *Mycobacterium leprae* drug resistance mutations and strain types

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Running title: *M. leprae* drug resistance screen and strain typing

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ABSTRACT

Drug resistance surveillance and strain typing of *Mycobacterium leprae* are necessary to investigate ongoing transmission of leprosy in endemic regions. To enable wider implementation of these molecular analyses, novel real time-PCR-high resolution melt (RT-PCR-HRM) assays without allele specific primers or probes and post PCR sample handling were developed. For the detection of mutations within drug resistance determining regions (DRDRs) of *folP1*, *rpoB* and *gyrA*, targets for dapsone, rifampicin and fluoroquinolones, real-time PCR-HRM assays were developed. A reference panel of wild type and drug resistant mouse foot pad derived strains which included three *folP1*, two *rpoB* and one *gyrA* mutation types were tested. RT-PCR-HRM correctly distinguished the wild type from the mutant strains. In addition, RT-PCR HRM analyses aided in recognizing samples with mixed or minor alleles and also a mislabeled sample. When tested in 121 sequence characterized clinical strains, HRM identified all the *folP1* mutants representing two mutation types, including one not within the reference panel. The false positives (<5%) could be attributed to low DNA concentration or PCR inhibition. A second set of RT-PCR HRM assays for identification of three previously reported SNPs that have been used for strain typing were developed and validated in 22 reference and 25 clinical strains. Real-time PCR-HRM is a sensitive, simple, rapid and high throughput tool for routine screening known DRDR mutants in new and relapsed cases, SNP typing and detection of minor mutant alleles in wild type background at lower costs than current methods and with potential for quality control in leprosy investigations.

INTRODUCTION

Leprosy is an infectious disease of skin and nerves caused by *Mycobacterium leprae*. The disease remains endemic in many parts of the world, and is now listed as a neglected tropical disease (27) by the World Health Organization. The drug dapsone was introduced in 1950, and administered in the form of long term monotherapy for treatment of leprosy; unfortunately, drug resistance emerged during the 1960s and 1970s (4). For this reason, in 1982, the World Health Organization (WHO) formally recommended multidrug therapy (MDT) which includes dapsone, rifampicin and clofazimine for the treatment and control of multibacillary (MB) leprosy (43). Sporadic reports of clinical resistance to dapsone and rifampicin started appearing in several countries such as Vietnam, Mexico, India and Philippines (1, 8, 13, 19, 24, 25, 26). Noncompliance and inadequate therapy may be the causes, particularly for MB leprosy. The drug targets and the mutations in the coding genes *folP1*, *rpoB* and *gyrA* that lead to clinical resistance to dapsone, rifampicin and the fluoroquinolones (used in an alternative leprosy drug regimen) respectively have been identified and characterized (5, 10, 14, 39). The *in vivo* drug susceptible or resistance phenotypes of various mutations seen in clinical strains in patient skin biopsies have been determined empirically by the traditional mouse foot pad inoculation assays (3, 20, 34). Mice are given drugs (in diet or by gavage) at different concentrations, and bacterial growth in the foot pads is measured at different time points. These assays have corroborated that clinical resistance to rifampicin, dapsone and oxfloxacin highly correlates (almost always) with detection of specific mutations within *M. leprae rpoB, folP1* and *gyrA* genes (21). For *M. leprae*, mutations have been reported in one or several codons located within short DNA regions in each of the target genes, thus defining the drug resistance determining regions (DRDRs). Although, it is possible that there are mutations outside the DRDRs of *rpoB, folP1* and *gyrA* or in other genes; or alternative mechanisms of resistance exist, these have yet to be identified. The MFP assays are labor, time and cost intensive; moreover the results are not available in time to

66 influence treatment options. Therefore, the MFP methods have given way to molecular methods
67 for screening proven resistance related mutations (42).

68 Furthermore, despite global MDT programs, the new case detection rates have not declined as
69 expected in many of the highly endemic countries (41), which indicates continued transmission
70 of the pathogen. In 2001, the first reference *M. leprae* genome of the 'TN' strain from a Tamil
71 Nadu, India leprosy patient was sequenced, offering new insight and opportunities for
72 development of tools in investigating bacteriology, pathogenesis and epidemiology. Mapping
73 polymorphic loci, such as 'variable number tandem repeats' (VNTRs) (7, 15) and single
74 nucleotide polymorphisms (SNPs) have applications in strain typing for tracing transmission of
75 leprosy. Four *M. leprae* lineages (SNP type 1, 2, 3 and 4) have been described, on the basis of
76 unique haplotypes derived from three SNPs which were identified by comparative genome
77 sequencing following the availability of TN strain genome sequence (28, 29).

78

79 Although it has become possible to perform amplification of the target loci by PCR followed by
80 DNA sequencing of the amplicons for detecting genetic variants (10, 11, 12, 32, 38, 40), the
81 labor and costs involved in PCR-DNA sequencing are still limiting factors for routine drug
82 resistance surveillance and SNP strain typing. Several surrogate methods such as single strand
83 conformation polymorphism (SSCP), reverse hybridization on membranes or microarrays have
84 been developed for allele specific detection of DRDR mutations from clinical specimens (23,
85 33), which require dedicated reagents or kits. PCR-Restriction fragment length polymorphism
86 assays developed by us expedited SNP typing by eliminating the sequencing steps, and replacing
87 them with conventional DNA electrophoresis for distinguishing the variants (31). DRDRs are not
88 suitable for PCR-RFLP, as there are different mutations at one or more positions. Numerous

89 PCR assays exist for allele discrimination, but often require multiple PCRs, allele target specific
90 primers or additional expensive probes (35).

91

92 In this context, we exploited the emerging real-time PCR technologies which can eliminate post
93 PCR procedures for genotyping any *M. leprae* genomic target of interest, particularly those
94 suitable for leprosy epidemiology applications. Real-time PCR-high resolution melt (HRM)
95 analysis is a novel simple post-PCR step that exploits thermal characteristics of the amplicons for
96 detection of sequence variants. This report describes the method development and validation of
97 real-time PCR-HRM assays for two applications: global drug resistance surveillance and SNP
98 based strain typing of *M. leprae*. These will be referred to as DRDR and SNP typing assays in
99 this study. The technical and practical considerations of the methods, and the advantages and
100 current limitations are discussed in the context of leprosy.

101

102 **MATERIALS AND METHODS**:

103 ***M. leprae* Reference and Clinical Specimens:**

104 *M. leprae* clinical strains maintained in mouse foot pad (MFP) system and in armadillo animal
105 systems were utilized as reference strains. Eighteen MFP strains were obtained from the Leprosy
106 Research Centre (LRC), National Institute of Infection Diseases, Tokyo, Japan (22); these strains
107 are therefore designated as MFP-LRC in this study. Suspensions of bacilli (10^8) were preserved
108 in 1ml of 70% ethanol. The armadillo derived *M. leprae* (ADML) clinical strains NHDP63,
109 Br4923, and 3039/210 obtained from infected tissues have been described previously (15).

110

The clinical skin biopsy samples used in this study were obtained from patients consulting at the Cebu Skin Clinic, Leonard Wood Memorial Leprosy Research Centre, Philippines as previously described (n=121) (18, 31), and at the Anandaban Hospital, Kathmandu, Nepal (n=25, during 2000-2010). The Philippine samples were stored in 70% ethanol at the time of collection. The Nepal samples were obtained from a repository of homogenized skin biopsies stored frozen in phosphate saline buffer containing 0.1% BSA. An aliquot of the homogenate was transferred to a fresh vial and suspended in 70% ethanol. All of the procedures involving biological sample collections and testing were performed following approval from the governing human research ethical committees and informed consent procedures as necessary.

DNA extraction from reference *M. leprae* cells and clinical tissue specimens:

As template DNA quality affects the amplification and the analysis by HRM, all the DNAs were prepared under uniform conditions using the DNeasy® tissue kit (QIAGEN, Valencia, CA) as described previously (15). The method involves proteinase K digestion and spin column chromatography. DNA was eluted in 100-200 µl AE buffer provided in the kit. For use as DNA concentration standards and as reference genotypes for the DRDR and SNP typing assays, approximately 1 mg of purified cells NHDP63 *M. leprae* purified from infected armadillo tissues as described previously were processed by DNeasy® tissue kit. These cells were prepared and obtained *via* the Leprosy research support-NIH-NIAID Contract at Colorado State University. The DNA was eluted in AE elution buffer. The DNA concentration was estimated by measuring UV light absorbance at wavelength of 260 nm. An aliquot of the stock DNA was adjusted with AE buffer to a final working stock of 10µg/ml, from which the DNA standards (1ng/ µl, 100pg/µl, 10pg/ µl, 1 pg/ µl and 0.1pg/µl) were prepared by 10 fold serial dilutions in

AE buffer. The DNA derived from MFP-LRC *M. leprae* (~10^8 cells) was diluted 1:100 in AE buffer; typically 1 µl of DNA was sufficient for one PCR.

Primers for real-time PCR-HRM DRDR and SNP typing assays:

Primer sets used for amplification of the DRDRs in *rpoB*, *folP1* and *gyrA* genes for real-time PCR-HRM are shown in **Figure 1**. The primers for the SNP typing assays are shown in **Table 1**. The primer sequences and nucleotide numbering system refer to those of the genome sequence of the TN strain (http://genolist.pasteur.fr/Leproma/). Primers for target loci were designed per the recommended amplicon size (less than 200-bp) for optimal real-time PCR-HRM genotyping using the freely available software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (20, 37). The target sequences were analyzed by MFOLD software to select the products with least secondary structure (http://mfold.rna.albany.edu/?q=mfold) (45).

Real-time PCR conditions and DNA template quantitation:

The real-time PCR (20 µl) was composed of 10 µl 2X Precision Melt Supermix (Bio-Rad Laboratories, Hercules, CA), forward and reverse primers (except for *rpoB* DRDR, 0.5 µl each, from 10 µM working stocks was used for the other five PCR amplicons; for *rpoB* 0.4 µl of each primer was used), nuclease-free water (8 or 8.2 µl) and DNA extracted from clinical and reference materials (1 µl). The reactions were set up in triplicate, in 96 well PCR plate and run on a CFX96® Real-Time PCR system (Bio-Rad). The CFX Manager™ software (Bio-Rad) was utilized to set up the sample arrangement on the PCR plate, to define PCR conditions, to monitor

the amplification in real time, to view melting curves and to calculate DNA concentrations and other PCR parameters.

The cycling parameters of PCR were as follows: 95 °C for 2 min followed by 45 cycles of 95 °C for 10 sec, 60 °C for 30 sec, 72 °C for 30 sec, and then a hetero-duplex formation step including 95 °C for 10 sec and 60 °C for 1 min. After the PCR amplification steps, melt curves for the products were generated by heating in 0.2 °C increments at a rate of 10 sec/step for the temperature range 65-95 °C.

DNA quantitation of templates was based on a standard curve developed with the five NHDP63 standards, each tested in triplicate for each target. The results were presented as C(t) values and starting quantity (SQ).

Real-time PCR-HRM analysis for cluster detection:

Post-PCR HRM analyses of the melt curves were performed using Precision Melt Analysis ™ software (Bio-Rad) which analyzes the temperature and shapes of the melting curves. The software classifies the data into different clusters. Data that are similar to each other are 'clustered' by the software and assigned a cluster number. The melt curves corresponding to each cluster are color coded for easy visualization. For each of the three targets, in DRDR (*folp1*, *rpoB* and *gyrA*) and SNP typing (locus 1, 2 and 3) assays, NHDP63 DNA was used to select the reference cluster. Clustering results are influenced by the melt region selected and cluster detection settings. The melt region can be auto-detected or manually defined by selecting the pre- and post melt temperature ranges. The cluster detection settings include melt curve shape sensitivity (default value of 50% clustering) and Tm difference threshold (default of 0.15

177 degrees). These settings can be adjusted to determine the stringency of the clusters. The
178 instrument and software manual indicates that for most applications, default settings produce
179 acceptable results; these were used as the starting points for analyzing melt curves obtained for
180 each of the six amplicons and mutation types within the reference and clinical samples.

181

182 **RESULTS**

183 **Development of DRDR assays based on real-time PCR-HRM analysis and assay validation**
184 **with reference specimens**

185 First, the performance characteristics of the real-time PCR assays using the primers, reagents and
186 reaction conditions were investigated using NHDP63 DNA. As shown in **Table 2**, it can be seen
187 that all three DRDRs were reliably amplified using the primer sequences shown in **Figure 1,**
188 with high quality performance values for PCR efficiency and correlation of determination (see
189 foot note, **Table 2**). A standard DNA curve (0.1 to1000 pg) using NHDP63 DNA was
190 established to enable the estimation of the *M. leprae* DNA in test samples and also used for assay
191 evaluation. The corresponding C(t) values ranged from 39 to 22. The cycle quantification C(t)
192 values obtained for the three DRDRs are comparable.

193

194 Next, the melt curves were analyzed by HRM. For the five concentrations of NHDP63 tested in
195 triplicate, the melt curves clustered together as a tight group; occasionally the standard with the
196 least input DNA (0.1 pg) separated into a different cluster. The normalized relative fluorescence
197 units (RFU) and differential RFU curves produced by the HRM Precision Melt Analysis software
198 in the melt region are shown (**Figure 2**). These results indicated that the PCR assay conditions

199 are suitable for testing other DNA samples, and that cluster classification for amplicons produced
200 at C(t) values of >35 may not be reliable and should be individually analyzed, repeated and
201 assessed by other tests.

202

203 The objective of the real-time PCR HRM assays was to determine if the samples that have
204 DRDR mutations of interest could be identified. By using NHDP63 DNA, which is of 'wild
205 type' sequence for the DRDRs in *folP1, rpoB and gyrA*, melt curves from the test samples that
206 do not 'cluster' with NHDP63 have putative sequence variations occurring anywhere in the
207 amplicon (hence designated as Cluster V), while those that cluster are of wild type sequence
208 (hence designated as Cluster WT). Prior to testing HRM assays on samples derived from leprosy
209 patients, a panel of 18 mouse foot pad (MFP) and one or more armadillo derived *M. leprae*
210 (ADML) of known DRDR mutations were utilized for validating the assays (**Table 3).**

211

212 The reference MFP-LRC strains that were available for this study represented several wild type
213 and two, three and one unique sequence variant(s) for the *folP1, rpoB and gyrA* amplicons
214 respectively (**Table 3)**. Furthermore, in this panel, one or multiple isolates represented each of
215 these genotypes. The DNA quantitation and HRM cluster classification (wild type, WT or
216 variant, V) are shown in **Tables 2 and 3**. The HRM cluster classifications were concordant in 8
217 WT and 10 mutant *folp1*; 12 WT and 6 mutant *rpoB;* and 14WT and 4 mutant *gyrA* DRDRs
218 (**Table 3**). Four situations are of note: 1) the strain Airaku-2 was expected to carry mutations in
219 both *rpoB* and *folP1*. Repeated HRM assays did not indicate these mutations, as was confirmed
220 from the amplicon DNA sequences. Therefore, VNTR strain typing was performed for all MFP-
221 LRC strains to verify strain identity (14, 44). The VNTR strain type of the sample received as

222 Airaku-2 was indeed not consistent with the expected VNTR (44) (data not shown). 2) For strain
223 Zensho-4, the *gyrA* amplicon was separated into a different cluster (blue curve in **Figure 2**, *gyrA*
224 panel) when compared to other strains that carry the expected mutation type. To verify if this
225 was due to a sample quality or quantity artifact, the PCR products was sequenced, which
226 confirmed presence of the mixed alleles at codon 91 (see **Figure 3)**. 3) For strain Zensho-5, the
227 HRM clustered the *folP1* and *gyrA* amplicons , as being different from NHDP63 and another
228 strain (Kusatsu-6) with the expected mutation type (blue and orange curves in **Figure 2,** *folP1*
229 and *gyr*A panels). Closer inspection of the sequence detected mixed alleles (in 53 and 55 codons
230 in *folP1* and codon 91 in *gyrA*) as shown in **Figure 3.** 4) For Zensho-9, HRM detected a variant
231 for *folP1*, which when sequenced verified the presence of a *folP1* DRDR mutation.

232

233 Altogether, these results validate that real-time PCR-HRM is suitable for distinguishing wild
234 type strains from those that carry some of the known mutations in the DRDRs of *folP1*, *rpoB* and
235 *gyrA*. Furthermore, HRM clustering can be sensitive to the presence of multiple alleles.

236

237 **Performance of the real-time PCR-HRM DRDR assays on clinical samples:**

238 Having validated that wild type and several mutation types in the DRDRs can be identified in
239 reference strains, the real-time HRM-PCR assays were tested on clinical samples. For this, 121
240 DNA samples extracted from clinical biopsies from Philippine patients that were analyzed
241 previously by conventional PCR-DNA sequence based mutation surveillance of *folP1* and *rpoB*
242 DRDRs were selected (18). The *gyrA* DRDR had not been sequenced. This sequence based
243 survey detected mutations in *folP1,* but not in *rpoB* DRDRs (18). The *folP1* mutation types