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Detection of Mutations in *folp1*, *rpoB* and *gyrA* genes of *M. leprae* by PCR- direct sequencing – A rapid tool for screening drug resistance in leprosy

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Summary
Introduction: Conventional Mouse foot-pad (MFP) assay for screening drug resistance in *M. leprae* is cumbersome and time-consuming (approximately 6 to 12 months). Molecular targets for different anti-leprosy drugs have been well defined. Molecular tools for rapid detection of drug resistance in *M. leprae* have been standardised. A study to compare molecular methods with MFP assay in determining the drug susceptibility of *M. leprae* was carried out.
Methods: Forty Bacteriological Index (BI) positive patients of leprosy with clinical features of relapse (25), new cases (11) and defaulters (4) were included in the study. A skin biopsy was done and the samples were processed using both MFP assay and Molecular method. PCR assays were carried out to amplify, 388 bp of *folP1*gene for dapsone resistance, 305 bp of *rpoB* gene for rifampicin resistance and 342 *bp* of *gyr*A gene for ofloxacin resistance, followed by direct DNA sequencing.
Results: Significant growth in the MFP test was obtained in only 28 out of 40 biopsies processed (70%). Ten of these isolates were dapsone resistant; one isolate showed combined resistance against dapsone, rifampicin and clofazimine. Amplification for all three genes was successful in all the 40 (100%) samples. Among *folP1* products sequenced, six isolates showed mutations at 53 (or) 55 amino acid positions. Those strains which showed high-level resistance with two log growth

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in MFP test, and/or showed growth in passage had mutations in *folp1* gene. No mutation was detected in *rpoB* and *gyrA* products. Thus no molecular evidence of Rifampicin resistance was found in the DNA isolated from biopsies.
Conclusion: Thus PCR-direct sequencing – the rapid and high sensitive molecular technique can be applied for detection of resistance against dapsone, rifampicin and ofloxacin in *M. leprae*, to over come the limitations of the conventional MFP assay.

Introduction

Multidrug therapy recommended by the World Health Organisation (WHO) in 1982[1] for treating leprosy is designed to prevent the spread of drug-resistant *M. leprae*. However drug resistance has been reported since 1964 for dapsone[2] in 1976 for rifampin[3] and also for ofloxacin – an alternative-therapeutic choice since 1996.[4] The conventional mouse footpad (MFP) assay carried out for screening drug resistance in leprosy is cumbersome, time-consuming – requires at least 6 months and relatively large numbers of bacteria. To identify leprosy patients with Multidrug Resistance (MDR), rapid methods for detection of drug resistance in *M. leprae* need to be established. Resistance to anti-leprosy drugs – dapsone, rifampicin and ofloxacin, evolves by amino acid substitution at the site of action of these drugs. Recent studies have identified point mutations in the *folP1* gene that encodes dihydropteroate synthase (DHPS) in dapsone-resistant *M. leprae*.[5,6] Rifampin resistance is associated with mutations in the *rpoB* gene that encodes the B subunit of RNA polymerase.[7] Resistance to ofloxacin is known to be associated with mutation in *gyrA* gene encoding the A subunit of DNA gyrase of *M. leprae*.[8,9] No molecular target has been defined for clofazimine. The susceptibility testing of *M. leprae* strains to these drugs is now made possible by a rapid DNA based PCR- direct sequencing method.

We carried out a study of cross sectional comparison of conventional MFP assay with molecular methods in the detection of drug resistant *M. leprae*, in the skin biopsies obtained from bacteriologically positive leprosy patients with relapse, new case and cases of defaulters with recurrence of disease. These three groups were selected as they are more likely to have drug resistance *M. leprae* strains. We examined the frequency of *M. leprae* mutations in the drug resistance determining region (DRDR) of *folp1, rpoB* and *gyrA* genes by molecular methods and compared with the performance of the *in-vivo* MFP assay. The hypothesis of the study is that the molecular methods may overcome the limitations of MFP assay in the detection of drug resistance.

Materials and Methods

Forty Bacteriological Index (BI) positive leprosy patients, who reported to the Central Leprosy Teaching and Research Institute (CLT&RI) from 2005 to 2007 were included in the study. The clinical status of the study patients were: relapse (25), new cases (11) or defaulters with recurrence (4) (Table 1).

A case of 'Relapse' is defined as '. . .a patient who successfully completed an adequate course of treatment but who subsequently developed new signs and symptoms of the disease either during surveillance period (or) there after.' Generally these patients had an increase of BI of 2+ or more, compared to their BI when they stopped their MDT.

Table 1. Details of the study cases

Clinical diagnosis	R-J classification	BI at RFT	Present BI	Duration of Treatment
Mono-Relapse	LL	0·0	5·3	DDS – 10 yrs
	LL	1·5	5·5	DDS – 7 yrs
	Histoid LL	0·0	3·0	DDS – 8 yrs
	LL	0·5	3·0	DDS – 10 yrs
	LL	NK	4·67	DDS – Duration – NK
	LL	0·0	3·3	DDS – 10 yrs
	BL	NK	5·16	DDS – 10 yrs
	LL	0·0	2·0	DDS – Duration – NK
	BL	0·0	2·0	DDS – 7 yrs
PB-MDT Relapse	LL	0·0	4·5	PB-MDT –Duration – NK
MB-MDT Relapse	BL	0·0	2·0	MB-MDT – 1 yr
	LL	0·0	3·0	MB-MDT – 1 yr
	LL	0·0	2·17	DDS – 6 yrs; DDS + CLF – 1 yr
	LL	NK	5·0	MB-MDT – 15 months
	BL-LL	0·0	5·5	MB-MDT – 6 months
	BL-LL	0·0	2·67	MB-MDT – 6 months
	LL	0·0	2·0	MB-MDT – 1 yr
	LL	0·17	4·67	MB-MDT – 1 yr
	LL	0·0	3·0	MB-MDT – 2 yrs
	BL	0·0	2·0	MB-MDT – 1 yr
	LL	0·83	5·67	MB-MDT – 1 yr
	LL	0·0	5·67	MB-MDT – 1 yr
	LL	0·0	4·5	MB-MDT – 1 yr
	LL	3·5	4·17	MB-MDT – 1 yr
	LL	NK	2·0	MB-MDT – 1 yr
New Case	BL-LL	NA	2·17	Nil
	LL	NA	4·5	Nil
	LL	NA	4·5	Nil
	LL	NA	3·5	Nil
	LL	NA	3·5	Nil
	LL	NA	2·0	Nil
	LL	NA	3·83	Nil
	LL	NA	4·83	Nil
	LL	NA	5·83	Nil
	LL	NA	5·0	Nil
	BL	NA	3·0	Nil
Defaulter	LL-Histoid	NA	4·5	MB-MDT – 3 months
	LL	NA	3·5	DDS-2 yrs
	LL	NA	5·67	MB-MDT – 3 months
	BL-LL	NA	4·17	MB-MDT – 6 months

NK – Not Known; NA – Not Applicable.

A ‘New case’ is a patient reported to the health facility with active signs and symptoms of disease without any history of previous treatment. These patients included in the study had a BI of 2+ or more. Since occurrence of primary drug resistance against the drugs of MDT, was reported,[10] smear positive new cases were also included in this study. A ‘Defaulter with recurrence’ is a patient who started treatment but has not received the full course – they could have taken at least 3 months of the course – and reported with new active lesions (or) worsening of the existing disease, requiring restarting of treatment. As irregular treatment is

a known potential cause for drug resistance, the defaulters who had incomplete treatment were included in the study.

Skin biopsies were obtained from the study patients after getting due consent. The biopsy samples were apportioned equally for MFP assay and molecular assay. A part of the biopsy samples were processed for histological examination to confirm clinical diagnosis. Samples for molecular assays were stored in deep freezers at – 20°C before processing. All the biopsy samples for MFP assay were processed within 48–72 hours by Rees' Method[11] at the Schieffelin Institute of Health Research and Leprosy Centre, Karigiri, Tamil Nadu, India, by following the national CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals) guidelines.

The biopsy samples were minced and ground in glass tissue grinders. A smear was prepared from the suspension and AFB were counted to enumerate the concentration per ml of suspension using conversion factor. The suspension was diluted to a final concentration of 10^4 AFB per 0·03 ml and the same volume was inoculated in to each of hind footpad of 27 normal CBA mice. The inoculated normal mice were segregated into control groups – fed with normal diet (three mice) and drug groups – fed with diet mixed with anti–leprosy drugs in different concentrations – viz:- dapsone three concentrations (0·01%, 0·001%, 0·0001%), rifampicin two concentrations (0·03%, 0·003%) and clofazimine three concentrations (0·01%, 0·001% and 0·0001%).

Harvest of the hind feet was carried at 6, 9 and 12 month intervals and the organism in each foot were enumerated. Tenfold (1 log) increase of growth in test mice when control mice showed 50 fold (1·5 log) increase was considered as 'Significant growth.'

If the bacterial count in the biopsy was less, additionally three Thymectomized-irradiated mice (Tr mice) were inoculated. If significant growth was observed in Tr mice, the recovered organisms were passaged into Tr mice and inoculated into normal mice for drug susceptibility testing. All growths found in test groups were passaged into normal mice and fed with respective concentration of drugs to confirm the drug resistant characteristics and to propagate the mutant strains.

All the samples were processed for DNA extraction as per the standard method of Herman *et al.*[12] Essentially the biopsy samples were minced with TE buffer and the cells were lysed with lysozyme followed by treatment with proteinase K and sodium dodecyl sulphate. Proteins and macromolecules were precipitated using NaCl and hexadecyl-trimethylammonium bromide-NaCl solutions. Nucleic acids were recovered from aqueous phase after extraction with chloroform and isoamyl alcohol. DNA was further precipitated overnight with isopropanol at −20°C. The pellet was washed with ethanol and later reconstituted in TE buffer.

The target regions of the *folp1*, *rpoB* and *gyrA* genes were amplified in a thermal cycler (Eppendorf) in 50 µl volume containing genomic DNA, 200 µM dNTPs, 1 TU of Taq polymerase and 10 µM of each primers, which were designed according to the sequence of the *folP1* (accession no. AL023093), *rpoB* (Z14314), and *gyrA* (Z70722) genes of *M. leprae*. Primers with the following sequence were used:-*folp1* F 5′-GCTTCTCGTGCCGAAGCG-CTCG-3′ and *folp1* R 5′-CCATCGCGGGATCTGCTCGCCC A-3′; *rpoB* F 5′-GACGCT-GATCAATATCCGT-3′ and *rpoB* R 5′-ACGGTGTTTC GATGAACCCG-3′; *gyrA* F 5′-ATGACTGATATCACGCTGCCA-3′and *gyrA* R 5′-ATAACGCATCGCTG CCGGTGG-3′.

Amplification of the target region of the *folP1* gene, the cycling conditions used were 95°C for 30 seconds, 60°C for 2 minutes, and 72°C for 3 minutes for 35 cycles, which yielded the product of 388 bp. (Figure 1).

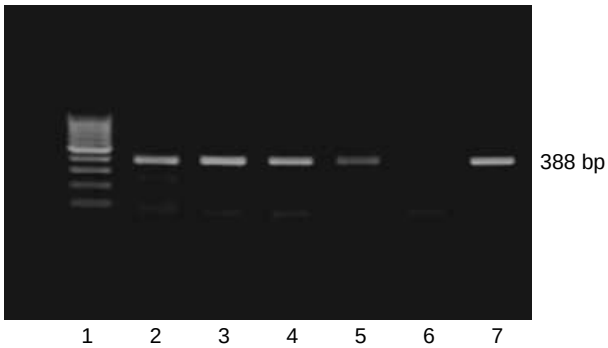

Figure 1. Agarose gel electrophoresis of PCR Product of the *folp1* gene (388 bp) – Lane 2,3,4,5 & 7- folp1 gene products; lane 1- 100 bp ladder; lane 6 – Negative control.

For amplification of *rpoB* and *gyrA* genes was carried out with a programme of 30 seconds at 95°C, 2 minutes at 50°C, and 3 minutes at 72°C for 40 cycles, which yielded products of 305 bp and 342 bp respectively.[9] (Figures 2 and 3).

All the PCR products for sequencing were recovered from low melting agarose gels using Qiagen MiniElute PCR purification kit after electrophoresis. DNA Sequencing was done using Bigdye terminator v 3.1cycle sequencing kit (Applied Bio system) in ABI Prism 310 Genetic Analyser at the Tuberculosis Research Centre, Chennai, India. The sequences were analysed with the Sequencing Analysis software. The DNA sequences of the PCR products of three genes studied were compared with the Gene sequence data base of 'Leproma'. (http://genolist.pasteur.fr/Leproma/)

The Study was approved by the institutional scientific ethics committee of the Central Leprosy Teaching & Research Institute, Chengalpattu, Tamil Nadu, India. Informed consent was obtained prior to the collection of bacterial samples.

Results

The diagnosis of 25 cases of relapse included in this study was made on clinical suspicion and supplemented with evidence of relapse by histological examination – they were LL-19,

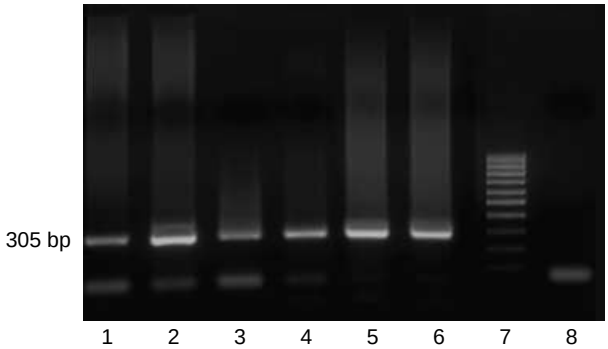

Figure 2. Agarose gel electrophotesis of PCR Product of the *rpoB* gene (305 bp) – Lane 1–6- *rpoB* gene products; lane 7- 100 bp ladder; lane 8- Negative control.

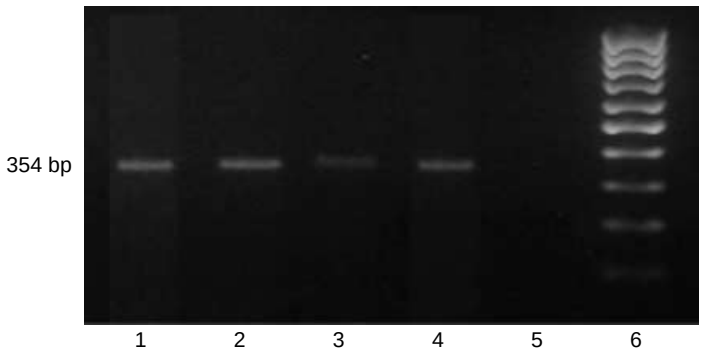

Figure 3. Agarose gel electrophoresis of PCR Product of the *gyrA* gene (354 bp) – Lane 1–4- *gyrA* gene products; lane 5-Negative control; lane 6- 100 b ladder.

BL-4 and BL-LL-2. Fifteen of them relapsed after MDT treatment (PB-MDT-1 and MB-MDT-14) and nine after monotherapy with DDS and one case had DDS for 6 years followed by combination of DDS + clofazimine for 1 year. In 21 relapse cases the BI result at the time of release from treatment (RFT) was available. Sixteen of them had BI = 0, at RFT and five had BIs ranging from 0·17 to 3·5. At the time of relapse their BI ranged from 2 to 5·67. The 11 new cases (LL-9; BL-1 and BL-LL-1) included in this study had BIs ranged from 2 to 5·83. Among the four defaulters included in this study three of them had incomplete MB-MDT treatment for 3–6 months; two with LL leprosy and one with BL-LL leprosy. One of the four defaulters was a case of LL leprosy and had incomplete monotherapy with DDS for 2 years. All of them reported back with new active lesions or worsening of the disease (Table 1).

Among 40 cases investigated using the MFP assay, 28 (70%) of the biopsies, had conclusive growth of *M. leprae* distributed among 16 (64%) of 25 cases of relapse, nine (81·8%) of 11 New cases and three (75%) of four defaulters. In 12 biopsy samples (nine cases of relapse, two new cases and one defaulter) no significant multiplication was observed in the control group – suggesting that the biopsy samples had no (or) fewer viable bacilli. This condition was observed in higher proportion among cases of relapse (36% (9/25) (Table 2).

Analysis of susceptibility of 28 cases which showed conclusive results revealed 18 strains of *M. leprae* were dapsone–sensitive and 10 isolates resistant to dapsone. The 10 resistant strains were distributed among six cases of relapse, three new cases and one defaulter. Five (50%) of the isolates showed high degree resistance (R100); four isolates showed

Table 2. Drug susceptibility testing by MFP assay

Clinical status	No growth	DDS Sen	Total DDS Res	DDS Res R100	DDS Res R10	DDS Res R1	RMP Sen	RMP Res (0·03%)	CLF Sen	CLF Res (0·01%)
Relapse (25)	9	10	6	4	1	1	15	1	14	2
New case (11)	2	6	3	0	3	0	9	0	8	1
Defaulter (04)	1	2	1	1	0	0	3	0	3	0
Total (40)	12	18	10	5	4	1	27	1	25	3

Note: DDS- Dapsone; RMP- Rifampicin; CLF- Clofazimine; Res- Resistant; Sen- Sensitive.

intermediate degree resistance (R10) and one isolate showed low degree resistance (R1). A higher proportion of isolates from cases of relapse were R100 strains (four of six (66·7%). All the three isolates from new case were R-10 and the one isolate from the defaulter was R100 strain (Table 2).

One isolate from a case of relapse which showed R100 resistance to dapsone also showed combined resistance to rifampicin and clofazimine at higher concentrations. In addition two isolates showed resistance to clofazimine only. No other rifampicin or clofazimine resistance was observed among the isolates studied.

PCR assays for *folp1*, *rpoB* and *gyrA* gene showed amplifications from all the 40 (100%) samples investigated. The sequencing of the amplified PCR products of all the three genes revealed conclusive results (either wild (sensitive) or Mutated (resistant)) in all (100%) the samples processed.

PCR assay for *folp1* gene revealed mutations in six out of 10 dapsone-resistant strains (60%) and in the remaining 34 isolates no mutation was observed, including all those dapsone sensitive strains and all the strains showed no growth in control group of mice by MFP assay (Table 3).

A comparison of MFP results with mutation detection in *folp1* gene showed that mutations were observed in four out of five (80%) R100 strains; two out of four (50%) R10 strains and nil in the one R1 strain. All the mutations in *folp1* were missense mutations at codon 53 (2 strains) (Thr53Arg-1; Thr53Gly-1) (or) at codon 55 (4-strains); (Pro55Leu-2; Pro55Arg-2) (Table 3).

No mutations were observed in *rpoB* gene in all 40 strains, including the one strain which showed rifampicin resistance along with dapsone and clofazimine in MFP assay. Also no mutations were seen in *gyrA* gene in all the 40 strains sequenced.

Out of 40 study patients, 23 cases could be followed-up, after their completion of treatment. They were 14 cases of relapse (six – mono relapse; eight – MDT relapse), eight new cases and one defaulter. Among them, nine were sensitive to all the drugs by MFP assay and sensitive to dapsone and rifampicin by molecular method, five showed no growth in MFP assay but were found sensitive to dapsone and rifampicin by the molecular method, seven were resistant to dapsone by MFP assay of which five were found resistant by molecular method, and two were resistant to clofazimine by MFP assay. All these patients were started

Table 3. Comparison of dapsone susceptibility by MFP assay and detection of mutation in *folp1* gene by molecular methods

MFP Assay			PCR- Direct sequencing		
Drug concentration	Type of resistance	No. of Strains	Mutation found	Amino acid substitution	Mutation not found
DDS (0·01%)	High Resistant (R100)	05	04 (80%)	Pro55Leu-2 Pro55Arg-1 Thr53Arg-1	01
DDS (0·001%)	Intermediate Resistant (R10)	04	02 (50%)	Pro55Arg-1 Thr53Gly-1	02
DDS (0·0001%)	Low Resistant (R1)	01	0	0	01
Total		10	06	06	04

on MB-MDT, immediately after investigation and responded well to the treatment both clinically and bacteriologically.

Discussion

The limitations of MFP assay for studying the susceptibility of *M. leprae* to different anti-leprosy drugs, and the developments in designing molecular tools for the rapid detection of drug resistance gave an impetus to study the feasibility of applying these techniques for rapid detection of drug resistance in leprosy.

Among 40 cases investigated MFP assay showed conclusive results in 28 (70%) strains, whereas molecular methods showed conclusive results in all the 40 (100%) strains. MFP assay revealed 10 strains resistant to dapsone (R100-5, R10-4, R1-1) – molecular methods detected mutation in six out of 10 dapsone resistant strains (60%). Of which four out of five (80%) were of R-100 and two out of four (50%) were of R-10 strains. Thus mutation in *folp1* gene was associated with high level DDS resistance – as shown by others.[6,13]

All the six mutations in *folp*1gene were missense mutations either at codon 55 (four) - Pro55Leu-02; Pro55Arg-02) – most of which (three out of four (75%)) belonged to secondary dapsone resistance or at codon 53(2)- Thr53Arg-01; Thr53Gly-01)-1 each belonged to secondary & primary dapsone resistance. Thus mutation at codon 55 was more frequently seen in R100 resistant strains as observed by others.[6]

No mutation was detected in *folp1* gene among 18 of dapsone susceptible (100% specificity) strains and among 12 of those strains that showed no growth in MFP assay. No strain showed mutation in *rpoB* and *gyrA* gene. No molecular marker has been identified yet for clofazimine, hence the molecular method cannot be adopted to detect clofazimine resistance.

Since the daily dosage of dapsone in MDT is 100 mg, only the high level resistance (R100) is clinically relevant.[13] In our study, except for one case of relapse (four out of five) all with high level resistance (R100) showed mutations in *folp1* gene. Two case of relapse (one with IR and another with LR) and one new case with IR did not show mutation. Further, these three cases attained less than 2 log growth in the harvest and the passage did not show any growth (Table 4).

The only case of relapse with high level resistance to DDS & RMP, that did not show mutation in *folp1* and *rpoB* genes, was a case of PB-MDT relapse who had irregular treatment after starting MB-MDT. The harvest of MFP showed less than 2 log increase of growth and passage to confirm the resistant characteristic did not show growth against DDS, RMP and CLF. When reported after 2 years, clinically the patient had regression of nodules. The BI showed no significant increase. Generally we observed that those strains with high-level resistance attaining around 2 log growth in MFP harvest, and / or showed growth in passage had mutations in *folp1* gene (Table 4). Similar observation of loss of concordance between MFP assay and molecular methods has been expressed, pointing weakness in the protocol for administering dapsone to mice.[14] This prompts us to suggest that the definition of 'significant growth' (i.e. 1 log increase) in drug group or the protocol of administering dapsone may have to be reviewed.

Apart from being cumbersome and time consuming, the MFP assay requires expensive facilities and sustained expertise. In the aftermath of decline of the prevalence of leprosy globally, most of the laboratories which had expertise in MFP inoculation have

Table 4. Comparison of mutations in *folp1* gene with growth in the MFP during harvest and passage

		MFP results in DDS group			
Clinical Status	Previous Treatment	Type of resistance	Harvest Count	Growth in passage	*folp1* mutation
1. Relapse	PB-MDT	HR	$1{\cdot}4 \times 10^5$	No growth	None
2. Relapse	Mono	HR	2×10^6	Growth (+)	Pro55Leu
3. Relapse	MB-MDT	HR	$2{\cdot}8 \times 10^6$	Growth (+)	Pro55Arg
4. Relapse	MB-MDT	HR	$1{\cdot}1 \times 10^6$	Growth (+)	Pro55Leu
5. Deflt/recur	MB-MDT Irregular	HR	1×10^6	Growth (+)	Thr53Arg
6. New case	Nil	IR	3×10^5	Growth (+)	Thr53Gly
7. New case	Nil	IR	$7{\cdot}31 \times 10^5$	Growth (+)	Pro55Arg
8. Relapse	MB-MDT	IR	$1{\cdot}8 \times 10^5$	No growth	None
9. Relapse	MB-MDT	LR	2×10^5	No growth	None
10. New case	Nil	IR	$5{\cdot}6 \times 10^5$	ND	None

Note: PB-MDT- Pauci-bacillary Multi-drug therapy; MB- MDT- Multi-bacillary Multi-drug therapy; HR- High degree resistance; IR- Intermediate degree resistance. LR- Low degree resistance, SEN- Sensitive; Deflt/recur- Defaulter with recurrence. ND- Not done.

disappeared.[15] Further the success of MFP assay is largely dependent on the biopsy containment and the elapsed time until mouse inoculation – this warrants the transport of the biopsy sample in wet ice and inoculation should be carried out within 48–72 hours.

The molecular method which comprises of DNA extraction, PCR amplification and product purification will require only few days. The sequencing of PCR products at a central facility may take few more days. Thus the molecular method requires about a week or even less. PCR – the basic molecular facility – is becoming more feasible and affordable in many laboratories even in developing countries like India. Sequencing facility which requires higher capital expenditure can be centralised. Molecular assays in addition to its rapidity, high sensitivity also offers solutions to the inherent problems of MFP assay. Since this PCR-based assay requires only DNA as the starting material, the problem of transporting the sample and the time delay until the processing of the sample have no limitation on the success of this technique. Considering all these factors, WHO has recently initiated sentinel surveillance for drug resistance in leprosy using molecular techniques.[14]

Although the finding of our study is not new, it is a first report of its kind in this region and also it emphasis the potential of molecular techniques in rapid detection of drug-resistant *M. leprae.* However, the gene-based detection of resistance has few inherent limitations like the mechanism of resistance not mediated by target gene mutations cannot be detected, or the gene may be present but not necessarily translated. Further studies are required to explore the application of these rapid molecular methods in routine investigations of drug resistance in multi bacillary leprosy.

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