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Original Research Article

Comparison of merits of DNA sequencing, PCR-SSCP and MFP assays in the detection of drug resistance in *Mycobacterium leprae*

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Abstract

Purpose: *To compare the merits of conventional Mouse Foot Pad (MFP) assay, Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP) analysis, and Direct-DNA sequencing in identification of drug resistance in M. leprae.*
Methods: *A total of 41 leprosy cases were investigated for drug resistance using MFP assay, whereby growth results were obtained in 23 cases. The DNAs extracted from these samples were amplified in polymerase chain reaction (PCR) using specific primers in order to determine the drug resistance-loci. Furthermore, PCRSSCP analysis was carried out using PCR amplicons, while gel electrophoresis was performed to identify any shift in mobility in any one of the DNA strands, as well as the pattern of mutation.*
Results: *A total of 5 isolates exhibited the highest degree of resistance R100, whereas 4 isolates showed intermediate level of resistance R10. In contrast, the least resistance was seen in R1. There were no mutations in 4 out of 10 strains which were dapsone-resistant, i.e., 1 HR, 2 IR and 1 LR. Moreover, there was no mutation in 305 bp sequence of the rpoB gene. DNA sequencing sensitivity was 60 %, whereas the sensitive isolates tested in the experiments exhibited no mutation, resulting in 100 % specificity.*
Conclusion: *These results indicate the advantages of molecular techniques over conventional MFP technique. The study has revealed that PCR-SSCP analysis, a rapid, specific and less expensive method, has greater potential for use in routine testing of the susceptibility of M. leprae to rifampicin and dapsone, than the other tests.*

Keywords: *Molecular techniques, Mycobacterium leprae, Lapromatous, Leprosy, Drug resistance, Mouse Foot Pad (MFP) assay, Polymerase Chain Reaction-Single Strand Conformation Polymorphism (PCR-SSCP)*

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INTRODUCTION

Leprosy, an infectious disease which is predominant in Asian countries, imposes serious health burden [1,2]. Due to dapsone resistance in the treatment of leprosy, rifampicin and clofazimine were developed and added to form a new treatment regimen designated as Multi-Drug therapy (MDT) for leprosy [3,4]. The global spread of leprosy is associated with appearance

of resistant strains of *M. leprae*. Drug resistance screening in *M. leprae* is a time-consuming process. In contrast, MFP is the only gold standard for confirming *M. leprae* [5,6]. Molecular techniques for elucidating drug resistance have gained immense importance in recent times, probably due to their technological advantages [7].

Dapsone resistance in *M. leprae* occurs through dihydropteroate synthase (*folP*) gene with two genetic homologues (*folP1* and *folP2*) which possess notable sequence homology [8]. The gene is encoded by five-point mutations at 53 and 55 codon positions [9,10]. Resistance to rifampicin occurs in a conserved region in the *rpoB* gene which encodes the β subunit of RNA polymerase in *M. leprae*. This resistance possibly impacts on the codons 401–427, predominantly involving substitution of Leu for Ser425, and substitution of Met and Phe for Ser425 [11,12]. Different quick molecular techniques have been used to screen the drug susceptibility of *M. leprae* so as to find out its resistance to dapsone, rifampicin and fluoroquinolone [13].

The PCR-SSCP technique is touted to be an easy qualitative molecular method in which the wild-type mutant target DNA is denatured first, followed by electrophoresis in a non-denaturing polyacrylamide gel. This method is based on the fact that the single-stranded DNA in solution possesses a defined secondary structure under specific conditions, so that the mutations in rpoB and folp1 genes of *M. leprae* can be identified [14].

It is known that DNA sequencing is a gold standard for all mutation studies, and it remains highly advantageous because it is fast and precise in detection of mutation locus and identification of the nature of mutations. However, it cannot be applied routinely since it is a cost-consuming process. To the best of our knowledge, there is no single study till date that compared the performance of the available methods for determining the drug resistance of *M. leprae*. Therefore, the current study was aimed at comparing the advantages of conventional MFP assay with those of PCR-SSCP analysis and Direct-DNA sequencing in the elucidation of drug resistance of *M. leprae*.

EXPERIMENTAL

Ethical statement and approval

All procedures used in this study were carried out in accordance with the Declaration of Helsinki (1964) and its later amendments. Written informed and formal consent was obtained from each of the patients and participants.

Personal information and history of medical condition were also obtained from each subject using a standard questionnaire. All experimental procedures and protocols were approved by the Ethical Research Board and Committee.

Susceptibility test

The study included a total of 41 Bacteriological Index (BI)-positive leprosy patients who exhibited clinical features of mono-relapse and MDT-relapse, as well as new cases and defaulters. Skin biopsy was performed, and the samples were processed using MFP assay as well as molecular methods. Each skin biopsy sample was divided into two portions: one portion was used for molecular assays, whereas the other portion was used for MFP assay. The whole biopsies for MFP assay were conducted within a timeline of 48 - 72 h to ensure that MFP inoculation was done as per Rees method. The experiments were conducted at our Hospital Research Centre in accordance with CPCSEA standards. Each biopsy was briefly minced and a smear was applied to prepare a 1-mL suspension using a conversion factor. The suspension was then diluted to achieve a concentration of 10^4 AFB/0.03 mL which was inoculated into hind foot pads of 27 CBA mice. In the control group, mice were fed normal diet, whereas those in the drug groups were fed diet combined with different concentrations of anti-leprosy drugs. At 6, 9 and 12 h, samples were taken and subjected to enumeration of *M. leprae*. Growth was measured and found to be 10 ten times higher in test mice, when compared to 50-fold increase observed in control mice i.e., significant growth achieved.

PCR-SSCP analysis

Total DNA was extracted from all samples using standard method [15]. The biopsy samples were minced in TE buffer, after which the cells were lysed using lysozyme. This was followed by treatment with proteinase K and SDS. The DNA pellet obtained after extraction with chloroform isoamyl alcohol was immediately kept in isopropanol at -20 ^oC. Prior to use, the pellet was washed with ethanol and then reconstituted with TE buffer. The DNA was amplified in PCR using primers in order to determine the drug resistance-loci. The PCR products were purified using Qiagen Mini Elute PCR purification kit, after which the DNA was sequenced using ABI

310 genetic analyser (Applied Biosystems, Life Technologies Corporation, CA). The resultant genetic sequences were cross-verified with TN reference strain sequence database with the help of sequence analyzer software. Furthermore, the data were compared with the gene sequence data base of Leproma.

Following PCR amplification, PCR-SSCP was carried out with the help of PCR amplicons according to the method of Mani *et al* [16]. Each PCR-amplified fragment was denatured at 95 ℃ for 5 min in a micro-centrifuge tube with an equal amount of stop buffer (2 mM EDTA, 95 % formamide and 0.05 % bromophenol blue). Ice was used to snap-cool the reaction mixture, followed by immediate loading of the mixture in 10 % polyacrylamide gel. Then, electrophoresis was carried out in a vertical slab gel apparatus (Amersham Pharmacia Biotech, China) in which 1X Tris-Borate-EDTA was used as the running buffer.

Electrophoresis was carried out for 17 h at 60 V at 4 ℃, and the DNA bands in the gel were visualized via silver staining process as follows: after treating the gel with 10 % ethanol for about 5 min and 1 % nitric acid treatment for 3 min, 0.2 % silver nitrate solution containing 1ml of 10 % formalin was used to stain the gel for 20 min. After washing the gel thrice with distilled water (3 min for each wash), the gel was exposed to the developing stain (3 % sodium carbonate solution containing 10 % formalin). The reaction was arrested with the addition of 100 ml of 10 % glacial acetic acid. The shift in mobility that occurred in any one of the DNA strands in the test sample was contrasted with the mobility of relevant strands of the control sample.

Statistical analysis

All values are expressed as mean ± standard deviation (mean ± SD). Statistical analysis was carried out using SPSS 21.0 software. Student's *t*-test was performed for comparison between the two groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

A total of 41 different leprosy cases were investigated using MFP assay, and the results showed *M. leprae* growth in 23 cases. When these 23 cases were analysed, a total of 13 strains were dapsone-sensitive, whereas 10 isolates (6 relapse cases, 3 new cases and 1 defaulter) were resistant to dapsone. A total of 5 isolates exhibited highest degree of resistance R100 (HR), whereas 4 isolates showed intermediate level of resistance R10 (IR). Least resistance (LR) was seen in R1. Results from PCR assays of the *folp1* and *rpoB* genes revealed that they were amplified in all the 23 growth isolates investigated in the study.

Conclusive results were achieved from the sequencing of the amplified PCR products of *folp1* and *rpoB* genes for the 23 growth samples investigated, as shown in Table 1. The DNA sequencing on 388-bp amplified fragment which extended up to DRDR of *folp1* gene in the 41 *M. leprae* isolates, showed mutation in only 6 out of 10 strains: 4 HR and 2 IR dapsone-resistant strains with the amino acid substitutions Pro55Leu-2, Pro55Arg-2, Thr53Arg-1 and Thr53Gly-1 (Table 1).

There were no mutations in 4 out of 10 strains which were dapsone-resistant (1 HR, 2 IR and 1 LR), and no mutation was found in 305 bp sequence of the rpoB gene in complete 23 growth strains. The DNA sequencing sensitivity was 60 %, whereas the sensitive isolates which were tested in the experiment exhibited no mutation, resulting in 100 % specificity. The rest of the isolates exhibited absence of mutation in all the strains, without any growth in MFP assay. The PCR-SSCP profiles of the 23 *M. leprae* strains were obtained.

Altered mobility was observed in DNA strands in 6 dapsone-resistant isolates which were identified in the sequencing process, when compared to DNA strands of the reference strain. The PCR-SSCP results were contrasted with those from traditional MFP assay and DNA (Table 1). The current study had a total assay sensitivity of 60 %, while achieving 100 % specificity. The results from the PCR-SSCP analysis were obtained within 24 h post-DNA extraction. Figure 1 and Figure 2 show the PCR-SSCP profiles of *folp1* gene and *rpoB* gene, respectively, as obtained from gel electrophoresis.

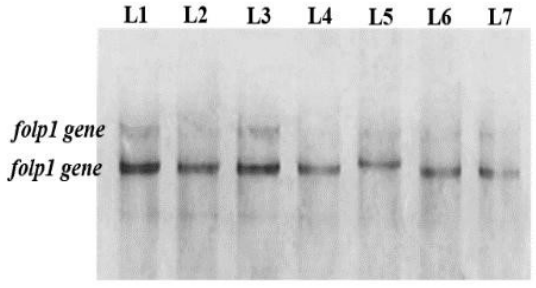

Figure 1: Gel electrophoresis of PCR-SSCP products of the *folp1* gene showing the pattern of mutation band

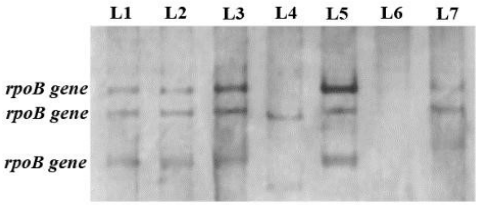

Figure 2: Gel electrophoresis of PCR-SSCP products of the *rpoB* gene showing the pattern of mutation bands. No mutation was observed in Lane 3 which was a mixture without amplicons

DISCUSSION

The use of molecular techniques has been advocated for confirmation of clinical diagnosis of leprosy since confirmation through MFP assay is difficult and laborious. In general, molecular tools are not required for identification of multi-bacillary groups associated with high lesions. However, molecular tools are required to ease assessment of bacterial viability and resistance in difficult clinical cases such as pure neural leprosy, and indeterminate and pauci-bacillary leprosy.

In leprosy transmission studies as well as MDT resistance studies, molecular tools have proven to be efficient in the identification of *M*. *leprae* infection among house-hold contacts and other high-risk groups [14].

Table 1: Clinical features and results of susceptibility to dapsone in the 23 cases

No.	Isolate code	Clinical features	Susceptibility	Mutation	MFP	Sample site (how obtained)
1	PHGD-21	Mono-relapse	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
2	PHGD-32	Mono-relapse	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
3	PHGD-35	Mono-relapse	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
4	PHGD-38	Mono-relapse	Dapsone-sensitive	No Mutation	Susceptible	Arm (smear)
5	PHGD-41	Mono-relapse	Dapsone-sensitive	No Mutation	Intermediate Resistant	Earlobe (smear)
6	PHGD-54	MDT-relapse	Dapsone-resistance	Pro55Arg	High Resistant	Skin (smear)
7	PHGD-56	MDT-relapse	Dapsone-resistance	Pro55Leu	High Resistant	Skin (smear)
8	PHGD-58	MDT-relapse	Dapsone-sensitive	No Mutation	Susceptible	Arm (smear)
9	PHGD-59	MDT-relapse	Dapsone-resistance	Thr53Arg	Intermediate Resistant	Earlobe (smear)
10	PHGD-67	MDT-relapse	Dapsone-sensitive	No Mutation	High Resistant	Arm (smear)
11	PHGD-68	MDT-relapse	Dapsone-resistance	Pro55Leu	High Resistant	Skin (smear)
12	PHGD-69	MDT-relapse	Dapsone-sensitive	No Mutation	Susceptible	Earlobe (smear)
13	PHGD-81	MDT-relapse	Dapsone-resistance	Thr53Gly	Intermediate Resistant	Skin (smear)
14	PHGD-83	MDT-relapse	Dapsone-resistance	No Mutation	Susceptible	Skin (smear)
15	PHGD-85	MDT-relapse	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
16	PHGD-91	MDT-relapse	Dapsone-sensitive	No Mutation	Least Resistant	Skin (smear)
17	PHGD-93	New cases	Dapsone-resistance	No Mutation	Susceptible	Skin (smear)
18	PHGD-102	New cases	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
19	PHGD-105	New cases	Dapsone-resistance	Pro55Arg	High Resistant	Skin (smear)
20	PHGD-121	New cases	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
21	PHGD-123	New cases	Dapsone-resistance	No Mutation	Susceptible	Skin (smear)
22	PHGD-126	New cases	Dapsone-sensitive	No Mutation	Susceptible	Skin (smear)
23	PHGD-131	Defaulter	Dapsone-resistance	No Mutation	Intermediate Resistant	Skin (smear)

Molecular tools with simple techniques that incur low costs can be made easily accessible for routine laboratory diagnosis and surveillance purposes in endemic countries [17]. Nucleotide target alterations in chromosomal genes are the primary causes of drug resistance in *M. leprae* and other mycobacterial species, rather than plasmid acquisition or transposons of other bacterial species. The genotypic techniques are efficient only with respect to accurate information on the mutations that are associated with drug resistance [18].

Out of the 41 cases under investigation in this study, the MFP assay produced conclusive results in 23 strains (56 %), whereas conclusive results were obtained for all the 41 strains using the molecular methods i.e., 100 %. Dapsone resistance of *M. leprae* in mouse footpad can be segregated into low, intermediate and high degrees. Five isolates showed high degree of resistance, while 4 isolates fell under intermediate degree of resistance. Only 1 isolate exhibited low degree of resistance.

Studies have showed that during DNA sequencing of *folp1* gene, 41 *M. leprae* isolates were accurately identified due to mutations in *folp1* gene and *rpoB* gene [19,20]. In the current study, the 6 missense mutations observed in dapsone-resistant strains involved 3 strains with secondary DDS resistance which had mutations in folPl codon 55: CCC-CGC Pro55Arg (2 strains), 53: ACC-AGG Thr53Arg- 1 (1 strain), and three strains with primary DDS resistance mutations at codon 55 CCC-CTC Pro55Leu (2 strains) and ACC-GGC Thr53Gly (1 strain). It has been reported that it is difficult to clearly establish whether low-degree dapsone resistance with corresponding mutation of ACC to GCC at codon 53 is true resistance or not [21]. However, published reports have identified such mutation in *M. leprae* [19,20]. In contrast, low resistance mutation was not identified in the current study. Moreover, 4 out of 10 (40 %) dapsone-resistant isolates showed no mutation in DRDR. The presence of mutations in any part of the gene, or the availability of alternative resistance mechanism such as membrane permeability of efflux pump would have resulted in cryptic resistance of the isolates. There were no amino acid substitutions at DRDR in *fop1* gene among the 13 isolates which were sensitive to dapsone [22]. Furthermore, mutation was absent in 18 strains (43.9 %) which exhibited nil growth in MFP assay.

In a previous study, it was established that the pattern of mutations that occur in *rpoB, folP1* and *gyrA genes* of South American *M. leprae* isolates made the strains resistant to rifampin and dapsone [23]. The mutations identified in the current study are in agreement with the pattern obtained in studies in other nations. When PCR products are directly sequenced, the results are definite, and they quickly identify the resistant cases. However, the main disadvantages are the high costs associated with experimentation and sequencing, which are quite unaffordable for developing countries. In order to avoid these disadvantages in DNA sequencing, the PCR-SSCP technique was developed for identifying mutants. Kim *et al* utilized PCR-SSCP to identify the point mutations in *folp1* and *rpoB* genes of *M. leprae* with 100 % specificity [24]. In the current study, results from PCR-SSCP were contrasted with those from the traditional MFP and direct DNA sequencing. The results were in agreement with results obtained using direct DNA sequencing. The assay showed 60 % sensitivity and 100 % specificity which were similar to corresponding values obtained from DNA sequencing.

Although, molecular tools have gained importance in *M. leprae* drug resistance studies, the sensitivity of the techniques is poor, as a result of which the MFP technique is still a gold standard for assessing the growth and drug resistance of *M. leprae*. However, MFP assay is an expensive, cumbersome and time-consuming process which also requires expertise in MFP inoculation of *M. leprae*. Furthermore, the results depend on biopsy microbial load and time interval between biopsy and inoculation.

The current study showed that with respect to sensitivity and specificity, PCRSSCP is comparatively more efficient, faster and less expensive than MFP assay. With 48 - 72 h of turn-around time, the primary harvest of the culture in this technique seems to be promising. Although DNA sequencing can be completed within 48 - 72 h, it is a cost-consuming process, when compared to PCR-SSCP. Furthermore, technical expertise and automated DNA sequencer are required to perform direct DNA sequencing, and the efficiency of the assay is completely based on the frequency of accessible resistance-associated genomic mutations.

However, PCR-SSCP has a drawback, viz, poor sensitivity, when compared to DNA sequencing. Indeed, at least 15 % of the total population needs to show resistance to the drug. Moreover, being an easy and simple qualitative technique, PCR-SSCP cannot be used to differentiate silent mutations. In spite of these drawbacks, PCR-SSCP completely satisfies the requirements of

developing countries in terms of simple equipment with easy operating procedures.

CONCLUSION

The PCR-SSCP technique is an easy-to-adopt process that has inherent advantages and can be used in developing countries that have limited infrastructure, to detect drug resistance in *M. leprae*. Although comparison of the three methods, as was done in the current study, is not novel; it is the first-of-its-kind in this region. Furthermore, the study shows the advantages of molecular techniques over conventional MFP technique. The findings of this study suggest that PCR-SSCP analysis is a rapid, specific and affordable method with great potential for use in routine tests for susceptibility of *M. leprae* to rifampicin and dapsone, than other tests.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Fan Long and JiuLong Li designed all the experiments and revised the paper. Fan Long, JiuLong Li, Jiedeng Jia performed the experiments and wrote the manuscript. Fan Long and JiuLong Li contributed to this work equally.

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