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Molecular detection of *Mycobacterium leprae* using RLEP-PCR in post elimination era of leprosy

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ABSTRACT

Leprosy is considered as a contagious disease and is still a health problem in several countries including India. Diagnosis of leprosy is based either on clinical findings or on acid fast bacilli staining. Due to low sensitivity of acid fast bacilli staining most of the leprosy cases were remained undetected. The present study aims to assess the efficacy of RLEP-PCR in the field condition where majority of the patients are acid fast bacilli negative and have early disease. A total of 80 suspected leprosy cases were recruited. Slit skin smear samples were taken for microscopy and molecular experimentation. DNA was extracted and RLEP-PCR was executed for all the 80 samples. To establish the statistical correlation χ^2 test and Fisher's exact test were made. To elucidate the sensitivity of the test Receiver Operating Characteristic (ROC) was drawn. These 80 leprosy patients comprised of 38 paucibacillary and 42 multibacillary leprosy cases. Of 80 leprosy patients 18 (22.5%) were AFB positive while 53 (66.25%) leprosy cases were RLEP-PCR positive. The results of test of significance (P=0.0001) and Cohen's kappa coefficient (κ) (0.614) indicated that the RLEP-PCR is a better diagnostic tool over AFB microscopy in case detection of leprosy. From the findings we concluded that RLEP-PCR could be used for the definitive detection of leprosy cases in accordance with the clinical findings in the field condition in the post elimination era of leprosy.

Keywords: Leprosy; RLEP-PCR; Paucibaccilary; Multibacillary

INTRODUCTION

Leprosy is an infectious disease caused by *Mycobacterium leprae*. In the severe state it leads to permanent deformity of skin, nerve, limb, eye etc. Infection without symptom can continue for 5 to 20 years [1]. Approximately 220 000 new cases of leprosy are diagnosed worldwide and this incidence rate has been essentially stable over the last decade [2]. Leprosy is

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clinically characterized by one or more of the three cardinal signs: hypo-pigmented or erythematous skin patches with loss of sensation, thickened peripheral nerves, and acid-fast bacilli detected on skin smears or biopsy material.

Leprosy is transmitted via aerosols in people with close contacts, in particular within household contacts, having the highest risk of acquiring the infection [3, 4]. *M. leprae* is an obligate parasite and cannot be cultured in any artificial medium. This property leads to the non effective and non definitive diagnosis of leprosy. The diagnosis of leprosy based either on clinical findings or on acid fast bacilli (AFB) staining. Interestingly all the paucibacillary (PB) cases and some multibacillary (MB) cases essentially in borderline (BB) cases were found to be negative by AFB staining [5, 6]. Laboratory based tools like slit skin smears (SSS) and histopathology are less sensitive tool for definitive case detection of leprosy. This leads to difficulty in early diagnosis of leprosy by clinical criteria alone. Thus in clinical practice, the detection of *M. leprae* by PCR in patients with negative baciloscopy or inconclusive histopathology would be of great value to define leprosy diagnosis. The use of repetitive sequence as a PCR target provides the advantage of higher sensitivity over other targets in the DNA because of its presence at multiple copies in genomic DNA. The polymerase chain reaction (PCR) has been applied to detect *M. leprae* in different clinical samples, such as slit-skin smear [7, 8], nasal cavity [9], skin tissues [10]. In the present study we addressed the use of RLEP-PCR for definitive diagnosis of leprosy in the field condition where majority of the patients are AFB negative and have early disease.

MATERIALS AND METHODS

Ethical statement: The study has been approved by Institutional Ethical Committee (IEC) before initiation of the study. Inform consent was taken from each of the patients at the time of sample collection. Participants who were children, informed consent was obtained from parent/guardian.

Collection of samples: A total of 80 leprosy cases were recruited in the study. The cardinal signs and symptoms of all patients were noted carefully before taking the slit skin smear (SSS) samples. Details of demographical data were given in the Table 1. SSS samples were collected from the ear lobes and from the edge of the active skin patches using sterile surgical blade. One sample from the ear lobe of each patient was put in the 1 ml of TE buffer and labeled for patient ID, village name and date of collection. All the samples were transported to laboratory and stored at -20^{0}C for further experimentation.

Table 1: Age wise distribution of leprosy cases in relation to spectrum of the disease

Leprosy Type	Age Group						
	1-14	15-24	25-34	35-44	45-54	>55	Total
BT	1	6	9	11	9	2	38
BB	4	3	6	5	6	4	28
BL	1	2	0	3	4	4	14
Total	6	11	15	19	19	10	80

Extraction of *M. leprae* Genomic DNA: DNA extraction was done by the method described previously [11]. In brief, TE buffer containing slit-skin samples was centrifuged at 21,000 rpm for 10 min, TE buffer was discarded, and 700 µl of extraction buffer (700 mM NaCl, 50 mM Tris-HCl [pH-8], 10 mMEDTA, 1% β- 2mercaptoethanol and 1% cetyl-trimethyl ammonium bromide) was added to the pellet and mixed by vortexing. The mixture was incubated at 65°C for 1 h, and an equal volume of chloroform-isoamyl alcohol (24:1) was added, vortexed, and centrifuged for 10 min at 10,000 g. The aqueous phase was precipitated with cold isopropanol and centrifuged at 10,000 g for 10 min. The pellet was washed with 70%

ethanol, air-dried, and resuspended in 10 µl of TE buffer. The DNA samples were quantified in a BioPhotometer (Eppendorf) for the absorbance at 260 nm (for DNA), 280 nm (for protein), and 230 nm (for RNA). Samples were diluted to 15 ng/µl for conducting RLEP-PCR experment.

Amplification of DNA by PCR (RLEP-PCR): Stringent precautions were taken to avoid cross-contamination. In all these experiments sterile tubes and plugged tips were used. PCR reactions were performed in 25 µl reaction mixture consisting of 5 µl of DNA template, 0.2 m mol deoxynucleoside triphosphate, 0.5 mol l^{-1} primers and 1U Taq polymerase (Bangalore Genei, Bangalore, India). The 129 bp fragment was amplified by using primers and procedure described by Donoghue *et al* [11]. The forward primer for RLEP was, 5-TGC ATG T CA TGG CCT TGA GG3'and the reverse primer was, 5'CAC CGA TAC CAG CGG CAG AA3'. RLEP PCR was performed at 95^0C for 2 min (initial de-naturation) and then for 45 cycles in 94^0C for 30 sec, 58^0C for 2 min and 72^0C for 2 min, 72^0C for 8 min (final extension) and hold at 4^0C. Each amplification reaction was analysed on 2% agarose. Gels were stained with ethidium bromide and photographed using gel documentation system. A 100bp DNA marker (Bangalore, Genei) was included on every gel for comparison purpose.

Data analysis: The statistical significance of the differences in sensitivities among RLEP-PCR and AFB microscopy were measured by means of Chi-square test and Fisher's exact test. SPSS (version 21; Chicago, IL) was used for statistical analysis. In medical science analysis of inter-rater agreement data often provides a useful of assessing the reliability of a rating system [12]. Thus Cohen's kappa coefficient (κ) was used to measure the inter-rater reliability agreement between two tests (AFB and RLEP-PCR) using GraphPad Prism software.

RESULTS

A total of 80 leprosy patients were recruited in this study. These 80 leprosy patients comprised with 38 PB, 42 MB cases. All the 38 PB cases were fall in the category of borderline tuberculoid (BT) leprosy cases while 42 MB cases constituted 28 BB and 14 borderline lepromatous (BL) leprosy cases. In the PB category 16 patients were found to have 1 active patch while 9 were having 2 active patches and 13 were having 4 active patches. Likewise incase of MB category 27 were having 6-9 active patches and rest 15 were having 10-12 active patches. Of these 80 patients 18 (22.5%) were AFB positive while 62 cases (77.5%) were AFB negative on SSS examination by ZN staining (Table 2). In case of PB spectrum all the BT leprosy affected persons were AFB negative while in MB spectrum 18 were AFB positive, including 7 BB and 11 BL type leprosy patients.

Table 2: Comparative positivity of the AFB and RLEP-PCR in the spectrum of leprosy

Positivity	Type of leprosy cases			Total
	BT	BB	BL	
AFB	0	7/28 (25%)	11/14 (78.57%)	18
RLEP-PCR	19/38 (50%)	20/28 (71.42%)	14/14 (100%)	53

DNA was extracted from suspension of slit smear scrapings; RLEP PCR was done as explained above. A band of 129 bp was observed on 2% agarose gel (Fig. 1). The positivity of RLEP-PCR was 53 (66.25%) leprosy cases out of 80 cases while AFB positivity was 22.5%. These 53 RLEP-PCR positive leprosy cases included 34 MB (20 BB and 14 BL type of leprosy patients) and 19 PB leprosy cases of BT type leprosy (Table 2).

The comparative statistical analysis between AFB microscopy and RLEP-PCR yielded significant P value for the Fisher's exact test (0.0001). The χ^2-test value for the comparison of

two tests was 29.27 (P=0.0001). Estimation of inter-rater reliability Cohen's kappa coefficient (κ) was calculated as 0.614 with sum of error (SE) of kappa 0.072 and 95% confidence interval from 0.372 to 0.656.

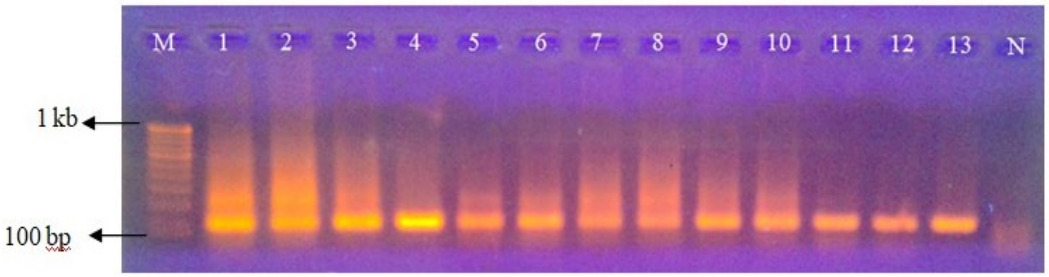

Figure 1: RLEP-PCR amplification showing 129 base pair amplified product in 2% agarose gel. Lane M: 100 bp ladder, Lane 1-2: Positive control, Lane 3–13: Positive slit skin scrapping samples of leprosy patients, Lane N: Negative control.

DISCUSSION

Leprosy is a public health problem in several countries including India [13]. Although multibacillary leprosy can be diagnosed easily using clinical examination and microscopy, the diagnosis of early disease, indeterminate forms, and cases in which the bacilli are difficult to demonstrate, continues to be a problem. Mainly the PB cases with faint clinical signs put a hurdle in the leprosy case detection.

For the past two decades, PCR-based detection of *M. leprae* DNA in clinical samples has become increasingly important in the field of molecular diagnostics of leprosy. A potential PCR assay capable of detecting DNA as low as 3-6 fg with high specificity, corresponding to 1-2 *M. leprae* cells [14].

The diagnosis of leprosy is based on microscopic detection of AFB in tissue smears, in combination with histopathological and clinical evaluation. But acid-fast staining requires at least 104 organisms per gram of tissue for reliable detection and AFB has a low sensitivity, particularly for the PB patients [15, 16]. Thus the need of hour is a PCR based DNA detection method which could be widely used for detection of *M. leprae* from clinical specimens [17, 18]. In this study we are able to amplify a specific repetitive sequence of *M. leprae* genome known as RLEP sequence and are present in 37 copies in the genome of *M. leprae*. In our study we assessed the efficacy of RLEP-PCR in field condition where most of the leprosy cases were AFB negative. In this study we found that RLEP-PCR is more sensitive than AFB smear. We found that only 22.50% samples were found to be AFB positive while the molecular detection method RLEP-PCR efficiently detected leprosy cases upto 66.25% almost 3 times better than AFB. Furthermore we found that 50% of the PB cases were detected by RLEP-PCR those were negative for AFB staining. Eight MB leprosy cases in the spectrum BB were RLEP-PCR negative, this could be due to presence of less number of bacteria in the slit scrapings. This was also evident from the AFB negative results of these 8 leprosy cases. Our result also corroborates with results published by other researchers worldwide [19-21]. Furthermore the P values of Fishers's exact test (P=0.0001) and χ^2-test (P=0.0001) gave a strong positive correlation that the RLEP-PCR is more sensitive than AFB smear microscopy in case detection of leprosy. The calculated Cohen's kappa coefficient (κ) was 0.614 with a confidence interval from 0.372 to 0.656 showed substantial agreements between AFB and RLEP-PCR. The Cohen's kappa coefficient (k) indicated that RLEP-PCR is substantially advantageous over AFB staining. Thus RLEP-PCR could be used for the detection of leprosy cases in accordance with the clinical findings in the field condition in the post elimination era of leprosy.

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Conflict of Interest: The authors declare that they have no conflict of interests.

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