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Original Article

RLEP PCR as a Definitive Diagnostic Test for Leprosy from Skin Smear Samples in Childhood and Adolescent Leprosy

R Kamal[1], R Dayal[2], K Gaidhankar[3], S Biswas[4], SB Gupta[5], N Kumar[6], R Kumar[7], R Pengoria[8], DS Chauhan[9], K Katoch[10], VM Katoch[11], PK Singh[12]

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The present study was conducted to evaluate the results of RLEP *M leprae* PCR using slit skin scraping samples (SSS) & compare the results with routine slit skin smears for AFB. Seventy three cases of both sexes (42 males and 31 females), 4 to 18 years of age (up to 5 years 4; 6-10 years 17; and the rest 52 between 11 to 18 years), clinically diagnosed cases of leprosy with hypopigmented / erythematous lesions associated with partial/total loss of sensation and/or presence of thickened nerves, diagnosed and classified by the criteria of IAL (1982), were included after obtaining their informed written consent. Skin smears from 30 non-leprosy cases of pulmonary tuberculosis, meningitis, skin diseases etc were also tested for *M leprae* RLEP PCR following the same protocol, after their informed consent and were included as controls. After clinical examination & clinical categorization two skin smears were taken, one for Z-N staining for AFB & another for *M leprae* RLEP PCR. After DNA extraction & amplification, electrophoresis was done on 2% agarose gel. Presence of 129bp fragment amplicon (RLEP of *M leprae*) was considered as positive result for the presence of *M leprae* DNA. Acid fast bacilli (AFB) positivity in smears after ZN staining was observed in 17/73 (cases (23.3%) and RLEP PCR positivity in 56/76 cases (76.71%). All controls showed negative results with *M leprae* RLEP PCR. The RLEP PCR technique had a significantly greater positivity ie more than three times that of AFB positivity on ZN staining ($p< 0.001$). The test can be easily performed and is a less invasive technique than biopsy for establishing the definitive diagnosis of leprosy.

Key words: Leprosy, *M leprae* RLEP PCR, skin smear, AFB

[1] R Kamal, MD, Scientist D, Head ,Department of Clinical Medicine, National JALMA Institute for Leprosy & Other Mycobacterial Diseases (NJILOMD-ICMR) , Agra
[2] R Dayal, MD, Professor and Head Department of Paediatrics, Sarojini Naidu Medical College, Agra
[3] K Gaidhankar, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[4] S Biswas, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[5] SB Gupta, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[6] N Kumar, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[7] R Kumar, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[8] R Pengoria, Department of Paediatrics, Sarojini Naidu Medical College, Agra
[9] DS Chauhan, PhD, Scientist E, Department of Microbiology and Molecular Biology, NJILOMD–ICMR, Agra
[10] K Katoch, MD, Former, Head Medical Unit I and Scientist G & Director, NJIOLOMD-ICMR, Agra
[11] VM Katoch, MD, Former Director, NJILOMD-ICMR & Secretary DHR and DG, ICMR, New Delhi
[12] PK Singh, MD, Prof and Head, Department of Skin, STD & Leprosy, Sarojini Naidu Medical College, Agra.
Corresponding author : Dr Raj Kamal, MD, Scientist-D(Medical) Head, Department of Clinical Medicine, NJIL & OMD, Agra
e-mail: rajushikamal@rediffmail.com

Introduction

In India the Leprosy Elimination has been achieved at the National level, but, some pockets of endemicity still remain at district and sub district level, and new cases continue to be detected at nearly the same rate in last 10-12 years (NLEP Annual Report 2014-2015). In absence of a confirmative test/culture demonstration of *M leprae,* diagnosis is still based on clinical suspicion and physical examination. It is diagnosed by the presence of at least two of the three cardinal signs mentioned or the last one independently: (i) Loss or impairment of cutaneous sensation, (ii) Thickening of peripheral nerves, iii) Demonstration of presence of AFB in skin (Dharmendra and Chatterjee 1978). However, in early cases the clinical diagnosis is sometimes difficult and histopathological examination is confirmatory in only 35% of early cases (Fine et al 1986). After the successful cloning of *M leprae* gene and the availability of amplification methods, newer methods targeting *M leprae* specific DNA sequences have been used to detect nucleic acid sequence specific to the pathogen for the definitive diagnosis of leprosy. However, due to large size, amplicons of most of the PCR based methods like 65kDa, 18kDa, 36kDa, which undergo damage/fragmentation during the procedure, these are not widely used (van Soolingen et al 1993). This does not occur with RLEP amplicon of *M leprae*, is specific for the organism, differs from other mycobacteria, and moreover, it is a repetitive sequence repeated 28 times in the *M leprae* chromosome and more sensitive than other PCR based methods (Donoghue et al 2001, Kang et al 2003).

Material and Methods

The study was conducted in Department of Pediatrics, in collaboration with Department of Skin, STD & Leprosy, S N Medical College, Agra & National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra. Clinically diagnosed and classified (Indian Association of Leprologists 1982), 73 cases of leprosy up to 18 years of age, of both sexes were included in the study after obtaining their Informed Written Consent. After eliciting a detailed history and a thorough clinical examination, cases were classified into Indeterminate (Ind), TT (Tuberculoid), BT (Borderline Tuberculoid), BB (mid Borderline), BL (Borderline Lepromatous) and LL (Lepromatous leprosy) types clinically. Size, location and number of lesions, loss of sensation & peripheral nerve enlargement were recorded. Two slit skin smear specimens were collected: one for Z-N staining for AFB & another for RLEP PCR, using the same blade from the same site to make the smears. Smears were taken from atleast three sites ie from the lesion as well as from both the the ear lobes. These were labeled, heat fixed and transported to NJIL & OMD for AFB staining and RLEP PCR. One SSS was stained by ZN stain as per the standard protocol, examined and recorded.

DNA extraction from the second skin smear samples was done using the method described by van Embden et al (1993). RLEP-PCR was done using the primers F-'TGCATGTCATGGCCTTGAGG3' and R-5'CACCGATACCAGCGGCAGAA3'to find out the presence of *M leprae* in the skin smear samples. Amplicon – a band of 129 bp on 2% Agarase gel electrophoresis was considered as a positive result (Donoghue et al 2001).

Results

In present study, there were 42 (57.5%) male cases and 31 female cases (42.5%). Four cases were of the age group 4-5 years (5.5%), 17 cases were of the age group 6 to 10 years (23.3%) and the rest 52 belonged to the age group of 10 to 18 years (71.2%). Maximum number of cases (31/73; 42.46%) were of BB type followed by BT (27/73; 36.9%), BL(12/73; 16.4%), TT (2/73; 2.7%), and 1

Table 1 : Results of AFB staining of skin smears and RLEP PCR positivity

Clinical classification of cases	Total No. of cases	Skin Smear positive for AFB	RLEP PCR positive
Ind	1	Nil	Nil
TT	2	Nil	1
BT	27	Nil	21
BB	31	10	25
BL	12	6	9
Total	73	17 (23.3%)	56 (76.7%)

X^2-39.570; P < 0.001 D.F. =1

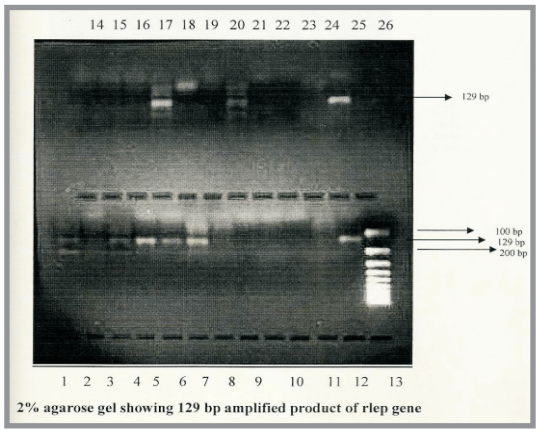

Fig 1 : Showing the appearance of RLEP PCR 129 bp amplicon of *M leprae* on 2% Agaraose gel electrophoresis

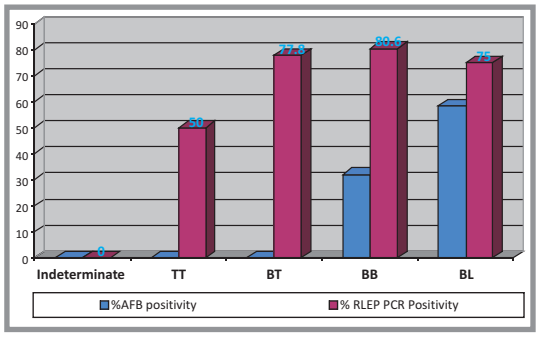

Fig 2 : Showing the comparative percentage positivity between AFB positivity and RLEP PCR positivity in skin smears

of Indeterminate type (Table 1). Most of the cases had both skin lesions & nerve involvement (61/73; 83.6%) at the time of presentation, while 12/73 (16.43%) cases presented with only skin lesions and no nerve involvement. None of the cases had only nerve involvement with no skin lesions. Five of the 73 cases (6.8%) had single skin lesion, 27 had 2-5 lesions (36.9%), and 45 had more than 5 lesions (56.3%). It was observed that by counting the number of skin lesions for operational classification of the disease 32/73 cases were of the paucibacillary type and the rest 41 cases were MB cases. On the other hand by the IAL classification (1982), there were 30 PB cases (Ind + TT + BT) and 43 MB cases (BB +BL). These discrepancies were due to only considering the number of lesions and not taking into consideration the satellite lesions and /or nerve enlargements.

Skin smear for AFB ware positive in 17/73 (23.28%) cases (Table 1). *M leprae* specific RLEP PCR in skin smears was positive in 56/73 (76.71%) cases. Figure 1 shows the snap shot of 2% agarose gel electrophoresis and RLEP positivity at 129bp region. All controls (non leprosy cases) showed negative results for RLEP PCR for *M leprae.* RLEP PCR technique was significantly better than ZN staining ($p < 0.001$) and more so in Indeterminate, TT, BT, BB and BL cases (Fig. 2).

Discussion

In the present study, maximum number of cases (31/73; 42.46%) were of BB type which is comparable to observation made by Dayal et al (1997 & 2007) and Kamal et al (2006) who also reported a higher percentage of BB cases in their series. Majority number of children (71.23%) belonged to adolescent age group which may be indicating a long incubation period (Harrison 2012, Kamal et al 2010 and 2013) of the disease, as well as change in the hormonal pattern in the host body which may be modulating the manifestation of the disease. Male predominance has been previously also observed by (Dave et al 1987, Dayal et al 2007, Nigam et al 1977). More number of cases (83.6%) presented with both skin & nerve involvement as reported previously by Dayal et al (2007) and Kamal et al (2006, 2010 and 2013). Therefore, the population of children included in the present study is similar to reported by several workers.

Identification of gene components of *M leprae* and use of amplification technology (PCR), which can magnify small amount of *M leprae* components present, has been used for diagnosis and monitoring of disease activity in leprosy. Primers of these specific amplicons have been prepared and several of them have been tested to ascertain the usefulness in the diagnosis of leprosy. After comparing the different primers in clinical specimens (Donoghue et al 2001, Kang et al 2003), found that RLEP PCR was a more sensitive method. The RLEP sequence is repeated 28 times in the *M leprae* genome and would, therefore, theoretically will be more sensitive than the other probes tested.

Acid fast staining of slit skin smears in leprosy is still considered as a specific test for leprosy specially if found positive and is still used in referral centres. It is less invasive than biopsy and is well tolerated by the patients. The present study evaluated the use of RLEP PCR after extracting the DNA from routine SSS to observe its use in leprosy diagnosis.

Slit skin smear for AFB was positive in 23.3% cases with most of the cases belonging to BB and BL type. None of the PB cases were positive for AFB in SSS. RLEP PCR was positive in 56/73(76.71%) cases {TT 1/2 BT 21/27(77.77%); BB 25/31 (80.64%); BL 9/12 (75%) of cases (Fig. 2); In the present study the results of RLEP PCR were similar to those of Kang et al (2003) who observed 73% positivity of RLEP PCR in their study. Donoghue et al (2001) and Martinez et al (2011) observed positivity of 87% and 100% respectively. RLEP PCR from SSS was confirmatory in about 78% of cases clinically diagnosed as leprosy. Ideally speaking all clinically diagnosed cases should be positive but this was not so and therefore some issues do remain about optimization of methods. However, this test was more sensitive than AFB in skin smears as well as also as reported for histological studies.

Conclusion

RLEP PCR detection in skin smear is more sensitive than AFB and can serve as a good, minimally invasive diagnostic tool for diagnosis of leprosy.

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