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# DETECTION OF MYCOBACTERIUM LEPRAE USING RLEP PCR IN CHILDHOOD LEPROSY AND IT'S COMPARISON WITH IS1081 PCR

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**ABSTRACT** The present study was conducted to evaluate the results of M leprae RLEPPCR& IS1081 PCR using slit skin scraping samples (SSS) & compare the results with routine slit skin smears for AFB. Forty cases (24 males and 16 females) clinically diagnosed 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 40 non-leprosy mycobacterial cases of pulmonary tuberculosis, meningitis, skin diseases etc were also tested for M leprae RLEP PCR following the same protocol, 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& IS1081 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 lepraeDNA. Acid fast bacilli (AFB) positivity in smears after ZN staining was observed in 11/40 cases (27.5%). RLEP PCR positivity was found in 27/40 cases (67.5%) and IS1081 PCR was found positive in 4/40 cases (10%). All controls showed negative results with M leprae RLEP PCR. The IS1081 PCR showed very low positivity in leprosy cases. The RLEP PCR technique had a significantly greater positivity than that of IS1081 PCR & AFB positivity on ZN staining ($p< 0.05$). The test can be easily performed and is less invasive than biopsy for establishing the definitive diagnosis of leprosy.

**KEYWORDS :** Leprosy, M leprae RLEP PCR, IS1081 PCR skin smear, AFB.

**Introduction-**

Leprosy continues to be a communicable disease of concern in the post elimination era. Different parts of the country depict the unturned curves in the epidemiology of childhood leprosy in its endemic pockets which mirrors active transmission and delayed diagnosis in this age group. This alarms the need to strengthen contact screening, early case detection, and referral activities in the pediatric population to sustain elimination[1].

Leprosy is more common in adults; however, the outbreak of cases in children and adolescents shows the active circulation of bacillus, with its continued transmission. Among patients under 15 years of age, the most affected age group is children between 10 and 14 years of age, although cases of patients of younger than 1 year of age have also been reported[2]. 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 as in last 10-12 years (NLEP Annual Report 2014-2015). The diagnosis of the disease is primarily clinical and is dependent on the presence of two cardinal signs[3], namely, the presence of typical skin lesions with hypoaesthesia, and/or the presence of acid-fast bacilli (AFB).

Over recent years, molecular diagnosis by using PCR has been increasingly used as an alternative for its due to its higher sensitivity. A study showed PCR to be more sensitive than SSS microscopy in diagnosing leprosy. DNA was extracted from SSS and PCR was carried out to amplify 129 bp sequence of *M. leprae* repetitive element. Sensitivity of SSS and PCR was 18% and 72% respectively. Improvement of 54% case detection by PCR clearly showed its advantage over SSS[4].

However, due to large size, amplicons of most of the PCR based methods(like 65kDa, 18kDa, 36kDa), undergo damage/ fragmentation during the procedure,so these are not widely used [5]. This does not occur with RLEP amplicon of *M.leprae.* Moreover, it is specific for the organism, differs from other mycobacteria and it is a repetitive sequence repeated 28 times in the *M. leprae* chromosome and more sensitive than other PCR based methods[6][7].

In a study involving 73 patients, Z-N staining for AFB was positive in 17/73 (23.28%) cases and RLEP PCR in 56/73 (76.71%) cases. All 30 controls showed negative results. RLEP PCR technique had a significantly greater positivity (especially in early stages of leprosy) than ZN staining ($p< 0.001$).[8]

R Kamal found that 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[9]

A PCR test based on insertion sequence *IS1081* was developed to detect *Mycobacterium tuberculosis* complex organisms in the peripheral blood. The method was applied to blood samples from immunocompetent individuals with localized pulmonary tuberculosis. Seven of 16 (43.75%) blood samples were found to be positive for the circulating DNA copies of *M. tuberculosis* complex.[10]

Dick Van Soolingen et al. investigated the host range of insertion element *IS1081* among the various pathogenic and nonpathogenic mycobacterial species and the genetic polymorphism associated with the putative transposable element. They demonstrated that this IS element is exclusively present in *M. tuberculosis* complex species and that *IS1081* can be used to reliably differentiate *M. bovis* BCG from other *M. tuberculosis* complex bacteria[11].

In the current study we investigated the utility Of RLEP PCR in diagnosing leprosy patients and compared it with *IS1081*PCR and slit skin smear for AFB.

**Material and methods-**

The study was conducted in Department of Pediatrics, S.N. Medical college Agra, in collaboration with National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra.

40 cases of leprosy, up to the age of 18 years of either sex were included in the study after obtaining Informed Written Consent from their parents. After eliciting a detailed history and a thorough clinical examination, cases were classified into TT (Tuberculoid), BT (Borderline Tuberculoid), BB (mid Borderline), BL (Borderline Lepromatous) and LL (Lepromatous leprosy) types. 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 and *IS1081* PCR, using the same blade from the same site to make the smears. Smears were taken from atleast three sites i.e, from the lesion as well as from both the ear lobes. These were labelled, 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 to find out the presence of *M. leprae* in the skin smear samples. Amplicon – a band of 129 bp on 2% Agarose gel electrophoresis was considered as a positive result (Donoghue et al 2001).

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|---|---|
| RLEP-PCR was done using the primers | F-5'TGCATGTCATGGCCTTGAGG3' |
| | R 5'CACCGATACCAGCGGCAGAA3' |
| IS1081 PCR was done using primer | F- 5'-CGACACCGAGCAGCTTCTGGCTG-3' |
| | R-5'-GTCGGCACCACGCTGGCTAGTG-3' |

**The primers were synthesized by GCC Biotech.**

Results-In present study, there were 24 male cases (60%) and 16 female cases (40%). Twelve cases were of the age below 10 years (30%) and 28 cases belonged to the age group of 11 to 18 years (70%). Maximum number of cases (15/40; 37.5%) were of BT type followed by TT (13/40; 32.5%), BB (8/40; 20%). BL, LL cases were present in5% cases. (Table 1).Most of the cases had both skin lesions & nerve involvement (36/40; 90%) at the time of presentation, while 4/40 (10%) cases presented with only skin lesions and no nerve involvement. None of the cases had only nerve involvement with no skin lesions. 8 of the 40 cases (20%) had single skin lesion, 22 had 2-5 lesions (55%), and 10 had more than 5 lesions (25%). It was observed that by counting the number of skin lesions for operational classification of the disease, 30/40 cases were of the paucibacillary type and the rest 10 cases were multibacillary cases.Skin smear for AFB ware positive in 11/40 (27.5%) cases (Table 1). M lepraespecific RLEP PCR in skin smears was positive in 27/40 (67.5%) cases.IS1081 PCR was positive only in 4 cases (10%). All controls (non-leprosy cases) showed negative results for RLEP PCR for M leprae.Thus IS1081 does not provide much utilityin detection of leprosy cases, (p value=0.291). In the present study RLEP PCR can detect a higher number of cases (67.5%) in comparison to IS1081 and AFB which is positive in 10% and 27.5% respectively. It clearly demonstrates advantage of RLEP PCR over both IS1081 PCR and AFB for the diagnosis of leprosy.

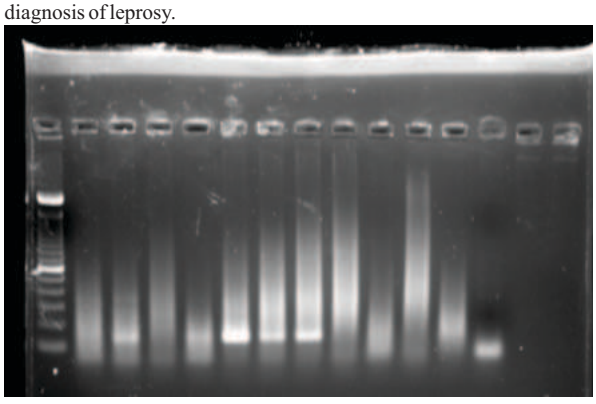

**Fig 1:** Showing the appearance of RLEP PCR 129 bpamplicon of M leprae on 2% Agarose gel electrophoresis.

**Table 1: Results of AFB staining of skin smears and RLEP PCR positivity and IS1081 PCR positivity.**

| Clinical types | No of cases tested | Smear positive | Positive for IS1081PCR | Positive for RLEP PCR |
|---|---|---|---|---|
| TT | 13 | 0 | 0 | 5 |
| BT | 15 | 1 | 0 | 12 |
| BB | 8 | 6 | 2 | 6 |
| BL | 2 | 2 | 1 | 2 |
| LL | 2 | 2 | 1 | 2 |
| Total | 40 | 11 | 4 | 27 |
| % | 100 | 27.5 | 10 | 67.5 |

(X2 = 4.96 D.F = 4 p-value = 0.291)

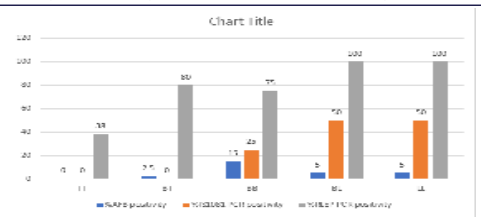


**Fig 2** Showing comparative percentage positivity between AFB positivity, RLEP PCR positivity and IS1081 positivity in skin smears.

**Table-2: IS1081 PCR detection in new leprosy patients**

| | Positive for IS1081 PCR | Negative for IS1081 PCR |
|---|---|---|
| Paucibacillary | 1 | 30 |
| Multibacillary | 3 | 6 |
| Total | 4 | 36 |

(X2 = 7.03 D.F = 1 probability = 0.008)

**Table-3: Reactivity to RLEP PCR in new leprosy patients**

| | Positive for RLEP PCR | Negative for RLEP PCR |
|---|---|---|
| Paucibacillary | 20 | 13 |
| Multibacillary | 7 | 0 |
| Total | 27 | 13 |

( X2=4.09 D.F = 1 p-value = 0.043 )

**Table-4: Statistics of IS1081 detection in patients and controls**

| Statistic | Value | 95% CI |
|---|---|---|
| Sensitivity | 10.00% | 2.79% to 23.66% |
| Specificity | 97.50% | 86.84% to 99.94% |
| Positive Likelihood Ratio | 4.00 | 0.47 to 34.24 |
| Negative Likelihood Ratio | 0.92 | 0.82 to 1.04 |
| Disease prevalence (*) | 50.00% | 38.60% to 61.40% |
| Positive Predictive Value (*) | 80.00% | 31.85% to 97.16% |
| Negative Predictive Value (*) | 52.00% | 49.14% to 54.85% |
| Accuracy (*) | 53.75% | 42.24% to 64.97% |

**Table-5: Statistics of RLEP PCR detection in patients and controls**

| Statistic | Value | 95% CI |
|---|---|---|
| Sensitivity | 67.50% | 50.87% to 81.43% |
| Specificity | 100.00% | 91.19% to 100.00% |
| Positive Likelihood Ratio | 16 | 1.35 TO 16.5 |
| Negative Likelihood Ratio | 0.32 | 0.21 to 0.51 |
| Disease prevalence (*) | 50.00% | 38.60% to 61.40% |
| Positive Predictive Value (*) | 81% | 71.4% to 96.5% |
| Negative Predictive Value (*) | 75.47% | 66.31% to 82.79% |
| Accuracy (*) | 83.75% | 73.82% to 91.05% |

**Discussion-**

In the present study, maximum number of cases (15/40; 37.5%) were of BT type. Majority of children (70%) belonged to the adolescent age group which may be an indicator of a long incubation period of the disease, as well as change in the hormonal pattern in the host body modulating the manifestation of the disease[12].As in the present study, male predominance has been previously also observed by (Nigam et al 1977)[13].Greater number of cases (90%) presented with both skin & nerve involvement as reported previously by Dayal et al (2007)[14] . Therefore, the age and sex pattern 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, we found that RLEP PCR was a more sensitive method[15]. The RLEP sequence is repeated 28 times in the M lepraegenome 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 and IS1081 PCR after extracting the DNA from routine SSS to observe its use in leprosy diagnosis. RLEP PCR was found to be 100% specific. Slit skin smear for AFB was positive in 27.5% cases with most of the cases belonging to BB and BT type. RLEP PCR was positive in 27/65(67.5%) cases [TT 5/13 BT 12/1, BB 6/8, BL 2/2 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 and specific than IS1081 PCR and AFB in skin smears.

## CONCLUSION-

Thus we can conclude that RLEP PCR detection in leprosy is more sensitive and specific than IS1081 PCR and AFB smear examination and can be a useful tool to confirm early cases of leprosy, where skin smear was negative. It is 100% sensitive in lepromatous leprosy cases and multibacillary cases. It serves as a good, minimally invasive diagnostic tool for diagnosis of leprosy. However, since number of cases on our study was small, there is need for further studies to validate the importance of IS1081 PCR in leprosy cases.

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