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Original Article

Evaluation of diagnostic role of *in situ* PCR on slit-skin smears in pediatric leprosy

R Kamal[1], M Natrajan[1], K Katoch[1], VM Katoch[2]

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A large proportion of early cases of leprosy in children remain AFB negative in skin smears. Such cases required additional techniques to confirm the diagnosis. *In situ* PCR on slit- skin smears is minimally invasive and less cumbersome as compared to skin biopsies. This study was initiated in our institute with the objective to evaluate the diagnostic value of *in situ* PCR on slit- skin smears in pediatric leprosy. A total of 25 cases of leprosy below 16 years of age were included in the study. After detailed history and thorough clinical examination, informed consent was obtained from the parents of children for slit- skin smears from lesion sites for AFB staining and for *in situ* PCR technique. Cases were clinically categorized according to IAL classification into indeterminate (I), tuberculoid tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) and lepromatous (LL). Most of the patients (76%) were between 9-16 years of age and 64% of the cases had history of contact with leprosy patients within the family. Skin smears were positive for AFB in only 20% of the cases. On applying *in situ* PCR, it was observed that 62.5% cases of I/TT/BT/BB category and 88.8% of BL/LL category gave positive signals. Overall *in situ* PCR confirmed the diagnosis in 72% cases while by slit smears diagnosis was confirmed in only 20% of cases . Further, out of 20 skin smear negative cases, 13 were positive by *in situ* PCR. Specificity of the signals of *in situ* PCR was established by demonstrating the absence of signals in nonleprosy dermatological conditions of vitiligo and P.alba. This study supports the potential usefulness of *in situ* PCR on slit- skin smears of early pediatric leprosy cases. This strategy will be especially useful in cases where skin smears are negative and in those cases where skin biopsy can not be done either because of unusual locations of lesions or because of sensitive age of the patients.

Key words : Pediatric leprosy, Slit-skin smears, *In situ* PCR

Introduction

Leprosy is a chronic granulomatous disease which primarily affects the skin and peripheral nervous system and also the mucous membrane and various other tissues (Katoch 2004). According to WHO, out of 231361 registered cases of leprosy in the world in 2007; 82801 were residing in India. While there has been a dramatic fall in the prevalence rate of leprosy in India, the new case detection rate has been reducing (WHO 2007).

Leprosy in children is mostly seen in the 5-14 years age group (Park 2006). Pediatric leprosy

R Kamal, MD, Scientist C (Medical)
M Natrajan, DD, Scientist F(Medical)
K Katoch, MD, Officer-In-Charge and Scientist F (Medical)
VM Katoch, MD, Secretary and Director General
[1]National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra-282 001, India
[2]Department of Health Research, Ministry of Health and Family Welfare, Government of India and Indian Council of Medical Research, New Delhi-110 029, India
Correspondence to : M Natrajan **Email:** mohunpath@rediffmail.com

reflects the transmission kinetics in the population. For eradication of the disease, it is important that leprosy cases should be diagnosed early and treated promptly so that deformity and spread of disease can be prevented. In the established form of the disease, the diagnosis relatively simple. However, now the established forms are decreasing in number due to widespread use of MDT and early disease is being reported more frequently. Clinical diagnosis in such cases may be difficult. The diagnosis of leprosy is primarily clinical. Anesthetic skin lesions with or without thickened peripheral nerves are virtually pathognomonic of leprosy. Routine histopathology can confirm the clinical diagnosis in only about 45 percent cases because of paucity of acid-fast bacilli (AFB) and absence of lymphocytic infiltration inside the dermal nerves which are considered essential criteria for diagnosis of leprosy (Binford 1971, Ridley 1974, Dayal et al 2005). Instead, they show non-specific histopathology in the form of chronic inflammatory cell infiltrate at various locations which is common to many dermatological conditions. A large proportion of clinically diagnosed cases of early leprosy, therefore, remain histologically unconfirmed (Ridley 1988, Agarwal et al 1990). Additional methods are, therefore, all the more imperative to confirm the diagnosis. Methods available include antigen demonstration by immunostaining or demonstration of nucleic acid sequences specific to the pathogen by *in situ* hybridization (ISH) and or *in situ* Polymerase Chain Reaction (*in situ* PCR). During the last few years, PCR based tests for detection of *M. leprae* in clinical specimens have been described (Katoch 1998, Haile and Ryonn 2004, Singh et al 2004). These gene amplification techniques can detect the specific presence of nucleic acids of *M.leprae* even from a specimen containing 1-10 organisms (Hartskeerl et al 1989). In comparison to *in vitro* PCR, *in situ* PCR procedure offers the following two advantages: (i) it permits the containment study of cytomorphology and (ii) the possibility of contamination by foreign DNA/RNA is minimized. Furthermore, as compared to *in situ* PCR on skin biopsy, *in situ* PCR on slit-skin smears is minimally invasive and less cumbersome. Slit-skin smears are much more convenient than skin biopsy. There is hardly any literature available on the diagnostic value of *in situ* PCR on slit-skin smears in childhood leprosy. With this background, we conducted this study to evaluate the diagnostic value of *in situ* PCR on slit-skin smears in cases of childhood leprosy.

Subjects and Methods

This study was conducted from May 2005 to September 2007 at the Department of Pediatrics, SN Medical College, Agra, India and National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Agra, India. A total of 25 cases below 16 years of age were included in the study after obtaining informed consent from their patients. A case of leprosy was defined by the presence of any one of the following cardinal signs (WHO 2010) :

i. Characteristic skin lesion with partial or total loss of sensation in the affected skin lesion or in the area of the skin supplied by the peripheral nerve involved with or without the presence of thickened nerves.

ii. Presence of AFB in skin smears.

Cases were clinically categorized according to Indian Association of Leprologists (1982) into: indeterminate (I), tuberculoid (TT), borderline tuberculoid (BB), borderline lepromatous (BL) and lepromatous (LL).

A detailed history and thorough clinical examination was done. Slit-skin smears were taken from lesion, site for AFB staining (Ziehl Neelsen method) and *in situ* PCR technique. Additionally, six cases (4 vitiligo and 2 Pityriasis alba) were taken as negative controls and *in situ* PCR performed on their skin smears.

***In situ* PCR:** Primers used in the study were 19 mer and 18 mer to sequence encoding 530 bp fragments of gene encoding the 36 kD protein of *M. leprae* (de Wit et al 1991) The sequences of primers were :

Forward primer: 5'CTCCACCTGGACCGGCCAT 3'

Reverse primer: 5'GACTAGCCTGCCAAGTCG 3'

The procedure of *in situ* PCR was performed in three steps as follows : (i) air dried smears were allowed to fix with 4% paraformaldehyde, rehydration was done with descending grades of alcohol, permeabilisation with 0.2 N HCl, proteolysis was done with pepsin and inactivated with glycine. (ii) amplification was performed using programmable Thermal Cycler (PTC 200, MJ Research, USA) having twin tower block designed for fitting microscopical glass slides. Amplification solution contained PCR buffer with $MgCl_2$, Fast *Taq* Polymerase, primer 19 mer and 18 mer, deionised water. (iii) post-amplification labeling and detection: In this step, an enzyme, substrate-chromogen combination (containing anti-digoxigenin antibody, alkaline phosphatase, NBT/BCIP) was added. Then, counterstaining with 2% neutral red was done. Finally, smears were mounted using DPX and visualized under light microscope for PCR signals.

The research approval for this study was taken from Institutional Ethical Committee (IEC). Data obtained were compared using *chi*-square test.

Results

In the present study, 25 patients under 16 years of age were included; majority (88 %) were males. Most of the patients (76%) were between 9-16 years of age (Table 1). Majority (64%) of the patients in this study had history of contact with a leprosy patient within the family. Clinically, cases were categorized according to IAL classification into I-3; BT-10; BB-3; BL/LL-9. None of the case belonged to TT group. Most of patients (60%) had hypopigmented lesions. Only 10% had erythematous lesions. 60% cases had well defined margins and 40% had ill defined margins.

Majority (60%) of the cases had lesions on both covered and uncovered body areas whereas 32 % and only 8% had lesions on uncovered and covered body areas respectively. Nerve thickening was observed in 68% cases. As age

Table 1 : Distribution of cases according to age

Clinical types	No. of cases in different age groups			
	4-8 years	9-12 years	13-16 years	Total
I	2 (8%)	1 (4%)	0 (0%)	3 (12%)
BT	4 (16%)	3 (12%)	3 (12%)	10 (40%)
BB	0 (0%)	2 (8%)	1 (4%)	3 (12%)
BL/LL	0 (0%)	2 (8%)	7 (28%)	9 (36%)
Total	6 (24%)	8 (32%)	11 (44%)	25 (100%)

Table 2 : Correlation of clinical types, results of skin smears for AFB and *in situ* PCR

Clinical types	No. of cases	Slit-skin smears for AFB		*In situ* PCR	
		Positive	Negative	Positive	Negative
I	3 (12%)	0 (0%)	3 (12%)	2 (66.6%)	1 (4%)
BT	10 (40%)	0 (0%)	10 (40%)	6 (60.0%)	4 (16%)
BB	3 (12%)	1 (4%)	2 (8%)	2 (66.6%)	1 (4%)
BL/LL	9 (36%)	4 (16%)	5 (20%)	8 (88.8%)	1 (4%)
Total	25(100%)	5 (20%)	20 (80%)	18 (71.0%)	7 (19%)

Chi-square (χ^2) = 15.901, Degree of freedom (DF) = 1, P = 0.01

Table 3 : Correlation of results of *in situ* PCR with skin smears

In situ PCR	Slit-skin Smears		Total
	Positive	Negative	
Positive	5	13	18
Negative	0	7	7
Total	5	20	25

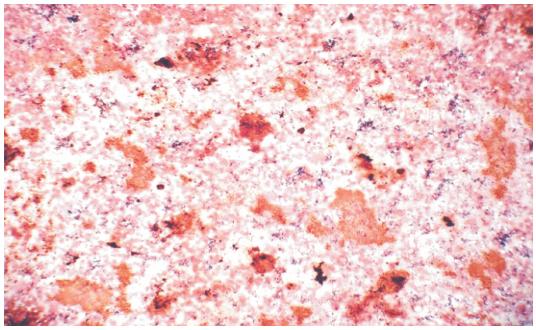

Figure 1 : *In situ* PCR performed on cell smear showing positive signals (black arrows). Counterstain : 2% neutral red. Original magnification : 240x.

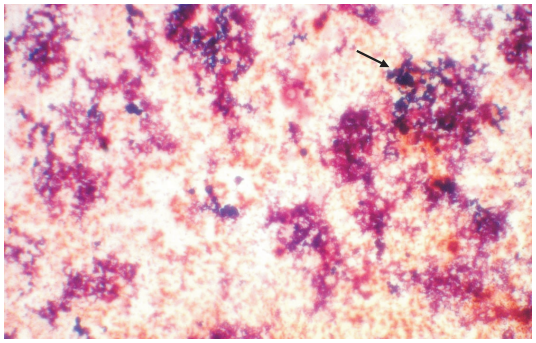

Figure 2 : *In situ* PCR performed on cell smear showing positive signals (black arrows). Counterstain : 2% neutral red. Original magnification : 400x.

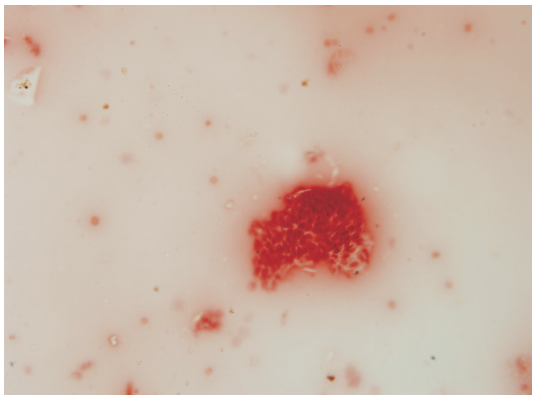

Figure 3 : *In situ* PCR performed on cell smear of P. alba showing absence of signals. Counterstain : 2% neutral red. Original magnification : 400x.

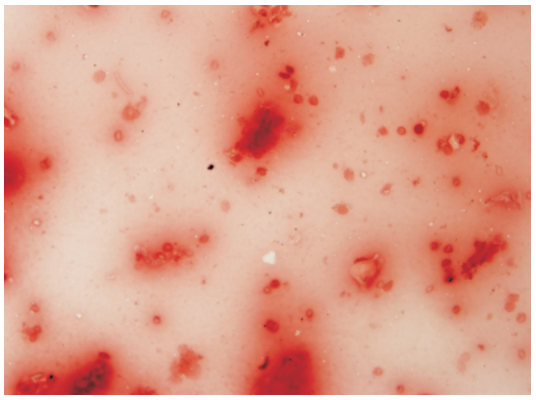

Figure 4 : *In situ* PCR performed on cell smear of vitiligo showing absence of signals. Counterstain: 2% neutral red. Original magnification : 240x.

advanced the disease moved from the tuberculoid end of the spectrum towards the lepromatous end. Skin smears were negative for AFB in most of the cases (80%); only 20% cases were positive for AFB (44.44% of BL/LL variety) (Table 2).

On applying *in situ* PCR, it was observed that 62.5% cases of I/BT/BB variety showed PCR signals as against 88.88% of BL/LL variety. Overall 72% cases were confirmed by *in situ* PCR (Figure 1 and 2, Table 2). Correlation of clinical types with results of skin smears for AFB and *in situ* PCR showed that skin smear for AFB was positive in 20% cases while *in situ* PCR was positive in 20% cases while *in situ* PCR was positive in 72% cases (Table 2). This value was statistically significant (P = 0.01) and proved that *in situ* PCR has better diagnostic value than skin smear for detection of AFB or DNA of *M. leprae.* Further, out of 20 skin smear for negative cases, 13 were confirmed by *in situ* PCR (Table 3). All cases of P. alba and vitiligo showed no signals with *in situ* PCR (Figure 3 and 4).

Discussion

In the present study, maximum number (60%) had hypopigmented lesions, present over both covered and uncovered parts of the body (60%). These results are comparable to the findings reported earlier (Dayal et al 1994, 1998). 64% of the cases had history of contact with a leprosy patient within the family which has higher than the previous study on *in situ* PCR on skin biopsy in our institute (20%). Majority (64%) of the cases in present study were of I/BT/BB variety of leprosy which showed increasing trend of reporting of early and suspected cases of leprosy. In these cases, the diagnosis may be difficult because of paucity of AFB and absence of lymphocytic infiltration inside the dermal nerves. Skin smears of majority (94.75%) of I/BT/BB variety were AFB negative while 44.44% cases of BL/LL were positive for AFB. The diagnosis of established forms of leprosy is easy in the presence of characteristic skin lesions and nerve thickening. However, in early cases of leprosy, sensitivity and specificity of cardinal signs becomes low and slit-skin smear for AFB is usually negative because of paucity of *M. leprae.* Histopathology if done, is inconclusive in most of the early cases of leprosy. Such cases require sophisticated and specific methods for diagnosis. Such modalities will become even more important in the post-elimination era. *In situ* PCR on slit-skin smears is of great significance in these cases. In our study we found that *in situ* PCR slit-skin smears confirmed the diagnosis in 65% skin smears which were AFB negative. Overall *in situ* PCR slit-skin confirmed the diagnosis in 72% cases while by slit-skin smears for AFB (Ziehl Neelsen staining) diagnosis was confirmed in only 20% cases. This showed an improvement of 52% which is statistically significant. Further, our results of 62.5% positivity in early leprosy, 88.88% positivity in advanced cases and 72% overall positivity are better than the results of another study from our institute using *in situ* PCR in skin leprosy. In the later study, 57.1% positivity was observed in early leprosy; 61.5% in advanced disease and overall positivity was 60% (Dayal et al 2005). The specificity of the signals in the study was further established by the absence of signals in the other dermatological conditions chosen namely P. alba and vitiligo.

Conclusion

To conclude, this pioneer study supports the potential usefulness of *in situ* PCR on slit-skin smears in early leprosy cases. This strategy will be specially useful when histopathology is not confirmative, in cases when skin smears are negative for AFB and in those cases where skin biopsy can not be done either because of unusual location of lesions or because age of the patient.

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