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Original Article

Histological Diagnosis of Early and Suspicious Leprosy by *In situ* PCR

M Natrajan[1], K Katoch[1], VM Katoch[2] Ram Das[3], and VD Sharma[1]

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Leprosy is a chronic mycobacterial disease whose diagnosis is primarily based on clinico-pathological examination and supported by slit skin smears for the presence of acid fast bacilli (AFB). However, definitive diagnosis of early leprosy and those suspected to have the disease but not histologically confirmed pose major public health problems. The present study reports the utility of the *in situ* Polymerase Chain Reaction amplification (PCR) directed at a 530bp fragment of DNA encoding the 36kd antigen of the causative *Mycobacterium leprae* for the diagnosis of such patients using skin biopsies of lesions. Twenty five adult patients (aged 15-50yrs) each from the clinical categories of Early and clinically Suspect leprosy were selected for the study after obtaining permission. They had solitary lesions, which were negative for AFB on slit skin smear examination. Routine histopathology confirmed the diagnosis of leprosy in 8/25 (32%) cases in the category of Early leprosy with AFB being seen in 2 biopsies, and in 5/25(20%) cases of Suspect leprosy with AFB being seen in a solitary case. The Direct *in situ* PCR procedure which was performed in the histologically unconfirmed cases improved the diagnosis with positive results observed in 12/17 (70.6%) cases of Early (p=0.001) and in 12/20 (60%) cases of Suspect Leprosy (p=0.005) indicating the usefulness of the Direct *in situ* PCR to establish the diagnosis of leprosy in histologically doubtful cases.

Key words : Early and Suspect leprosy, diagnosis, *in situ* PCR

Introduction

Leprosy is a chronic mycobacterial disease caused by *Mycobacterium leprae (M leprae)* which infects primarily, the peripheral nervous system and the skin. The diagnosis of the disease is essentially clinical, and is based either on the characteristic anaesthetic skin lesions, uniform skin infiltration, or, peripheral nerve trunk thickening along with the signs indicative of its damage (Pfaltzgraf and Bryceson 1985).

[1] M Natrajan, DD, PhD, Scientist-F
[1] K Katoch, MD, Scientist-G and Director
[2] VM Katoch, MD, Secretary and Director General
[3] Ram Das, PhD, Scientist-B
[1] VD Sharma, PhD, Scientist E
[1] National JALMA Institute for Leprosy and Other Mycobacterial Diseases (ICMR), Taj Ganj, Agra UP-282001 India
[2] Department of Health Research, Ministry of Health & Family Welfare, Government of India and Indian Council of Medical Research, New Delhi-110029
[3] National Institute of Malaria Research (ICMR), Sector-8, Dwaraka, New Delhi-110077
Correspondence to : M Natrajan email : mohunpath@rediffmail.com

In the limited form of the disease, the clinical diagnosis depends on the presence of one of 2 cardinal signs (Dharmendra and Chaterjee 1978, WHO 1998), namely – characteristic skin lesions showing a clear sensory deficit, and the presence of Acid Fast Bacilli (AFB) in a skin smear.

In the evolving stages of the disease, however, the cardinal signs are not manifest and one often encounters a situation where skin lesions are seen, which are visually suggestive of leprosy, yet the diagnosis cannot be made for want of the presence of these cardinal signs - a condition referred to as Suspect Leprosy (Ponnighaus et al 1993, Ramu et al 1996). In such situations one resorts to histopathology to resolve the clinical doubt. Histological confirmation of the diagnosis of leprosy requires the presence of infiltration *within* dermal nerves, and/or, the presence of AFB, and these features are often not seen in the early stages of the disease (Ridley 1985, 1988). The proportion of cases confirmed can be expected to vary from situation to situation in early and suspect leprosy but is invariably low and is in the order of 35% (Abalos 1973, Fine et al 1993). To the best of our knowledge, there is no similar data regarding histological confirmation of the diagnosis in these categories of cases in the Indian context and the present study gives an indication of the degree of confirmation that can be expected in this region.

The degree of confirmation of diagnosis can be augmented to a limited extent by the use of immunostaining procedures, which demonstrate mycobacterial antigens in tissue specimens, and several studies have reported the finding of antigens in AFB negative specimens (Mshana et al 1982, Barbosa et al 1994, Natrajan et al 1995). *In situ* hybridization is yet another alternative and a highly specific technique (Gall and Pardue 1969), which has been used to detect viral and bacterial nucleic acids in tissue sections (McNicol and Farquharson 1997). The present author has, however, found that the *in situ* hybridization procedure can contribute to the histological diagnosis of Early and Suspect leprosy (Natrajan et al 2004). In situ hybridization has also been applied in paediatric leprosy with positive results across the spectrum (Dayal et al 2007).

In situ PCR is a relatively recent technique of very high sensitivity and specificity (Komminoth and Long 1993, Nuovo 1996a) wherein nucleic acids are amplified within tissue sections to the point of detection, and has been used to demonstrate nucleic acid sequences in several viral infections (Haase et al 1990, Bagasra et al 1992, Chiu et al 1992). In our initial experience this approach has been observed to be promising in enhancing the diagnosis of leprosy in children (Dayal et al 2005). The present study tests the applicability of the procedure on buffered formalin-fixed, routinely processed paraffin sections, and, its usefulness in the histoconfirmation of clinically diagnosed early leprosy and clinically doubtful cases of leprosy which are not confirmed by routine histopathology.

Materials and Methods

Cases were chosen from amongst those attending the Out Patient Department of this Institute. New untreated cases were chosen using defined criteria. Early Leprosy was defined by the presence of discrete flat lesions with partly defined margins (early Borderline Tuberculoid (BT) leprosy), or, by lesions with vaguely defined borders (Indeterminate (Idt) leprosy) in which hypoaesthesia was *clearly* demonstrable. Clinically Suspect Leprosy was characterized by lesions similar to those described above, visually suggesting leprosy, but without a demonstrable sensory deficit. The clinical details of the selected cases were recorded and skin smears performed for examining the presence of AFB. Incisional skin biopsies were taken in 10% buffered formalin

after taking the patient's informed consent. The skin biopsies were processed for paraffin wax embedding, and were subsequently stained with Haematoxylin–Eosin for studying morphologic alterations, and, with the Fite-Faraco stain for demonstrating the presence of AFB (Ridley). Specimens which were diagnostically unconfirmed by such routine histopathologic examination, were subjected to Direct *in situ* PCR procedure for detection of 530bp fragment of DNA encoding the 36kD Ag of *M leprae*. Specimens positive for AFB on histopathological examination were also subjected to the Direct in situ PCR procedure.

The direct *in situ* PCR procedure

Glass slides coated with organosilane (APES, Sigma, Cat. No. A3648) were used such that the tissue specimens would remain adherent during the harsh conditions that would prevail during the performance of the *in situ* PCR procedure. Each set of slides stained contained, apart from the test slides, a positive control in the form of an AFB positive tissue section obtained from a patient clinically diagnosed as lepromatous leprosy. The negative control consisted of the same AFB positive specimen, on which the same procedure was performed as with the test slides but with the primers omitted. A section of normal (non-diseased) skin was additionally included.

The primers chosen were directed to amplify a segment encoding the 530bp fragment of the 36kDa protein of *M.leprae* (Haartskeerl et al 1989), and bearing the following sequences –

Forward primer 5' CTC CAC CTG GAC CGG CCA T 3'

Reverse primer 5' GAC TAG CCT GCC AAG TCG 3'

The primers used are well recognized to amplify sequences specific to *M. leprae* and have been used in several studies.

The Direct *in situ* PCR procedure was performed wherein labelling occurs simultaneously with amplification. Digoxigenin was chosen as the label as it is considered the most sensitive amongst the non-radioactive labels presently available (Kessler 1991).

The following pretreatments were performed on the tissue specimens prior to amplification -

(1) Sections were dewaxed with xylene, rehydrated with descending series of alcohols and deionised water, and, treated with 0.2N HCl for permeabilizing the tissue.

(2) Proteolysis was performed with pepsin (Sigma, P6887) at a concentration of 500mg/ml in 0.2N HCl at 37^{0}C for 20 min.; the reaction was stopped with 2% gylcine, and the specimens subsequently post-fixed with 4% paraformaldehyde.

Amplification

Amplification was performed on a PTC-100 thermal cycler (Model 60 MJ Research) which had provision for a Slide Griddle attachment. The Slide Griddle is designed to accommodate standard microscopic glass slides (25mm X 75mm) on which the amplification procedure is performed.

The Amplification Mix was constituted using the commercially available PCR DIG Labelling Mix procured from Roche Molecular Biochemicals (Cat. No. 1585550) the final concentrations of the individual constituents of the mix were as follows-

Deoxynucleotides of adenosine, cytosine, and guanidine (dATP, dCTP and dGTP) 200 µM each, deoxythymidine triphosphate (dTTP) 190 µM, Digoxigenin labeled Deoxyuridine triphosphate (d UTP) 10 µM, Primers 1 µM each, $MgCl_2$ 4.5 mM, Bovine Serum Albumin 0.06%, Taq polymerase 5U/50 µl, and deionised water 32.5 µl to get a reaction volume of 50 µl.

In performing the amplification procedure, the enzyme Taq polymerase was withheld from the amplification mix for addition at a later stage. The amplification mix thus prepared was applied onto the tissue specimens over which a polypropylene

coverslip (Hybri Slip, Sigma Z 36,591-2) was placed. The glass slides with the tissue specimens were then placed on the thermal cycler where a soak file of 70° C was run. Taq polymerase was added by gently lifting the coverslip when the temperature reached 55° C. Thereafter the coverslips were sealed with a thermal seal.

The above procedure – termed the Hot Start manoeuvre (Birch 1996) – was adopted to eliminate the non-specific pathways of amplification which are known to occur with the use of the *in situ* PCR procedure.

The amplification cycles were chosen from a menu of programmes provided in the cycler and consisted of the following steps -

STEP 1 92°C 1 Sec.

STEP 2 68°C 45 Sec.

STEP 3 GO TO STEP 1 24 TIMES

STEP 4 75°C 5 Min.

On completion of the amplification cycles, the slides were allowed to cool and the coverslips removed with thin forceps after a brief dip in Standard Sodium Citrate (SSC). Post- fixation with 4% paraformaldehyde was once again repeated , followed by washings with 2X SSC for 10-15 minutes at room temperature.

Detection of the amplification product by the sequential application of

1) Blocking Reagent (Roche, Cat.No. 1096176) at 37°C, for 30 min.,
2) anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche, Cat. No. 1093274) at a dilution of 1:150, also at 37°C, for 30 min., and
3) Substrate/chromogen NBT (Nitro blue tetrazolium at 0.4 mg/ml conc.)/BCIP (Bromo chloro indolyl phosphate at 0.19mg/ml conc.)

Colour development was watched for under the light microscope and was seen to first appear between 20-60 min. of incubation in different experiments. Once a satisfactory level of colour development was obtained the reaction was stopped with deionized water. 2% Nuclear Red was employed as the counterstain and the sections mounted with the polystrene synthetic resin, DPX, for viewing.

Brief dips in and alcohol and xylene and the use of the synthetic resin did not wash away the alkaline phosphatase - NBT/BCIP end product, as is often feared. Product verification from amplified specimens was verified by size on gel electrophoresis.

Results

Twenty-five cases from the clinical categories of Early leprosy (Idt = 15, BT = 10) and clinically Suspect leprosy were included in the study. The cases were all adults (age ranges = 15-50 yrs. for both categories), predominantly male, had solitary lesions and were negative for AFB on skin smear examination. Routine histopathology confirmed the diagnosis of leprosy (all histologically Indeterminate) in 8 (32%) of the cases of Early leprosy with AFB being seen in 2 cases; in clinically Suspect leprosy histological confirmation was possible in 5 (20%) cases with AFB being seen in a solitary case. The histological confirmation of leprosy required either the presence of infiltration *within* dermal nerves or the presence of AFB.

The Direct *in situ* PCR procedure which was performed on the specimens which remained histologically un-confirmed, yielded positive signals in 12/17 (70.6%) cases from the clinical category of Early Leprosy and in 12/20 (60%) cases from the clinical category of Suspect Leprosy. Positive signals were also seen in the three histopathologically AFB positive specimens (not shown in data table). The results are depicted in Table 2. Negative results were seen where the

Table 1 : Clinical data of cases of Early Leprosy and Suspect Leprosy chosen for the study

Parameter	Category Early leprosy	Category Suspect leprosy
Sex, Male: Female	22:3	20:5
Age (yrs) Range, Mean	14-60, 27	14-60, 35
Duration (mo) Range, Mean	1-36, 10	1-24, 8

Table 2 : Data resulting from routine histopathologic, and Direct *in situ* PCR study of specimens from Early and clinically Suspect Leprosy.

Parameter	Category Early leprosy	Category Suspect leprosy
N	25	25
histopathologically confirmed diagnosis	8 (32%)	5 (20%)
N AFB positive	2	1
N ISPCR positive	12 /17 (70.6%)	12/20 (60%)

N = number of cases
AFB = Acid Fast Bacillus,
IS PCR = *in situ* Polymerase Chain Reaction

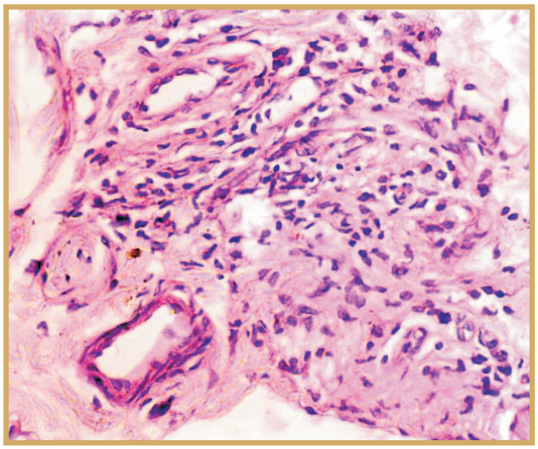

Fig 1 : Histology in Indeterminate leprosy shows infiltration into dermal cutaneous nerve but no granuloma formation. Haematoxylin-Eosin staining. Orig.Mag.100x.

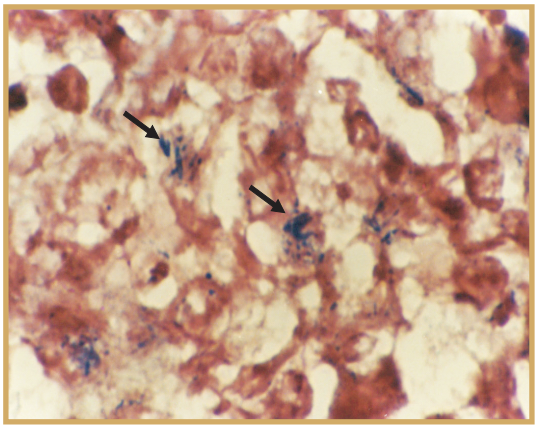

Fig 2 : *In situ* PCR positive signals (indicated by arrows)indicating amplification of 36kDa DNA fragment labeled with digoxigenin using NBT/Alkaline phosphatase system seen in a case of Early leprosy. End Product: deep blue Counterstain: Neutral Red. Orig. Mag. 670x

primers were omitted and in 8 cases of Pityriasis rosea chosen as non leprosy control.

The morphology of the tissues was fairly well preserved and the positive signals could be

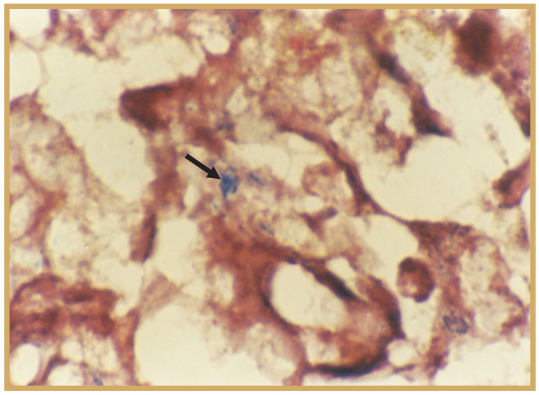

Fig 3 : *In situ* PCR positive signals (indicated by arrows) indicating amplification of 36kDa DNA fragment labeled with digoxigenin using NBT/ Alkaline phosphatase system seen in a case of Suspect leprosy. End Product: deep blue Counterstain: Neutral Red. Orig. Mag. 670x

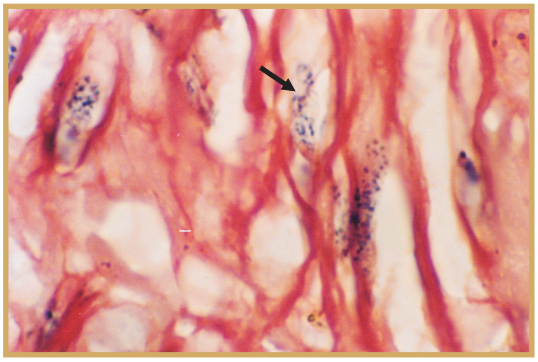

Fig 5 : *In situ* PCR positive signals (indicated by arrows) indicating amplification of 36kDa DNA fragment labeled with digoxigenin using NBT/ Alkaline phosphatase system seen in a case of Early leprosy showing variability in staining. End Product: deep blue Counterstain: Neutral Red. Orig. Mag. 500x

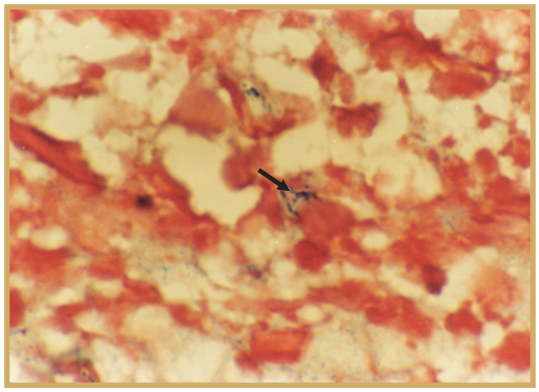

Fig 4 : *In situ* PCR positive signals (indicated by arrows) indicating amplification of 36kDa DNA fragment labeled with digoxigenin using NBT/Alkaline phosphatase system seen in a case of Early leprosy. End Product: deep blue Counterstain: Neutral Red. Orig. Mag. 500x

located in the context of contiguous pathological alterations. Nonspecific background staining was low and was mostly confined to the basal epidermis and the cells of the eccrine glands and hair follicles.

Positive signals were viewed as deep-blue deposits of the alkaline-phosphatase reaction and could be easily discerned against the red background of the Neutral Red counterstain.

The positive signals were found to be mostly located amongst the dermal infiltrates. A few signals could be seen at extracellular sites in the dermis which can be attributed to the 'out diffusion' of the end-products of amplification, a phenomenon known to occur with the use of the *in situ* PCR procedure and did not constitute a positive result. Any ambiguous staining was treated as a negative result.

Statistical evaluation of the results was done using the Z statistic for testing the significance of difference between 2 proportions. Significance testing of the increase in diagnosis following *in situ* PCR showed the difference to be significant in both the category of clinically Suspect leprosy

(z = -2.928, p = 0.001) and in the category of Early leprosy (z = -2.582, p=0.005).

Discussion

A decrease in the number of leprosy cases worldwide coupled with an increasing awareness about the disease, had already resulted in an increasing fraction of number of Early cases seen (WHO 2000). Concomitantly, there has also been a rise in the number of clinically Suspect cases seen, a situation where confirmation of the diagnosis is problematic as routine histo-pathologic examination of such lesions resolves the diagnostic doubt in only a low proportion of the cases. The need for additional means to augment the diagnosis is therefore acute. Technological advancements have presently made available, several techniques which can be put to gainful use in such situations. Immunohistochemical (IHC) staining procedures, particularly the multistep procedures (Hsu et al 1981), are highly sensitive and have been employed to augment the diagnosis to a certain extent where conventional histopathology fails. Antigenic presence has been demonstrated in AFB negative specimens with varying positivity rates (Mshana et al 1982, Barbosa et al 1994, Natrajan et al 1995). *In situ* hybridization (*IS*H) is a powerful technique, which has become more amenable for use beyond the research laboratory by virtue of the easy availability of known nucleic acid sequences and the advent of highly sensitive nonradioactive systems for labelling and detection. The technique has been successfully employed to detect viral and bacterial nucleic acid sequences in tissue specimens. The *in situ* hybridization procedure however has detection limits of the order of 10-20 copies of DNA/cell, or, 40kB of target DNA (Nuovo 1996b). Studies comparing the sensitivities of IHC and ISH have yielded conflicting data with the results obtained appearing to be dependent on the situation at hand. In the experience of the author, ISH is the more sensitive technique in the context of histological diagnosis of Early leprosy and Suspect leprosy (Natrajan et al 2004).

In situ PCR is a still more powerful technique, where nucleic acids in tissue specimens are amplified to the point of detection, and has been successfully employed to demonstrate the tissue presence viral nucleic acids. Studies which have employed both ISH and *IS*-PCR have invariably found the latter to be superior in terms of sensitivity despite the amplification not being exponential as is seen in solution phase PCR. In the present study, the procedure was employed to demonstrate *M leprae* specific nucleic acids.

The Polymerase Chain Reaction as a procedure has been widely used to detect *M leprae* in tissue specimens but has been performed on DNA/RNA extracted from the tissue specimens (Woods and Cole 1989, deWit et al 1991, Arnoldi et al 1992, Wichitwechkarn et al 1995, Sharma et al 1996, Misra et al 1996, Singh et al 2004). Studies employing the in situ Polymerase Chain Reaction have been performed at the Institute on paediatric group of patients with favorable results (Dayal et al 2005). An overall positivity rate of 57.1% was reported for Early leprosy and 36.3% positivity in cases showing nonspecific pathology. The present study in adults stresses on cases showing nonspecific pathology which leaves no diagnostic alternative, and, importantly, also includes clinically suspect cases an entity not studied in the previous report on paediatric cases. The results obtained are still more encouraging with higher positivities of 70.6% and 60% in the categories of early and suspect leprosy respectively.

A few technical aspects of the procedure performed, deserve special mention.

In situ PCR can be performed in one of two ways (Kommimoth and Long 1993). In the *Direct*

procedure employed in this study, labelling takes place during the amplification step, with one of the deoxynucleotides bearing the label, and, the end product is detected by immunocytochemical means. In the *Indirect* procedure, amplification is first performed and the amplified product subsequently hybridized and then detected immunocytochemically. The latter procedure is considered more sensitive but in this study the Direct procedure, which is less cumbersome, performed very well. The non-specific pathways of the PCR were prevented by using the Hot Start procedure (Birch 1996), and the occasional failure and tissue destruction encountered is more likely to occur with use of the Indirect procedure. Yet another aspect deserving mention is the occurrence of signals at extra cellular and unanticipated locations due to out-diffusion of the amplified product (Leary 1998), which however, is not difficult to discern from the artifacts formed by NBT/BCIP precipitates. The alkaline phosphatase NBT/BCIP system of detection employed is the most sensitive non-radioactive system presently available (De Jung et al 1985).

It was observed that the time taken for colour development varied between 20-60 minutes of incubation in different experiments. This variation in time may appear high but the reaction was observed under the microscope for the development of colour and the reaction was stopped at its very first appearance. The possibility of overstaining and false positives was therefore minimal.

These limitations not withstanding, the diagnostic yields far outweigh the effort expended. Further improvements in the state of instrumentation for performance of the procedure on glass slides, and availability of reagents like the Fast Taq polymerase which de-necessitates the use of the Hot Start procedure, has undoubtedly ease the technical difficulties of the procedure.

In the present state of art itself, this study has amply demonstrated the usefulness of the procedure in histologically unconfirmed cases of clinically diagnosed Early and Suspect leprosy.

Conclusion

The present study reports the utility of the Direct *in situ* PCR procedure in the histological diagnosis of Early leprosy and clinically Suspect leprosy, wherein routine histology is inconclusive. The histologic confirmation obtained with routine histopathology were 32% for Early Leprosy and 20% for clinically suspect leprosy. With performance of the Direct *in situ* PCR on the histologically unconfirmed cases, the positivity rates obtained were 70.6% and 60% respectively. The results of the study confirm the utility of the procedure in the diagnostically difficult situations of Early and Suspect leprosy, and proposes the procedure be employed in all situations of clinical doubt.

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