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Original Article

Use of RLEP-PCR as a Molecular Tool and Definitive Laboratory Test from Skin Smear Scrapings for Early Diagnosis of Leprosy in Field Situations

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The diagnosis of leprosy is based on clinical suspicion and demonstration of cardinal signs and symptoms of the disease due to limited availability of efficient laboratory investigations. Among the available laboratory tests for leprosy the most commonly used is AFB smear microscopy with ZN staining. Histopathology of biopsy specimen is useful, but is an invasive procedure and correlation with early clinical disease is often not definitive. The histology findings in such situations have often to be corroborated with clinical findings for confirmation of diagnosis. Molecular tests like polymerase chain reaction (PCR) using RLEP (*M leprae* repetitive element) in slit skin smear scrapings (SSS) is simple technique, can be done from the same skin smear specimen, and has been observed to be advantageous over SSS AFB both in terms of sensitivity as well as it was highly specific. The objective of the present study was to assess the applicability and sensitivity of RLEP-PCR from skin smear specimens in field conditions. After taking the informed written consent, the slit skin smears of 169 clinically diagnosed leprosy patients (2010-2012), living in Ghatampur tehsil were evaluated, using the standard technique for AFB microscopy. The blade used for making the skin smear slide was put in a sterile eppendorf with TE buffer, after making the skin smear, and transported at room temperature to the Microbiology and Molecular Biology Laboratory at NJIL & OMD, for molecular tests. *M leprae* DNA was isolated from the TE buffer solution in which the skin smear blade was dipped as per the method described by Sharma RK et al 1996. The isolated DNA was amplified using the standard protocol

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described by Donoghue et al (2001). The primers used in RLEP experiments were : F-5'TGCATGTCATG GCCTTGAGG3' & R-5'CACCGATACCAGCGGCAGAA3'. AFB were detected in skin smears in 14/169 patients (8.3%); RLEP-PCR positivity was observed in 84 (49.7%) in the same patients samples. This positivity was observed in both PB and MB patients; the difference in sensitivity was highly significant ($p<0.0001$). RLEP PCR on slit smear specimens can be done from skin smear samples. It is not invasive as a biopsy, more sensitive as well as specific test than AFB microscopy for the diagnosis of leprosy, well accepted by patients and can easily be undertaken in field conditions.

Key words : Polymerase Chain Reaction (PCR); Slit Skin Smear (SSS); Ziehl Neelson (ZN) staining; post elimination era; early diagnosis

Introduction

Mycobacterium leprae the causative organism of leprosy is a rod shaped acid fast bacilli and infects and affects primarily the skin and peripheral nerves. The organism is not culturable in artificial media and therefore the definitive diagnosis of the disease is not easy and at present is based on the clinical characteristics of the disease, especially in programme settings. Rapid, specific and sensitive tests are needed for early definitive diagnosis (Cox et al 1991, Wichitwechkarn et al 1995), and institution of effective treatment to further reduce the burden of the disease.

The advances made after the identification and deciphering of the *M leprae* genome (Cole et al 2001), has ushered in several molecular methods including RLEP based PCR, for the detection of *M leprae*/DNA/RNA in different clinical samples/ patient materials. These include slit-skin smears (Wichitwechkarn et al 1995, Phetsukisiri et al 2006, Jadhav et al 2006, Martinez et al 2011), blood, nasal mucosa and its secretions (Santos et al 2001, Torres et al 2003, Banerjee et al 2010), biopsy specimens (Kang et al 2003, Martinez et al 2006, Yen et al 2014) as well as from environmental samples (soil and water) in endemic settings (Lavania et al 2006, Turankar et al 2015). The aim of the present study was to assess the use of RLEP-PCR in the field conditions, where majority of the patients are diagnosed with early disease, are AFB negative and histology is not feasible. In paucibacillary and early stage leprosy, who harbor a lesser bacillary load, definitive diagnosis is difficult, as AFB staining of bacilli in skin smears requires at least $\sim 1\times10^4$ bacilli/ml to be detected by light microscopy, to make a definitive diagnosis. Histopathological findings can be non-specific and often need to be corroborated with the clinical findings (Cochrane 1961, Porichha et al 1993, Bhatia et al 1993) for making a definitive diagnosis.

India achieved leprosy elimination as a public health problem in Dec 2005, but pockets of endemicity, exist in some areas. In absence of specific laboratory tools, absence of a vertical programme & paucity of trained manpower, early and accurate diagnosis is an issue and needs to be tackled. Leprosy is known to spread by human to human contact after a long and variable period. However, during history taking it has been observed, (especially in the post elimination era)that most of the new cases have no previous contact with known leprosy patients. Most of the leprosy cases detected today are PB patients, AFB –ve, and having 2 to 3 lesions with or without nerve trunk involvement and discernable enlargement. Diagnosis is therefore made on basis of the clinical findings alone. In the present study we have used RLEP PCR of *M leprae* from skin smear scrapings, for the definitive diagnosis of leprosy patients under field conditions.

Material and Methods

All patients were clinically confirmed as leprosy by the Medical Officer, after examining the suspected cases identified by the field investigator/worker. These confirmed leprosy patients belonged to both the sexes, 4 to 74 years age group, with variable number of skin patches (one to innumerable) with impairment/loss of sensation, with or without thickened nerve trunks (Table 1). None of the patients were of pure neuritic and/or of LL type. Ethical Approval for the study was obtained from the Institutional Ethical Committee of NJIL & OMD. Slit smears were undertaken for all leprosy patients after explaining the procedure to each of them as well as their guardians, and obtaining their Informed Written Consent. RLEP-PCR was done from the DNA extracted from TE buffer solution containing the blade used for doing the slit skin smear sample as per the protocol.

Study Protocol

Sample Collection

A total of 169 leprosy patients, who were clinically diagnosed as suffering from leprosy, during the period 2010 to 2012 from Ghatampur MRHRU field area in Uttar Pradesh, were included in the study after obtaining their informed written consent. The slit skin smears of these patients and controls were taken for AFB as per the standard method, from an active lesion, and the blade used for making the smear was put in labeled screw capped Eppendorf tube containing TE buffer. This was labeled and transported to NJIL & OMD, Agra laboratory, at room temperature. Slit smear scraping samples were also collected from the skin patch of 15 patients having other skin problems like Pityriasis and Psoriasis from OPD of the Institute, and these acted as negative controls for the study.

Extraction of *M. leprae* genomic DNA

DNA was isolated from the TE buffer containing the patients slit skin scrapings by following a procedure as adapted from van Soolingen et al 1993 (Sharma et al 1996). Bacilli were disrupted by freezing and thawing, followed by enzymatic disruption by lysozyme (3mg/ml for 2 hours followed by proteinase K treatment (250 µg/ml for 6 hours). To this extract an equal volume of phenol : chloroform (I:I) was added and thoroughly mixed. After centrifugation at 8000g for 15 minutes, deproteinization was done with chloroform-isoamyl alcohol (24:1 v/v) and repeated 2-3 times till clear interphase was obtained. After centrifugation at 8000 g for 15 minutes, the upper phase was collected. DNA was then precipitated from the upper phase with 0.6 volume of isopropanol, washed with chilled ethanol, dried and re-suspended in 25 µl of (TE) Tris-EDTA buffer before being used for PCR amplification.

Amplification of DNA by PCR (RLEP-PCR)

Stringent precautions were taken to avoid cross-contamination and 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 l^{-1} deoxynucleoside triphosphate, 0.5 mol primers for RLEP gene and 1U Taq polymerase (Bangalore Genei, Bangalore, India). The 129 bp fragment was amplified by using primers and procedure described by (Donoghue et al 2001). The forward primer for RLEP was, 5-TGCATGTCATGGCCTTGAGG3' and the reverse primer was, 5'CACCGATACCAGCGGC AGAA 3'. PCR was performed using the following temperature cycles: at 95ºC for 2 minutes (initial denaturation); then for 45 cycles at 94°C for 30 seconds; 58°C for 2 minutes and 72°C for 2 minutes, followed by final extension at 72°C for 8 minutes and kept at 4°C till further use. A 100 bp marker (Bangalore, Genei) was included on every

gel to determine the RLEP-PCR product size of 129 bp. Each amplification reaction was analysed on 2% Agarose gel in TAE (mixture of Tris base, acetic acid and EDTA) buffer (pH 8.0). Gels were stained with 0.5 µg/ml ethidium bromide and photographed using transmitted UV light.

Data was analyzed using McNemar test to see the difference between two techniques i.e. Slit Skin Smear AFB and RLEP PCR from skin smear scraping/solution.

Results

A total of 169 clinically classified leprosy patients were included. These patients were inhabitants from MRHRU Ghatampur area and the period of study was from 2010 to 2012. The skin lesions and nerves were recorded on the body chart and written informed consent from each of the cases was taken for performing slit skin smears and testing for RLEP PCR. Besides the operational NLEP classification, the disease was also classified using IAL (Indian Association of Leprologists classification 1982), which was done on clinical examination and is known to correlate very well with the Ridley Jopling immune-histological classification (Indian Association of Leprologists Classification 1982).

These leprosy cases included 91BT patients (PB) and 78 MB (72BB and 6 BL) patients (Table 1). Among these there were 54 females and the rest 115 males. It was observed that 30/169 patients (17.8%) belonged to the age group of 4 to 19 years.

Ninety one of the 169 patients (53.8%) were of the BT type indicating that the majority of cases were of the early forms of leprosy, 72 patients were of BB type and only 6 patients were of BL type. Further, majority of cases belonged to the age groupings of 10-50 years and 17.8% were less

Table 1 : Classification of leprosy patients and their age wise distribution

IAL disease classification as assessed clinically	Age in years								Total
	Up to 9 years	10-19 years	20-29 years	30-39 years	40-49 years	50-59 years	60-69 years	≥ 70 years	
BT	1	22	16	23	15	8	5	1	91
BB	2	5	11	20	12	10	7	5	72
BL	Nil	Nil	1	2	3	nil	Nil	nil	6
Total	3	27	28	45	30	18	12	6	169

Table 2 : Comparative positivity of SSS-AFB and RLEP PCR from skin smear scrapings, according to the disease classification and in controls (non leprosy patients)

Diagnosis by	Disease classification			Total	Other skin diseases
	BT	BB	BL		
Clinical examination	91	72	6	169	15
AFB positivity observed in Slit SSS	3 (3.3%)	7* (9.7%)	4 (66.7%)	14 (8.3%)	Nil
RLEP PCR positivity in washings of blade of SSS	36 (39.6%)	44* (61.1%)	4 (66.7%)	84 (49.7%)	Nil

*1 patient was AFB positive, but RLEP-PCR negative

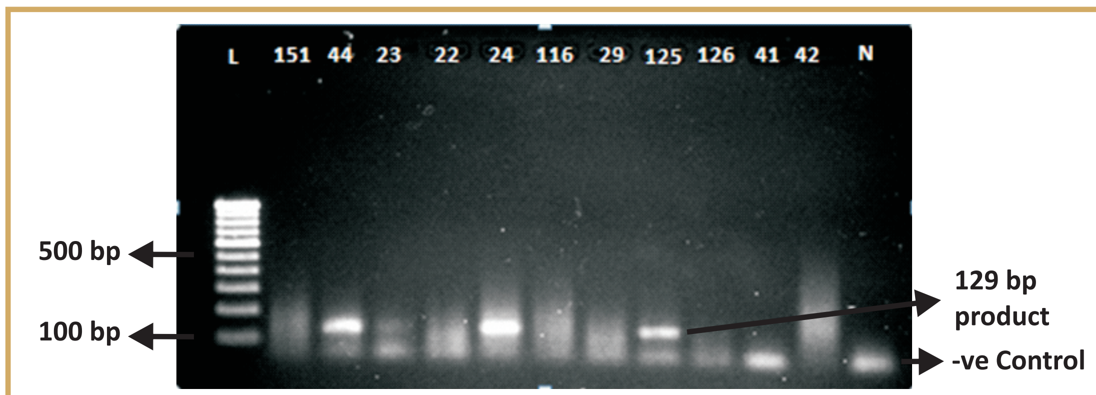

Fig 1 : PCR amplification of 129bp fragment of RLEP of *M leprae* on 2% agarose gel of the DNA isolated from washings of SSS blade.

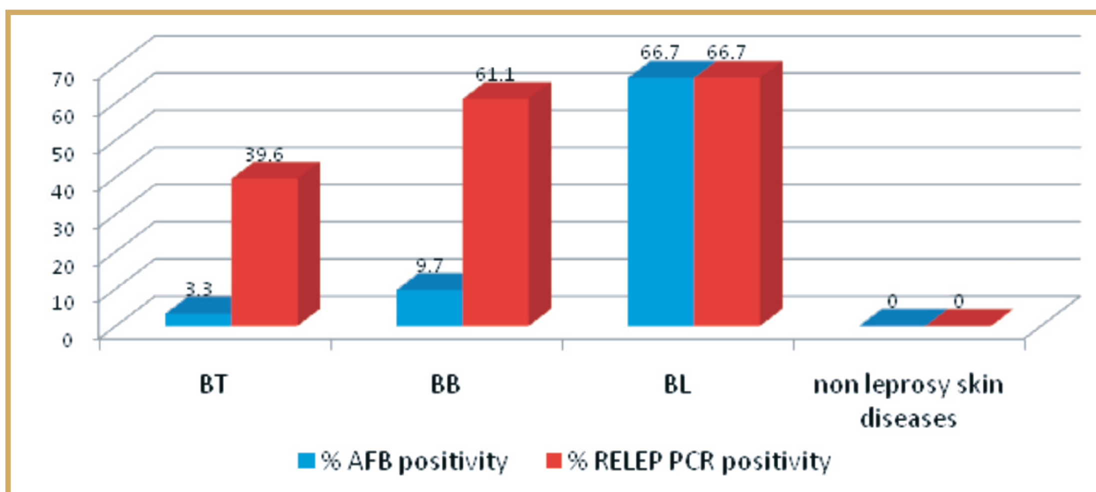

Fig 2 : Positivity rates of AFB skin smears and RLEP-PCR according to disease classification

than 19 years of age. Of these clinically diagnosed leprosy patients 14 (8.31%) were AFB positive on ZN staining, while the rest 155 cases were AFB negative on skin slit smear examination. These AFB positive cases included 5 females and 9 males. Figure 1 shows the 129bp amplification in RLEP PCR on agar gel electrophoresis. RLEP PCR positivity was observed in 84 specimens i.e. in 49.7% of leprosy cases. These 84 RLEP PCR positive leprosy cases (49.7%) included 36 PB cases and 48 MB cases (Table 2); SSS AFB positivity was observed in 8.3% of cases. The difference between the two tests was statistically significant ($p<0.0001$). Also this was more significant in BT type of patients.

It was observed that one patient from BB group who was SSS AFB positive was RLEP PCR negative. All the rest of the patients who were SSS positive were also RLEP PCR positive. RLEP PCR was positive in more number of early disease (BT disease) and this difference was statistically significant (p-value<0.0001). Moreover, none of patients with other skin diseases were RLEP PCR positive for *M leprae*.

Discussion

Leprosy is a chronic infectious disease caused by *M leprae.* Although India has been notified as a country which has achieved leprosy elimination in December 2005 the disease is still prevalent in some pockets of the country. New cases are being detected in nearly the same rate annually in programme conditions for the last 9-10 yrs. This implies that transmission is continuing and more tools/methods are required for early diagnosis and efficient treatment of the disease.

The diagnosis of leprosy is based to a major extent on clinical examination and due to dwindling trained manpower in the post elimination phase, a careful and correct diagnosis is important to detect and treat the identified cases. Among the available laboratory tests for diagnosis of leprosy the most commonly used is AFB smear microscopy by the ZN staining due to its high specificity. However this test has a limited sensitivity ranging from 10% to 50% (Report of the International Leprosy Association Technical Forum 2002). Histopathology is usually not undertaken in programme settings and moreover, especially in early forms of the disease it is often reported as non specific and has to be correlated with the clinical findings and follow-up (Porichha et al 1993, Bhatia et al 1993).

PCR amplification of DNA/RNA of *M leprae* have been used to develop diagnostic tests for early diagnosis, monitoring progress of patients as well as assessment of viability of *M leprae.* These have been demonstrated to be advantageous over smear microscopy both in terms of specificity and sensitivity by several researchers (Cox et al 1991, Phetsukisiri et al 1994, Jamil et al 1994, Kang et al 2003, Wichitwechkarn et al 2004, Martinez et al 2006, Bannerjee et al 2010, Yan et al 2014, Turankar et al 2015).

The genome of *M leprae* is 3,268,203 bp long and the G+C content of the genome is 57.8% (Cole et al 2001). This genome consists of several inter spread repeats of which RLEP (Repetitive element of *M leprae*) is a well known. This interspread repeat has 37 copies, and is highly specific to *M leprae* and is not present in the genome of any other bacterial species (Donoghue et al 2001). The use of a repetitive sequence RLEP as a PCR target provides an additional advantage of higher sensitivity over other targets in the DNA because it is present at multiple sites in the genomic DNA. Other gene targets such as 18 KDa protein, 16S rRNA, Ag85B, rpoT and sodA have also been evaluated for their ability to diagnose early leprosy (Yoon et al 1993, Jamil et al 1994, Kang et al 2003, Martinez et al 2011, Turankar et al 2015). On comparison of different gene targets and amplification with PCR, RLEP-PCR was found to be most sensitive (Jadhav et al 2005, Kang et al 2003, Martinez et al 2011, Yan et al 2014, Turankar et al 2015).

In the present series RLEP PCR was found to be positive in 49.7% of leprosy cases which included all patients being treated and followed up in 1-3 year period in the field area. This positivity was 39.6% (36/91) in BT patients; 61.1% (44/72) in BB patients and 66.7% (4/6) in BL patients (Fig. 2).

In the present series one patient of BB group was observed to be AFB positive but RLEP PCR negative. All other AFB positive cases were RLEP PCR positive. It could be possible that this patient was scarcely positive and the DNA was lost due to

stringent PCR conditions used. Moreover as RLEP PCR was done from skin scrapings after making the skin smears, the small amount of DNA present on the blade may have been lst on the slide and no DNA was detected in the TE buffer solution containing the blade.

The sensitivity of RLEP PCR in comparison with AFB smear positivity was highly significant p<0.000.1. A percentage of both PB and MB patients were negative for RLEP PCR thus indicating that *M leprae* (AFB positivity in SSS), as well as *M leprae* RLEP was not present in the specimens although clinically they were having the disease and being treated for it. This could be due to sampling error as only a small superficial skin sample is/was sampled from the lesion. Scrapings taken from different and more number of active lesions/areas could have yielded better results as reported by others (Kang et al 2003, Phetsuksiri et al 2006)., who undertook the PCR from multiple sites. As these patients were also on treatment it is possible that the disease may have subsided at the smear taking site in some of them. Also, the bacilli and its components (DNA) was not present in the superficial skin but could be present deeper down in the dermis. This has been reported and documented in histological examination in several studies (Natrajan et al 2004, 2012*),* and *in situ* demonstration of *M leprae* could be a more sensitive diagnostic tool than the slit skin smear scrapings. It is important to note that none of the non leprosy cases were positive for RLEP PCR of *M leprae* and is therefore the test is specific. Similar findings have also been reported by (Martinez et al 2011, Yen et al 2014). In the series of studies reported by several investigators the rate of positivity for RLEP PCR was more than that reported in the present study (Kang et al 2003, Phetsuksiri et al 2006, Turankar et al 2015). This could be due to inclusion of all type of cases (newly diagnosed and on treatment cases), in the present study, as well more early cases as these patients were detected in field conditions and were not reported patients from clinics/hospitals. It is also possible that some amount of *M leprae* DNA was lost during the DNA sample processing.

In the present post elimination era with dwindling trained manpower and decreasing availability of experienced clinicians, it is important to have definitive diagnosis of leprosy. In such scenario, RLEP PCR from skin smears provides a very good tool. Additionally it can be done from both skin smears as well as in biopsies of tissue specimens including *in situ* RLEP PCR demonstration in the tissues. Demonstration of *M leprae* specific DNA/RNA, RLEP can provide a better confirmative tool for early definitive diagnosis. With advancements like *in-situ* hybridization and *in-situ* PCR (Natrajan et al 2004, 2012) the sensitivity of detection has increased manifold and definitive diagnosis can be reached in substantial number of early and suspicious disease patients.

The present technique is simple, specific, well accepted by patients. It can be used in field conditions, is more sensitive than AFB staining and can be undertaken from routinely done skin smears and provides a definitive diagnosis of the disease in substantial number of cases.

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