Appropriately Selected Nerve in Suspected Leprous Neuropathy Yields High Positive Results for *Mycobacterium leprae* DNA by Polymerase Chain Reaction Method

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Abstract. Identification of *Mycobacterium leprae* DNA by polymerase chain reaction (PCR) is a reliable and an affordable method to confirm leprosy. DNA from 87 nerve samples (61 from paraffin blocks and 26 fresh samples) was extracted. *Mycobacterium leprae* DNA was amplified by PCR from 80/87 (92%) specimens. Patients were seen over a period of 11 years (2007–2019), and leprosy was diagnosed based on clinical and characteristic histopathology findings. The clinical diagnostic possibilities were as follows: leprosy neuropathy in 73/80 (91.3%), mononeuritis multiplex of unknown etiology in four (5.0%), vasculitic neuropathy in two (2.5%), and distal symmetric sensory motor neuropathy in one (1.3%). The biopsied nerves were as follows: superficial radial = 34 (42.6%), dorsal cutaneous branch of ulnar = 19 (23.8%), sural = 18 (22.5%), and superficial peroneal = 9 (11.3%), and corresponding neurological deficits were recorded in 77 (96.3%) cases. The histopathological diagnoses in total group were as follows: (borderline tuberculoid (BT) = 52, tuberculoid (TT) = 8, borderline lepromatous (BL) = 8, borderline borderline (BB) = 3, nonspecific inflammation = 3, healed/fibrosed = 4, and axonopathy = 2). Acid fast bacilli (AFB) was demonstrated in 11 (13.7%) samples. For comparison, 31 clinically and histopathologically defined non-leprosy disease control nerves (inherited neuropathy = 20, vasculitis = 8, and nutritional neuropathy = 3) subjected to PCR were negative for *M. leprae* DNA. In most instances, there are multiple thickened peripheral nerves in suspected cases of leprosy, but neurological deficits pertaining to the thickened nerve are not as widespread. The current findings emphasize the importance of selecting the most appropriate nerve for biopsy to obtain a positive PCR result. We infer that clinical, histopathological, and PCR tests complement each other to help achieve a definitive diagnosis of leprosy particularly in pure neuritic leprosy and in leprosy neuropathy with negative skin smears/biopsy.

INTRODUCTION

Leprosy or Hansen's disease, an ancient mycobacterial disease, results from the uncultivable pathogen *Mycobacterium leprae*.¹ Although the prevalence of leprosy in India is less than 1/10,000 since 2005, it still accounts for 62% of the total new cases reported worldwide,² and more than 81% of the new cases are reported from three countries—India, Brazil, and Indonesia.³

Since the implementation of multidrug therapy, the prevalence of leprosy has significantly reduced worldwide, but because of the occurrence of permanent disabilities and sequelae, leprosy still represents a serious health problem in the developing countries.⁴

The *M. leprae* bacillus causes mononeuropathy, multiple mononeuropathy, and symmetrical neuropathy, resulting in autonomic, sensory, and motor dysfunction. The peripheral neural impairment includes nerve trunks as well as distal cutaneous branches. Sensory symptoms often are the most common and initial complaints.^{5–8}

In addition, up to one-fourth develop neuropathy during or after treatment.⁹

Worldwide, more than three million people are living with disabilities due to leprosy.¹⁰ Early detection and correct treatment of neuropathy is imperative to prevent permanent neurological disabilities. It is also observed that when sensory impairment is clinically detectable, significant damage has

already occurred to the nerves.¹¹ Primary neural leprosy (PNL), also known as pure neural or neuritic leprosy form, was initially described in the Indian classification published in 1955.¹² Since then, it is considered a challenge to clinicians. This clinical form is characterized by absent skin lesions and negative slit skin smear (SSS) on bacilloscopy. Therefore, diagnosis is mainly based on supplementary tests, such as electroneuromyography, nerve biopsy, serology, and molecular analyses.^{13–18}

Presence of minimal symptoms at disease onset frequently leads to diagnostic errors and underdiagnosis/misdiagnosis of the neural form. In such cases, polymerase chain reaction (PCR) testing for *M. leprae* DNA helps in establishing an early diagnosis and timely treatment.^{13,19,20}

In the current study, extending over a period of 11 years (2007–2019), we procured 87 specimens of peripheral nerve tissue from clinical and histopathologically defined cases of leprosy. We report on the identification of *M. leprae* DNA from preserved as well as fresh nerve samples by PCR method (61:26). Here, we stress that selection of the peripheral nerve associated with corresponding neurological deficits results in good yield of *M. leprae* DNA by PCR.

MATERIALS AND METHODS

Study subjects. This study was conducted at the National Institute of Mental Health and Neurosciences (NIMHANS) Bangalore, located in the southern part of India. It is a quaternary care center for all neurological disorders. Here, cases with predominantly pure neuritic form of leprosy or leprosy neuropathy with negative SSS and those with neuropathy and unnoticed skin lesions attend the outpatient clinics.

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We scrutinized the neuropathology records with a diagnosis of leprosy maintained over a period of 11 years (2007–2019). The clinical criteria applied were as follows: presence of a sensory and autonomic/sensory motor and autonomic neuropathy with or without skin lesions. We identified 102 retrospective relevant histopathology reports. Of these, 61 paraffin blocks were available. Prospectively, 26 fresh nerve samples stored in RNAlater solution made up a total of 87 samples for the study. We also selected 31 non-leprosy nerve tissue samples as negative controls and subjected the tissue to PCR for *M. leprae* DNA.

Ethics statement. As part of the hospital requirement, all patients provide consent for minor procedures such as nerve and muscle biopsy during registration at the outpatient clinic. All patients included in the prospective group gave written informed consent for biochemical tests and biopsy procedure as part of diagnostic tests. Ethics approval from “NIMHANS ethics committee” (Institutional Review Board) was obtained to collect all clinical, biochemical, and histopathological characteristics from the hospital medical records and also for processing stored nerve tissue for PCR study.

Detection of *M. leprae* DNA by PCR. All samples of DNA isolated from the preserved nerve tissue and the fresh nerve tissue stored in RNAlater solution were tested at Stanley Browne Laboratory, the Leprosy Mission Trust India at New Delhi. Nerve tissue from paraffin blocks were subjected to FFPE DNA (M/s. Analytik Jena AG, Jena, Germany) kit method for DNA extraction. *Mycobacterium leprae*-specific DNA was amplified by PCR method. Of the 87 specimens tested, PCR amplified the *M. leprae* DNA from 80 samples, including the 26 fresh nerve tissue samples preserved in RNAlater solution.

Polymerase chain reaction methodology. Polymerase chain reaction-based gene amplification was performed using primers according to the guidelines of the WHO.

“Global Surveillance of Drug Resistance in Leprosy 2008” for the detection of mutation in *rpoB*, *gyrA*, and *folP1* genes in *M. leprae* genome. The PCR mixture contained 12.5- μ L Hot Start Taq polymerase PCR master mix (2 $\times$) (Qiagen, Hilden, Germany), 1.25 of μ L forward primer and reverse primer at a final concentration of 0.5 μ M, and 5 μ L of template DNA from the processed sample. The final volume of reaction mix was made up to 25 μ L with nuclease-free water. Primer sequences used in this study were as follows: *folP* primers *folPF* CTTGATCCTGACGATGCTGT; *folPR* CCACCAGACACATCGTTGAC, *rpoB* primers *rpoBF* GTCGAGGCGATCACGCCGCA; *rpoBR* CGACAA TGAACCGATCAGAC, and *gyrA* primers *gyrAF* ATGGTCTCAAACCGGTACATC; *gyrAR* TACCCGGCGAACCAGAAATTG. The reaction was cycled 40 times at 94°C for 1 minute, 60°C for 1 minute, and 70°C for 1 minute, which is preceded by initial denaturation at 95°C for 15 minutes and ended by final extension at 72°C for 10 minutes.

Each reaction setup contained one negative and one positive control. Detection of PCR product was performed on a 2% agarose gel, and amplicons were excised from the gel and purified by using the Qiagen Gel Extraction Kit.

RESULTS

All patients were referred to our center with symptoms of neuropathy and underwent nerve biopsy for confirming the aetiological diagnosis. Based on the clinical and nerve

histopathology findings, 87 cases of leprosy were selected for this study. DNA could be isolated from all the 87 nerve tissue specimens. *Mycobacterium leprae*-specific DNA could be amplified by PCR from 80 of the 87 (92%) specimens, and these were included for the final analysis. The seven patients' DNA samples not amplified by PCR were also diagnosed to have leprosy by histopathology: (healed leprosy = 2, lepra reaction = 1, and borderline tuberculoid [BT] = 4 [one showed AFB]). There were 56 drug-naïve cases, whereas 24 were on variable periods of MDT (range, 6 months to 2 years) at the time of biopsy. Among the 80 cases, the clinical diagnoses were as follows: mononeuritis multiplex suggestive of leprosy neuropathy in 73/80 (91.3%), mononeuritis multiplex with no definitive diagnostic signs of leprosy in four (5.0%), possible vasculitic neuropathy in two (2.5%), and distal symmetric sensory motor neuropathy in a HIV-positive case (1.3%). The 24 patients already receiving multi drug therapy (MDT) for leprosy had developed fresh signs of neuropathy and had no features to suggest lepra reaction.

For the entire group, the mean age at the onset of disease, age at presentation, and duration of illness was 35.4 ± 12.9 (10–71) years, 37.8 ± 13.3 (14–71) years, and 4.7 years (1 month to 24 years), respectively. Burning paresthesias in distal extremities was reported in 39 cases (48.8%), tingling paresthesias in 25 (31.3%), and numbness in 24 (31.2%). Ulnar nerve was the most prominently and commonly observed thickened nerve followed by superficial radial and sural. Painful tender nerves were present in 54 (67.5%) cases. Skin lesions were reported in 27 (33.7%), but examination revealed skin changes in 42 patients (hypopigmented macules in 42 [52.5%] patients, skin nodules in 11 [13.8%], and painless foot ulcers in 18 [22.5%]). Thus, patients had features of mononeuritis multiplex with predominant involvement of the upper limbs. Slit skin smears had been performed in 15 patients from the referring hospital and reported as negative for lepra bacilli. Skin biopsy performed by prior treating dermatologists was available in 18 (22.5%) cases; granulomas with no AFB were seen in four, nonspecific inflammation in seven, and no diagnostic pathology in seven cases. We had no access to the slides for review. The biopsied nerves in our cohort were superficial radial in 34 (42.6%), dorsal cutaneous branch of ulnar nerve in 19 (23.8%), sural nerve in 18 (22.5%), and superficial peroneal nerve in nine (11.3%) (Figure 1). The peripheral nerve with deficits in its area of supply was biopsied in 77 (96.3%) cases. The histopathological diagnosis were as follows: (BT = 52, TT = 8, BL = 8, BB = 3, nonspecific inflammation = 2, healed/fibrosed = 3, and axonopathy = 1), whereas only the thickened nerve was biopsied in three cases (healed leprosy = 1, nonspecific inflammation = 1, and axonopathy = 1) (Figure 2). AFB was demonstrated in 11 (13.7%) cases. One of these patients with mild axonopathy in the thickened superficial radial nerve had no corresponding deficits. However, fine needle aspiration material of the ulnar nerve abscess revealed *M. leprae* DNA. Histopathologically, perineural thickening was seen in 78 (97.6%), endoneural fibrosis in 75 (93.8%), lymphocytes in 74 (92.6%), perivascular inflammation in 73 (91.3%), histiocytes in 42 (52.5%), foam cells in 28 (35.1%), giant cells in 20 (25.1%), granulomas in 22 (27.5%), plasma cells in 19 (23.8%), and caseous necrosis in eight (10.0%) biopsies (Figure 3). Polymerase chain reaction performed in the disease control nerves was negative for *M. leprae* DNA in all 31 samples.

FIGURE 1. Selection of the nerve for biopsy.

DISCUSSION

There are a limited number of studies assessing the importance of identification of PCR-based *M. leprae* DNA from predominantly neural form and pure neural leprosy. This was an ambispective study on nerve tissue samples collected over a period of 11 years. The main aim was to identify *M. leprae* DNA by PCR technique in DNA isolated from biopsied peripheral nerve tissue preserved in paraffin block and fresh nerve tissue collected in RNAlater solution. The total number of DNA samples subjected for PCR was 87, and in 80 samples, *M. leprae* DNA could be amplified. We also studied 31 non-leprosy nerve tissues as negative controls.

Over the last two to three decades, detection and identification of *M. leprae* DNA in various tissues has proven to be a useful tool in diagnosing leprosy.^{21–23} Direct detection of AFB in slit-skin smears has a high specificity but low sensitivity, as approximately 50% of all leprosy patients are SSS negative.^{24,25} In the current cohort, SSS was negative in all cases when it was performed, and skin biopsy revealed non-specific granulomas in only 4/18 cases, and none showed

AFB. Thus, no definitive diagnosis of leprosy could be made from SSS and skin biopsy even in cases with skin lesions.

Leprosy patients lacking skin manifestations, but having involvement of one or more nerves, are afflicted with pure neural leprosy (PNL), which accounts for 3.9–8.2% of all leprosy patients in India.^{26–28} Generally, the diagnosis of leprosy is clinical and depends on the presence of hypoesthetic skin lesions, thickened peripheral nerves with sensory motor and autonomic neuropathy, and supplemented by demonstration of AFB in skin/nasal smears/nerve biopsies. Definite histopathological features showing pathological alteration in nerve specimens along with AFB are seen in only 35% of early cases.²⁹ In our cohort, such a definitive diagnosis was possible in only 11 (13.7%) cases, which is far less compared with the aforementioned report. In our highly biased cohort, PNL comprised 50% of cases (40/80), and four (10%) of the 40 were reported as healed leprosy/fibrosed nerve. These patients predominantly had symptoms related to neuropathy and hence consulted our neurology services. Curiously, several patients and the treating physicians did not notice the skin lesions.

FIGURE 2. Bar diagram showing the type of leprosy based on the nerve histopathology findings.

FIGURE 3. Bar diagram showing the histopathology findings as demonstrated on nerve biopsy.

There are very few reports in the English literature with regard to pure neural leprosy (PNL) and its diagnostic workup.^{27,28,30–33} Confirming the diagnosis in PNL is challenging even for experts in peripheral nerve disorders, and it is not uncommon to mislabel/wrongly treat patients because of nerve biopsy being reported as inconclusive/nonspecific inflammatory findings. In our cohort, PNL was diagnosed in almost 50% of the cases, and treatment was based purely on histopathology characteristics in the retrospective group.

The use of PCR for the detection of *M. leprae* DNA in nerves has proved to be very useful in confirming PNL.^{22,34–36} Polymerase chain reaction has been found to be especially valuable in paucibacillary disease and in patients with atypical clinical presentation and histopathological features compatible with leprosy.³⁷ Our prospective group benefited most with a definitive diagnosis of leprosy based on PCR confirmation and, thus, correct treatment for patients of leprosy neuropathy with negative AFB in nerve biopsy. Therefore, PCR-based *M. leprae* identification plays an important role in the diagnosis of neural form of leprosy. In our latest publication, we confirmed patients with peripheral neuropathy and central nervous system lesions to have leprosy through PCR on DNA from nerve tissue.³⁸

Histopathological confirmation is often difficult in PNL because of the low sensitivity and absence of AFB in the biopsy specimen, particularly in the tuberculoid type. In our cohort, 77 cases had biopsies of nerves that had deficits in its distribution. However, nine nerve samples showed no diagnostic features of leprosy neuropathy on histopathology, but did reveal *M. leprae* DNA by PCR. The amplification of *M. leprae* DNA in 80 of 87 tested samples indicates a high yield when compared with previous reports. Nevertheless, one AFB-positive nerve sample failed to reveal *M. leprae* DNA. This could possibly be due to improper DNA or loss of DNA during the cleanup process of PCR product. However, such discordance has been reported in a previous study.³⁹ Histopathological features similar to that observed in tuberculoid PNL can also be seen in chronic inflammatory demyelinating neuropathy, vasculitic/noninfective granulomatous neuropathy, and neurosarcoidosis. Clinically, the disease could also mimic hereditary sensory and autonomic neuropathy and needs to be differentiated. Furthermore, impaired kinaesthesia and variable stretch reflexes particularly those with hyperactive tendon reflexes may mislead clinicians toward a wrong diagnosis.⁴⁰ In this same study from our institute, 36/133 patients with biopsy-proven leprosy were suspected to have neuropathy other than leprosy,⁴⁰ implying a high degree of misdiagnosis at the bedside.

In clinically and histopathologically undefined neuropathy cases, it may be imperative to subject tissue samples for the presence of *M. leprae* DNA by PCR method. Although this technique is available in highly specialized centers, it is a simple and cost-effective diagnostic method, and its availability needs to be known to physicians.

In a report from Northern India, PCR performed in nerve tissue from 35 PNL cases revealed *M. leprae* DNA in 22 (62%). Polymerase chain reaction was positive in 12/13 AFB-positive cases and in 10/22 with no AFB.³⁹ In comparison among our 87 histopathologically diagnosed leprosy cases, 80 samples showed *M. leprae* DNA, and the four cases with fibrosed/healed nerve tissue and one with only axonopathy also demonstrated *M. leprae* DNA. However, isolating DNA for PCR in fibrosed/healed tissue may not be successful always. It is clearly evident that the PCR yield was significantly higher, including the group with BT and TT leprosy in our series.

In another study from this same group, in 67 nerve biopsies from PNL patients presenting as mononeuritis multiplex, histopathology showed epithelioid granuloma in 14%, AFB in 16%, and nonspecific inflammatory infiltrate and/or fibrosis in 39% of cases. Polymerase chain reaction for *M. leprae* was positive in 47% of 23 nerve specimens.³⁹ In comparison, in our patients, PCR amplified *M. leprae* DNA in more than 90% of samples. Subjecting nerve biopsies for PCR analysis has been reported several decades ago when *M. leprae* DNA was observed in all lepromatous leprosy patients, and the majority were paucibacillary cases.⁴¹ In our cohort, most had BT type leprosy, but all types demonstrated *M. leprae* DNA.

In a study from Brazil, in 58 patients of PNL, PCR was positive from 29 patients (50%).¹⁹ In comparison our PCR positive rate was more than 90% and was high even in the AFB negative group. Antunes et al., from Brazil identified that 28 of their 92 AFB negative nerve biopsy samples showed a positive PCR test while PCR was negative in all the non-leprosy samples emphasizing its high specificity for confirming *M. leprae* infection.⁴² Similarly among our 31 clinically and histopathologically defined non-leprosy cases PCR was negative for *M. leprae* DNA reiterating the specificity of PCR testing. Above authors also mention that the 'probable' PNL benefits most from an additional molecular investigation on the same biopsy tissue. Similar findings have been reported in earlier studies on nerve and skin biopsy specimens.^{19,43} In our cohort we found that PCR revealed *M. leprae* DNA in a higher percentage of non-AFB cases.

In the recent years, high-resolution sonography of peripheral nerves is projected as an informative and additional diagnostic tool for leprosy. Thickened epineurium noted in the ulnar nerve, which is the most commonly affected nerve, and altered echotexture of the nerves with increased epineurial blood flow on color Doppler may provide clues toward the diagnosis of leprosy.^{44,45}

CONCLUSION

On most occasions, there are multiple thickened peripheral/cutaneous nerves in suspected cases of leprosy, but neurological deficits pertaining to the thickened nerve are usually not as much widespread. It is important to select the nerve which has deficits in relation to its distribution. Polymerase chain reaction for the identification of *M. leprae* DNA should become a routine practice to confirm all suspected cases of leprosy so that delayed diagnosis and mislabeling and active transmission is reduced. It may be extended to all nerve biopsies in endemic regions. The limitations of the current study are as follows: samples included are from a highly biased set of patients who visit a quaternary care neurological center. Nevertheless, the findings have significant clinical, diagnostic, and therapeutic implications.

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