Use of Fine Needle Aspirate from Peripheral Nerves of Pure-neural Leprosy for Cytology and Polymerase Chain Reaction to Confirm the Diagnosis: A Follow-up Study of 4 Years

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Abstract
Background: Pure neural leprosy (PNL) still remains a diagnostic challenge because of the absence of sine qua non skin lesions of leprosy and a confirmatory diagnostic method. The authors had earlier described a simple yet objective technique of combining fine needle aspiration cytology (FNAC) coupled with a multiplex polymerase chain reaction (PCR) in a pilot study, wherein the technique showed promise of a reliable diagnostic tool. In the pursuit of further evidence, the authors carried out a 4-year study with PNL cases to find the efficacy and reliability of the said method in a larger sample size. Aim: This study was conducted to find the efficacy, reliability, and reproducibility of FNAC coupled with multiplex PCR and Ziehl-Neelsen (ZN) staining in identifying the cases of PNL. Materials and Methods: All cases that were suspected to be suffering from PNL, following evaluation by two independent observers were included in the study and were subjected to FNAC from the affected nerve, and the aspirates were evaluated for cytology, ZN staining, and multiplex PCR for Mycobacterium leprae genome. In addition, serum anti-PGL1 levels were also performed in all the study subjects. Fifteen non-PNL cases were also included in the control arm. Results: A total of 47 cases were included in the test arm and subjected to FNAC. Conventional ZN staining could demonstrate acid-fast bacilli (AFB) in only 15 out of 47 cases (31.91%) while M. leprae DNA could be elicited in 37 (78.72%) cases by the multiplex PCR. Only 13 (27.65%) out of 47 cases showed anti-PGL1-1 antibody positivity. On cytological examination of the nerve aspirates, only 11 (23.40%) cases showed epithelioid cells whereas nonspecific inflammation was seen in 26 (57.60%) cases. Conclusion: The results of this study conducted over a larger sample size corroborate with the findings of our pilot study. In a resource poor set up, FNAC in combination with ZN staining and multiplex PCR is a rapid, simple, and easily performed test, which can give a reproducible and objective diagnosis in cases of PNL.

Key Words: Acid-fast bacilli, Mycobacterium leprae, polymerase chain reaction, pure neural Hansen

What was known?
Diagnosis of pure neural leprosy was till very recently clinical and often equivocal. Earlier, criteria suggested by Jardim et al for the diagnosis of pure neural leprosy largely remained unpopular because of involvement of complex laboratory investigation and requirement of demanding nerve biopsy. Earlier the authors, showed a simple yet reliable technique of diagnosing pure neural leprosy by using fine needle aspiration and Polymerase chain reaction in a small group of patients; however it was needed to be validated with a larger number of study population.

Introduction
According to estimates, pure neural leprosy (PNL) affects over 5%–17.7% of all cases of leprosy, which even in today’s era of Leprosy elimination, still remains a good number especially in endemic countries like India. However, it is not only the sheer number that makes this variety of leprosy a perplexing problem. In the absence of simple, reliable, and reproducible diagnostic tests PNL remains a diagnostic challenge. Jardim et al. described criteria for PNL, which involved nerve biopsy, histopathology, polymerase chain reaction (PCR), and use of anti-PGL antibody.[2]
These tests and therefore the criteria suggested by Jardim et al. however, failed to gain popularity in this part of the world because of limited resources and lack of expertise and facilities for performing nerve biopsy. Moreover, nerve biopsy is an invasive procedure and limited to certain sensory nerves. Since Hansen’s disease involves motor and mixed nerves such as median, ulnar and peroneal, biopsy from these sites can lead to high rates of complications even in the hands of an expert. Another argument that makes nerve biopsy unfavorable is the fact that even in most ideal conditions, results can often be nonspecific.

In an effort to find a simple yet objective and test to diagnose PNL, the authors demonstrated in a pilot study that fine needle aspiration cytology (FNAC) when combined with Ziehl-Neelsen (ZN) staining and PCR gives a good diagnostic yield.\(^{[6-4]}\) FNAC being a less invasive test needs less skill and infrastructure and comes with lesser rates of complications and can be easily performed within the existing infrastructure. With this background and in pursuit of further evidence, the authors conducted this study over a larger sample size, for a longer duration to determine the reliability, efficacy, and reproducibility of FNAC combined with ZN staining and PCR in diagnosis of PNL.

**Materials and Methods**

**Patient selection criteria**

Patients who were clinically suspected as cases of PNL were included in the study after obtaining a written informed consent form. Two independent, experienced dermatology consultants examined the patients for evaluation. For clinical diagnosis, the definition of PNL was taken as thickening and/or tenderness of a peripheral nerve commonly involved by leprosy with sensory and/or motor functional impairment along the distribution of same nerve in the absence of characteristic skin lesion.

Patients who were diagnosed by both the clinicians independently as cases of PNL were included in a group designated as “Possible PNL.” Others who were diagnosed as PNL by one observer and non-PNL by the other observer were included in a group designated as “Doubtful PNL.” Patients who had no clinical sign of Hansen neuropathy, but had the easily palpable ulnar nerve, were included as the “Control group,” provided they signed the informed consent form.

**Nerve conduction studies**

All the patients were subjected to nerve conduction study (NCS) as per the standard protocols of our hospital in the department of neurosciences.

**Fine needle aspiration cytology**

FNAC was done as described by Theuvenet et al. and aspirates were subjected to cytological examination with Giemsa and ZN staining.\(^{[5-4]}\)

**Extraction of DNA from fine needle aspiration and multiplex polymerase chain reaction**

Genomic DNA was extracted from a portion of the FNA sample aspirates, collected aseptically with the standard precautions from ulnar nerve of study participants by standard Phenol Chloroform method after proteinase-K digestion as described by Banerjee et al.\(^{[7]}\)

A multiplex PCR for the rapid diagnosis of *Mycobacterium leprae* was performed as per Banerjee et al.\(^{[8]}\) based on the following oligonucleotide primers sets:

1. The repetitive sequence of the *M. leprae* DNA reported by Han et al.\(^{[9]}\) is very specific to *M. leprae* and not present in 20 other mycobacterial species other than *M. leprae*. (Primer LR1 and LR2) as shown in Table 1
2. A region flanking entire 21TTC repeat sequences, specific for multibacillary leprosy (MB) designed by Shin et al.\(^{[10]}\) The specificity and sensitivity of the primers: LR1 and LR2 and TTC-A and TTC-B had been already established in earlier studies.

Briefly, 100 ng genomic DNA was amplified with AmpliTaq Gold, (Applied Biosystems, Inc., [ABI], Foster City, CA, USA) in PCR reaction mixtures, containing 1x PCR buffer (Applied Biosystems), 2 mM MgCl2, 0.25 mM each dNTP, 20 picomoles primers LR1 and LR2, and TTC-A and TTC-B. The primer sequences, primer annealing temperature (Ta°C), and PCR product sizes are given in Table 1. The PCR reactions were performed in the following conditions: 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, Ta°C for the internal control as given in Table 1 for 1 min, 72°C for 1 min, and finally elongated at 72°C for 10 min. The amplified products were separated by electrophoresis on 2% agarose gel stained with 0.5 mg/mL ethidium bromide and visualized and photographed under an ultraviolet transilluminator.

**Anti PGL-1 antibody levels**

Anti-PGL-1 antibody levels were measured by radioimmunoassay as per the protocol.\(^{[11]}\)

| Table 1: Tm value and primer sequences and used in multiplex polymerase chain reaction |
|-------------------------------------------------------------------------------------------------|
| **Name of primer** | **Primer sequences (5’–3’)** | **Tm value** | **Product size** |
|----------------------|-----------------------------|-------------|-----------------|
| LR 1                 | CGG CGG GAT CCT CGA TGC AC  | 58°C        | 372 bp          |
| LR 2                 | GCA CGT AAG CTT GTC GGT GC  |             |                 |
| TTC A                | GCA CCT AAA CCA TCC GTG TT  | 201 bp      |                 |
| TTC B                | CTA CAG GGG GCA CTT AGC TC  |             |                 |
| Serial number | Sex | Age | Nerve involvement | Reaction | Clinical diagnosis | Group | Nerve conduction study | Cytology | AFB | PCR | bp amplified | Anti PGL 1 (pg/ml) |
|---------------|-----|-----|------------------|----------|-------------------|-------|-----------------------|----------|-----|-----|-------------|-----------------|
| PNL 1         | Male| 35  | Right ulnar, left ulnar and left popliteal | S/M Nil | Probable | Right ulnar, left ulnar and left popliteal | P, L | Negative | Positive | 372 bp, 201 bp | 0.178 pg/ml |
| PNL 2         | Male| 45  | Right ulnar     | S/M Nil | Probable | Right ulnar | P, L | Positive | 372 bp | 0 |
| PNL 3         | Male| 29  | Right ulnar     | S/M Nil | Probable | Right ulnar | P, L, E | Positive | 201 bp | 0 |
| PNL 4         | Male| 23  | Left ulnar, left popliteal | S Positive | Probable | Left ulnar, left popliteal | P, L | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 5         | Male| 31  | Right ulnar     | S/M Nil | Probable | Right ulnar | P, L | Negative | Positive | 372 bp | 0.164 pg/ml |
| PNL 6         | Male| 48  | Right ulnar and left ulnar | S Nil | Doubtful | Right ulnar, normal | Nil | Negative | Negative | Negative | 0 |
| PNL 7         | Male| 42  | Left ulnar     | S/M Nil | Probable | Left ulnar, Axon, demyl | P, L, E | Negative | Positive | 372 bp, 201 bp | 0.152 |
| PNL 8         | Male| 26  | Right ulnar     | S Nil | Probable | Right ulnar | P, L | Negative | Negative | Negative | 0 |
| PNL 9         | Male| 28  | Right ulnar and left ulnar | S/M Nil | Probable | Right ulnar, left ulnar | P, L | Negative | Positive | 201 bp | 0 |
| PNL 10        | Male| 39  | Right ulnar     | S/M Nil | Probable | Right ulnar, left ulnar | P, L | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 11        | Male| 36  | Right ulnar, left ulnar | S Positive | Probable | Right ulnar, left ulnar | P, L, E | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 12        | Female| 31 | Left ulnar     | S/M Nil | No | Left ulnar, Axon, normal | P, L, E | Negative | Positive | 201 bp | 0 |
| PNL 13        | Male| 24  | Right ulnar     | S/M Nil | Probable | Right ulnar, normal | Nil | Negative | Positive | 372 bp | 0 |
| PNL 14        | Male| 27  | Left ulnar, left ulnar | S/M Positive | No | Doubtful | Right ulnar, normal | Nil | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 15        | Male| 30  | Right ulnar, left ulnar | S Nil | No | Doubtful | Right ulnar, normal | Nil | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 16        | Male| 50  | Right ulnar     | S/M Nil | Probable | Right ulnar | P, L, E | Positive | 372 bp, 201 bp | 0.188 pg/ml |
| PNL 17        | Male| 21  | Right ulnar     | S/M Nil | Probable | Right ulnar | P, L, E | Negative | Positive | 201 bp | 0 |
| PNL 18        | Male| 19  | Right ulnar     | S Nil | Probable | Right ulnar, demyl | P, L, E | Negative | Positive | 372 bp, 201 bp | 0.098 pg/ml |
| PNL 19        | Female| 41 | Right ulnar, left ulnar | S/M Nil | Yes | Probable | Right ulnar, demyl | P, L, E | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 20        | Male| 27  | Right ulnar     | S/M Nil | No | Doubtful | Right ulnar, normal | Nil | Negative | Negative | Negative | 0 |
| PNL 21        | Male| 15  | Right ulnar     | S/M Nil | No | Doubtful | Right ulnar, normal | Nil | Positive | Positive | 372 bp, 201 bp | 0.162 |
| PNL 22        | Male| 21  | Right ulnar     | S Nil | Probable | Right ulnar | P, L, E | Positive | Positive | 201 bp | 0 |

Contd...
Table 2: Contd...

| Serial number | Sex   | Age | Nerve involvement | Reaction | Clinical diagnosis | Group | Nerve conduction study | FNA study | Anti PGL 1 (pg/ml) |
|---------------|-------|-----|-------------------|----------|--------------------|-------|------------------------|-----------|-------------------|
|               |       |     | Nerve Type        | Observation 1 | Observation 2 | Nerve involved Type | Cytology | AFB | PCR | bp amplified |
| PNL 23        | Male  | 36  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Positive | 372 bp | 0 |
| PNL 24        | Male  | 39  | Right ulnar       | S/M       | Positive           | Probable | Right ulnar Axon       | P, L, E   | Positive | Positive | 372 bp, 201 bp | 0 |
| PNL 25        | Male  | 45  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Positive | 372 bp, 201 bp | 0.158 |
| PNL 26        | Male  | 26  | Left ulnar        | S/M       | Nil                | Probable | Left ulnar Axon        | P, L      | Negative | Positive | 372 bp | 0 |
| PNL 27        | Male  | 19  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Negative | 372 bp, 201 bp | 0 |
| PNL 28        | Male  | 32  | Right ulnar and left ulnar | S/M       | Positive           | Probable | Right ulnar Axon       | P, L      | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 29        | Male  | 41  | Right ulnar       | S/M       | Nil                | Doubtful | Right ulnar normal Axon | P, L      | Negative | Positive | 201 bp | 0 |
| PNL 30        | Male  | 38  | Right ulnar       | S/M       | Nil                | Doubtful | Right ulnar normal Axon | P, L      | Negative | Positive | 372 bp, 201 bp | 0.127 |
| PNL 31        | Male  | 36  | Right ulnar and left ulnar | S/M       | Nil                | Probable | Right ulnar normal Axon | P, L      | Negative | Positive | 201 bp | 0 |
| PNL 32        | Male  | 31  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L, E   | Positive | Positive | 372 bp, 201 bp | 0.142 |
| PNL 33        | Male  | 27  | Left ulnar, right ulnar, right popliteal | S/M       | Positive           | Probable | Left ulnar Axon       | P, L      | Positive | Positive | 372 bp, 201 bp | 0 |
| PNL 34        | Female| 22  | Left ulnar        | S       | Nil                | Probable | Left ulnar Axon       | P, L      | Negative | Negative | 201 bp | 0.142 |
| PNL 35        | Male  | 26  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 36        | Male  | 43  | Right ulnar       | S       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Positive | 372 bp, 201 bp | 0 |
| PNL 37        | Male  | 36  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Positive | Positive | 372 bp | 0 |
| PNL 38        | Male  | 18  | Right ulnar, right popliteal | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Positive | Positive | 372 bp, 201 bp | 0 |
| PNL 39        | Male  | 25  | Right ulnar       | S       | Nil                | Doubtful | Right ulnar normal Axon | P, L      | Positive | Positive | 372 bp, 201 bp | 0.153 |
| PNL 40        | Female| 22  | Right ulnar, left ulnar and left popliteal | S/M       | Nil                | Probable | Left ulnar Axon       | P, L      | Positive | Positive | 372 bp, 201 bp | 0 |
| PNL 41        | Male  | 32  | Left ulnar        | S/M       | Nil                | Probable | Left ulnar Axon       | P, L, E   | Positive | Positive | 201 bp | 0.986 |
| PNL 42        | Male  | 33  | Right ulnar       | S       | Nil                | Probable | Right ulnar normal Axon | P, L      | Negative | Negative | 0 |
| PNL 43        | Male  | 38  | Right ulnar       | S/M       | Nil                | Probable | Right ulnar Axon       | P, L      | Negative | Negative | 0 |
| PNL 44        | Female| 39  | Left ulnar        | S       | Nil                | Probable | Left ulnar Axon       | P, L      | Negative | Negative | 0 |

Contd...
Results

The study included 47 cases as “study group” and 15 cases as “control group” [Table 2]. Out of the 47 suspected cases of PNL, 33 (70.21%) were kept in the “probable” group and 14 (29.78%) were kept in “doubtful” group according to the patient inclusion criteria of our study. The “control group” comprised of 15 patients out of whom 9 were daily laborers, 5 had diabetic neuropathy, and 1 patient was a heavy weight lifter.

Out of 47 clinically diagnosed cases of PNL, 5 were females and 42 were males. The mean age of the study group was 32.23 years with ages varying between 15 and 50 years. Both sensory and motor involvement was seen in 35 out of 47 cases (74.46%) while 12 (25.53%) cases had predominantly sensory involvement. Clinical signs of reaction were seen in 6 of the cases all of which belonged to the probable group [Table 3].

NCS was done on all the participants of the “study group.” Six (12.76%) out of 47 cases showed normal NCS. All of these were cases in the “doubtful PNL” group. All the participants of the “Probable PNL” group showed some form of Nerve conduction damage. Of the 41 (87.23%) participants who showed deranged NCS, all showed features of axonopathy while only 3 (6.3%) cases showed additional features of demyelination. Combining clinical and neurological data, we had 41 cases who showed neuropathy. Of these 29 (70.73%) had mononeuropathy and 12 (29.26%) had polineuropathy [Table 2].

FNAC revealed that all the participants in the “Probable PNL” group showed the presence of inflammatory cells. However, of the 14 in the “doubtful PNL” group no inflammatory cells were seen in 5 (35.71%) patients. Aspirates from 42 FNAC specimens that showed inflammatory infiltrate, it was seen that only 11 (23.40%) of them showed the presence of epithelioid cells while rest showed

| Table 2: Contd... |
|------------------|
| **Clinical diagnosis** | **Group** |
| **Observation 1** | **Observation 2** |
| **Type** | **Nerve involved** |
| **Sex** | **Age** | **Nerve involvement** |
| **Male** | **Female** | **Ulnar** | **Poplietal** | **Poly** |
| Probable | 33 | 29 | 4 | 13 | 6 | 9 | 6 |
| Doubtful | 14 | 13 | 1 | 14 | Nil | 3 | Nil |

| Table 3: Epidemiological and clinical data |
|-----------------------------------------|
| **n** | Male | Female | Nerve involvement | Reaction |
| **Ulinar Poplietal Poly neuropathy** |
| Probable | 33 | 29 | 4 | 13 | 6 | 9 | 6 |
| Doubtful | 14 | 13 | 1 | 14 | Nil | 3 | Nil |

| Table 4: Microbiological data |
|-------------------------------|
| **n** | Cytology | FNAC | Anti-PGL1 |
| **N, L, E, N, L, E, AFB, PCR** |
| Probable | 33 | 4 | 3 | 11 | 9 | 2 | 12 | 26 | 10 |
| Doubtful | 14 | 2 | 1 | Nil | 6 | Nil | 3 | 11 | 3 |

N: Neutrophils, L: Lymphocytes, E: Epithelioid cells, FNAC: Fine needle aspiration cytology, AFB: Acid-fast bacilli, PCR: Polymerase chain reaction, PGL-1: Phenolic glycol lipid
nonspecific infiltrate consisting of neutrophils and lymphocytes. Foam cells could not be visualized in any case [Table 4].

ZN staining of the aspirate was done for all 47 cases. It was seen that 15 (31.91%) cases out of 47 showed the presence of Acid-fast bacilli (AFB) [Figure 1]. Of these 15 cases, 12 (80%) belonged to the “probable PNL” group and 3 (20%) belonged to the “doubtful PNL” group [Table 4].

**Table 5: Total number of confirmed cases of pure neural leprosy by various techniques**

| Method                        | Number of confirmed cases (%) |
|-------------------------------|-------------------------------|
| Epitheloid cell positive      | 11 (23.4)                     |
| AFB on ZN staining            | 15 (31.91)                    |
| AFB + epitheloid cella        | 26 (55.31)                    |
| PCR                           | 37 (78.72)                    |

AFB: Acid-fast bacillus, ZN: Ziehl neelson, PCR: Polymerase chain reaction

**Table 6: Sensitivity, specificity, positive predictive value and negative predictive values individual method**

| Test                        | Sensitivity | Specificity | Positive predictive value | Negative predictive value |
|-----------------------------|-------------|-------------|---------------------------|----------------------------|
| FNAC cytology               | 29.63       | 100         | 100                        | 27.78                      |
| AFB                         | 43.21       | 100         | 100                        | 32.26                      |
| Clinical diagnosis          | 67.57       | 20          | 75.76                     | 14.29                      |
| Anti-PGL antibody           | 27.03       | 70          | 76.92                     | 20.59                      |

FNAC: Fine needle aspiration cytology, AFB: Acid-fast bacilli, PGL: Phenolic glycolipid

**Figure 1: The multiplex polymerase chain reaction analysis**

On performing multiplex-PCR on all 47 samples obtained, 37 (78.72%) cases were found to be positive for *M. leprae* genome [Figure 2]. Of these 37 cases, 27 cases (72.97%) belonged to the “Probable PNL” group and 10 (27.02%) belonged to the “Doubtful PNL” group [Table 4]. Out of these 37 cases, 22 showed positive bands for both 372 and 210 bp, while 15 showed positive bands only for either of the base pairs [Table 1].

We also performed serum anti PGL-1 antibody levels in all the participants of “case group.” The levels were positive in 13 (27.65%) of the cases. Out of these 10 were from the “probable PNL” group and 3 were from the “Doubtful PNL” group [Tables 1 and 4].

Statistical analysis of the results so obtained was performed [Tables 5-7]. It was seen that sensitivity
was highest for clinical examination (67.57) followed by AFB, Cytology, and anti-PGL-1 antibodies. Both AFB and Cytology were 100% specific. Clinical examination showed a poor specificity of 20% while anti-PGL1 levels were 70% specific. Since none of the tests had both high sensitivity and specificity a concordance analysis was performed with respect to PCR, which was considered to be highly specific and sensitive. On performing concordance analysis, it was seen that no single test or a combination of tests showed high concordance [Table 7].

Of the 15 participants in “control group,” only 1 showed inflammatory infiltrate consisting of neutrophils and lymphocytes. ZN, multiplex PCR for M. leprae and anti-PGL1 antibodies were negative in all the participants in control group.

Discussion

Even though science has made great strides in efforts to combat Leprosy, PNL still evades the clinicians’ eyes and remains a diagnostic challenge. Major work to lay down diagnostic criteria for diagnosing PNL came from Brazil, where Jardim et al. showed that high yield could be obtained if PCR was used to demonstrate mycobacterial DNA on nerve tissue samples obtained out of a nerve biopsy along with anti-pgl-1 antibody levels. [2-12]

These criteria failed to gain popularity in our part of the world, predominantly because of the fact that we do not have the adequate infrastructure or resources to perform a test as invasive as nerve biopsy which has a high rate of complications if not done in proper setting and with skill that requires training and precision. [13,14]

To simplify diagnosis of PNL and make it more objective, the authors demonstrated in a pilot study that FNA from peripheral nerves affected in patients of PNL can be combined with techniques such as ZN staining and multiplex PCR to improve the yield of diagnosis. The basic premise of the thought here was that FNA is a much simpler technique, which does not require a high level of skill and can be done routinely in OPD set up as a day care procedure. [3] However, the sample size in this study was only 13 patients. To check the reliability, reproducibility and precision of FNA combined with ZN staining and multiplex-PCR in the diagnosis of PNL, the authors performed these tests on a larger sample size of 47 patients over a span of 3 years in Dermatology OPD of a tertiary care center in eastern India.

Jardim et al. showed that in their study group 91% of patients had axonal neuropathy and only 8% showed demyelination on NCS. Our data corroborate with these findings. [2] Out of 47 study subjects, 41 (87.23%) showed axonal neuropathy and 3 (6.3%) showed demyelination [Table 2]. Although NCS gives valuable clues while choosing appropriate nerve for FNA, it, however, does not add much to diagnosis because the findings of this test are nonspecific for PNL and can be found in a host of other conditions.

FNA was performed from the affected peripheral nerves in the patients of PNL. The material so aspirated was subjected to cytology, ZN staining and multiplex PCR. Although Singh et al. [15] had documented that an entire spectrum of leprous neuritis can be ascertained on cytological evaluation of such aspirates, these claims were later refuted by Jardim et al. [2] They observed that histological criteria for the diagnosis of PNL should include the presence of epitheloid cells, epithelioid granuloma, or AFB and the presence of nonspecific infiltrate consisting of mononuclear cells could not be taken as a specific sign for PNL. They documented that though the presence of nonspecific inflammation was observed in about 71% of the patients, only 40% cases had definitive diagnostic features such as AFB or epithelioid granuloma or both. In our study, only 11 out of 47 patients (23.40%) showed the presence of epithelioid cells. [3] On performing ZN staining to demonstrate AFB, a total of 15 (31.91%) cases showed positivity of AFB. Of these 15 cases, 6 showed positivity for epithelioid cells. Therefore, on cytomorphological evaluation, PNL could be diagnosed (in patients who had either epithelioid cell or AFB or both) in 26 cases (55.31%) out of 47 [Table 5]. This correlates well with a study done by Jardim et al. and only adds emphasis to the point that cytomorphology has a limited role in the diagnosis of PNL.

Recently, it was seen that multiplex PCR could successfully demonstrate M. Leprae DNA in skin samples of patients with leprosy. Chemouilli et al. showed that by performing PCR on nerve biopsy specimens, diagnostic frequency of PNL could increase at least two times. Jardim et al. showed results, which were consistent with the study done by Chemouilli et al. [16]
However, nerve biopsy being the major deterrent in our efforts to diagnose PNL, we had conducted a pilot study where we demonstrated M. Leprae DNA in specimens obtained out of FNA done on affected peripheral nerves in 13 patients.[3,4] FNA, being a relatively less invasive and simpler procedure, could be done with ease in our outpatient set up. The diagnostic yield of FNAC-PCR was as high as 84.6% in the pilot study. In this study, 37 out of 47 (78.72%) of the cases could be diagnosed with FNAC-PCR. Of these 37 cases, 26 belonged to the probable group and 11 to the doubtful group. All the cases, which were in doubtful group, were negative for epitheloid cells and only one case was AFB positive [Tables 2 and 5]. It only goes on to prove that if we rely on cytology and clinical suspicion alone, there is a high possibility of missing a significant number of people suffering with PNL. These results show that FNAC PCR could reproduce high diagnostic yield in cases of PNL even on a larger sample size over a span of 3 years.

Out of 37 cases, which were positive on FNAC-PCR, 21 showed positivity for both 372 and 210 bp. This suggests that these cases had a higher bacillary load than the rest. It was also interesting to observe that out of 12 cases which showed polyneuropathy, 10 belonged to this group. It can only extrapolated at this point, that PCR could be an investigation that not only diagnoses PNL but also indicates the brunt of bacillary load and neuropathy thereof in these cases.

Anti-PGL-1 antibodies are considered to be a useful additional test in aid to PCR and histopathology in the diagnosis of PNL. Jardim et al. in a study showed that an antibody response was present in 14/67 (21%) of the patients diagnosed as PNL as compared with 3/34 (9%) of controls. Anti-PGL-I positivity was observed in 5/8 (63%) of the AFB positive cases.[2] Patients whose diagnosis was confirmed solely by M. leprae PCR on the nerve sample had 4/25 (16%) seropositivity. In addition, anti-PGL-I antibodies were detected in 9/40 (23%) of the PNL patients who were PCR negative for M. leprae DNA. In our study, the levels were positive in 13 (27.65%) of the cases. Out of these 10 were from the “probable PNL” group and 3 were from the “Doubtful PNL” group. None of the patients who were negative for PCR showed positivity for anti-PGL1 levels [Tables 2 and 5]. Therefore, while anti-PGL1 antibody testing may be a good adjunctive diagnostic test and an epidemiological tool, it may not contribute much in diagnosis of PNL in our setting.

PCR is a highly sensitive and specific test for diagnosis of PNL [Table 6]. In our study, clinical examination, AFB, and cytology failed to show both high sensitivity and specificity in the diagnosis of PNL. Even on concordance analysis, the results of these tests individually and in multiple combinations with each other showed poor concordance with PCR. In light of these results, it could be safely said, that PCR either by nerve tissue biopsy or FNAC can be considered to be the diagnostic gold standard in diagnosis of PNL.

Conclusion

PCR is considered to be a highly sensitive and specific test for detecting M. leprae in cases of PNL. FNA is a much simpler technique to collect tissue materials for PCR than nerve biopsy. The Pilot study done by us earlier and now with the current data over 47 patients of PNL, only confirms the fact that FNAC combined with PCR not only has high diagnostic yield, but it is much simpler, reliable, efficient, and precise instrument and in conjunction with ZN staining, it can help us to reduce the burden of PNL by decreasing subjectivity of the diagnosis. It enlightens our path and emboldens our vision of leprosy free society.

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Conflicts of interest

There are no conflicts of interest.

What is new?

Our study confirms that diagnosis of pure neural leprosy can be made objective, reliable, sensitive and specific by combination of FNAC and PCR. FNAC is a much simpler technique than nerve biopsy and PCR when routinely practiced can be made affordable to most diagnostic set up. We suggest readers to perform our method of diagnosis of pure neural leprosy more frequently, so that ambiguity of the diagnosis can be reduced.

References

1. Giridhar BK. Neuritic leprosy. Indian J Lepr 1996;68:35-42.
2. Jardim MR, Antunes SL, Santos AR, Nascimento OJ, Nery JA, Sales AM, et al. Criteria for diagnosis of pure neural leprosy. J Neurol 2003;250:806-9.
3. Reja AH, De A, Biswas S, Chattopadhyay A, Chatterjee G, Bhattacharya B, et al. Use of fine needle aspirate from peripheral nerves of pure-neural leprosy for cytology and PCR to confirm the diagnosis: A pilot study. Indian J Dermatol Venereol Leprol 2013;79:789-94.
4. De A, Reja AH, Biswas S, Bhattacharya B, Chatterjee G, Basu K, et al. Unique TTC repeat base pair loss mutation in cases of pure neural leprosy: A Survival strategy of mycobacterium leprae? Indian J Dermatol 2015;50:351-5.
5. Theuvenet WJ, Miyazaki N, Roche P, Shrestha I. Cytological needle aspiration for the diagnosis of pure neural leprosy. Indian J Lepr 1996;68:109-12.
6. Theuvenet WJ, Miyazaki N, Roche P, Shrestha I. Cytological needle aspiration of the nerve for the diagnosis of pure neural leprosy. Int J Lepr Other Mycobact Dis 1993;61:597-9.

7. Banerjee S, Sarkar K, Gupta S, Mahapatra PS, Gupta S, Guha S, et al. Multiplex PCR technique could be an alternative approach for early detection of leprosy among close contacts – A pilot study from India. BMC Infect Dis 2010;10:252.

8. Banerjee S, Ray D, Bandypadhyay D, Gupta S, Gupta S, Ghosal C, et al. Development and application of a new efficient and sensitive multiplex polymerase chain reaction (PCR) in diagnosis of leprosy. J Indian Med Assoc 2008;106:436-40.

9. Han YK, Nae CS, Kyeong LM. Evaluation of polymerase chain reaction amplification of Mycobacterium leprae specific repetitive sequence in biopsy specimens from leprosy patients. J Clin Microbiol 1993;31:895-9.

10. Shin YC, Lee H, Lee H, Walsh GP, Kim JD, Cho SN, et al. Variable numbers of TTC repeats in mycobacterium leprae DNA from leprosy patients and use in strain differentiation. J Clin Microbiol 2000;38:4535-8.

11. Brito Mde F, Ximenes RA, Gallo ME, Bührer-Sékula S. Association between leprosy reactions after treatment and bacterial load evaluated using anti PGL-I serology and bacilloscopy. Rev Soc Bras Med Trop 2008;41 suppl 2:67-72.

12. Jardim MR, Antunes SL, Simons B, Wildenbeest JG, Nery JA, Illarramendi X, et al. Role of PGL-I antibody detection in the diagnosis of pure neural leprosy. Lepr Rev 2005;76:232-40.

13. Prasad PV, George RV, Kaviarasan PK, Viswanathan P, Tippo R, Anandhi C, et al. Fine needle aspiration cytology in leprosy. Indian J Dermatol Venereol Leprol 2008;74:352-6.

14. Smith EW. Diagnosis of pure neuritic leprosy. Neurol J Southeast Asia 2002;7:61-3.

15. Singh N, Malik A, Arora VK, Bhatia A. Fine needle aspiration cytology of leprous neuritis. Acta Cytol 2003;47:368-72.

16. Chemouilli P, Woods S, Said G, Cole ST. Detection of mycobacterium leprae in nerve lesions by the polymerase chain reaction. Int J Lepr Other Mycobact Dis 1996;64:1-5.