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Improved Protocol for PCR Detection of *Mycobacterium leprae* in Buffered Formalin-Fixed Skin Biopsies[1]

TO THE EDITOR:

It is often difficult to diagnose early leprosy by clinical criteria alone. Frequently, the patients' skin smears are negative, clinical findings inconclusive, and history unreliable and subjective. In many such cases, routine histopathology may also be nonspecific as *Mycobacterium leprae* cannot be demonstrated in the tissues of many early lesions. Therefore, the demonstration of *M. leprae,* or any of its components in a tissue biopsy, is crucial and important to reach a definitive diagnosis.

Polymerase chain reaction (PCR) amplification has been successfully used to detect extremely low numbers of *M. leprae* in fresh unfixed human skin biopsy specimens, providing powerful direct and unequivocal tests for diagnosis of leprosy ([1, 2, 3, 4, 5, 7, 8, 10, 11]). However, these studies required the specimens to be processed and analyzed promptly, in addition to being properly stored and transported. Adoption of PCR technology for detecting *M. leprae* and/or its components in fixed tissues would give clinicians the option of examining biopsy specimens for the presence of *M. leprae*, which along with histology would help in arriving at a definite diagnosis. This PCR technology would be of great help to arrive at a quick and conclusive diagnosis, identify and treat early cases of leprosy, to differentiate leprosy from non-leprosy cases and also for epidemiological purposes.

While earlier reports have demonstrated that buffered formalin was good for both histology and PCR detection, formalin fixation of skin biopsy specimens for longer than 24 hr has been reported to have an inhibitory effect on PCR amplification ([2]). Therefore, we here report the development of improved protocol for PCR diagnosis of *M. leprae* from formalin-fixed specimens and report the results of a blind study using a large number of biopsies obtained from both paucibacillary and multibacillary leprosy patients.

MATERIALS AND METHODS

After obtaining informed consent and using aseptic precautions and techniques such as cleaning of skin with iodine solution and alcohol, 5 mm punch biopsies were taken from 78 patients of Indeterminate, polar tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline-lepromatous (BL), or polar lepromatous (LL) leprosy cases from the Ghatampur field area of Kanpur district, India. For controls, biopsies from 12 cases of other dermatological conditions (Pitrrysis alba, Tinea versicolor, and Vitiligo) from the same population were also taken and analyzed using the same protocol. These biopsies were fixed and transported in buffered formalin and processed in a blind manner after 5 to 7 days. The scientists performing the gene amplification assay were unaware of the diagnosis of the case, and that biopsies of other dermatological cases were also included in the study. Each biopsy was divided into two parts: one part was used for histopathology and the other for the gene amplification assay.

Initially, the biopsies were kept in 15 ml of sterile distilled water for 8 hrs, which had been determined to be optimal after testing with various time periods. The biopsies were then aseptically homogenized in 1 ml of sterile T. E. buffer (Tris 10 mM, EDTA 0.1 mM, pH 8.0) using pestle and mortar in a bio-safety hood.

A technique based on the principle of a combined physiochemical approach, first freeze-boiling and then treatment with lysozyme-proteinase K ([7]) was used for extraction of DNA. Briefly, the homogenates in T. E. buffer were frozen, thawed, and

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then enzymatically treated at 37°C, first with lysozyme (3 mg/ml) for 2 hr followed by proteinase K (250 ug/ml) treatment for one hour at 65°C ([9]). After de-proteinization with chloroform: isoamyl alcohol (24:1), DNA was precipitated with 0.6 volume of isopropanol. This was dissolved in T. E. buffer and stored at –20°C until further use. Primers and the gene amplification method as described by Hartskeerl, *et al*. ([3]) and as used by de Wit, *et al*. ([1]) and Singh, *et al*. ([8]) were followed for the amplification of 36kD gene of *M. leprae*. Primers were obtained commercially from Bioserve Biotechnologies (India) Ltd. In all cases, 35 cycles were used for the amplification and confirmation was done by the southern blot analysis with random digoxygenin (DIG) labeled amplified 36 kDa gene fragment of *M. leprae* as a probe. DIG labeling and detection was done by using a kit from Roche Diagnostics (Cat. No. 1093651).

RESULTS

The percentage positivity of biopsy samples for *M. leprae* by gene amplification is shown in The Table. It was observed that 48% of Indeterminate, 55% of TT/BT, and 83% of BB/BL were positive by gene amplification. The lone BB patient who was negative by this assay was smear-negative and had taken multi-drug therapy (MDT) for more than a year prior to the biopsy. None of the specimens of non-leprosy cases were positive by this method, demonstrating the specificity of the method.

For histologic investigations, tissue samples are mostly stored as formalin-fixed, paraffin-embedded blocks. Widening the applicability of amplification techniques to formalin-fixed blocks could improve the routine diagnosis of mycobacterial infections. This is particularly important in leprosy as *M. leprae* cannot be grown in any *in vitro* system. Definite diagnosis of early and suspicious cases of leprosy is required, so that the patients can be treated at an early stage to prevent development of deformities, rather than following them up closely and treating them when definite clinical signs appear. While large studies are necessary to gain more confidence, published information shows that with PCR technology, identification of *M. leprae* is sensitive, as well as and specific ([1, 3, 5, 7, 8, 10]).

THE TABLE. *Positivity rates for gene amplification targeting 36 kDa gene in biopsies collected and fixed in buffered formalin.*

Type of leprosy	No. tested	No. positive	% positivity
Indeterminate	25	12	48
TT/BT	47	26	55
BB/BL	6	5	83
Non leprosy cases	12	0	Nil

It would be ideal if the PCR could be performed on fixed specimens in which both the detection of *M. leprae* and/or its components, and correlation with the histopathology, can be made. Over the years, different fixatives have been tried for histology and the detection of RNA/DNA of the causative organisms. Fiallo, *et al*. ([2]) and deWit, *et al*. ([1]) have observed significant reduction in sensitivity of PCR for *M. leprae* DNA when Zenker's fluids (mercuric chloride) fixatives are used. Although Carnoy-Lebrun's fluid is recommended for fixation of tissues for optimal staining of glycogen and RNA, unfortunately this fixative has the potential for chemical modification, resulting in degradation and excessive depurination of the DNA due to the acidic nature of fixative. Ethanol was reported to be a good fixative for PCR analysis, but this causes excessive shrinkage of the tissues, and is not recommended if the same tissue is to be used for histology and PCR analysis. Neutral buffered formalin fixation has been reported to be satisfactory for subsequent PCR, but significant reduction in the level of PCR signals for *M. leprae* DNA was observed after fixation of 4 to 7 days ([2]). In the case of formalin-fixed, paraffin-embedded specimens of smear-positive tuberculosis, the highest sensitivity rates have been obtained by amplifying the highly repetitive IS 6110 insertion sequence, and the different primers tested showed a sensitivity ranging from 80% to 87%, whereas a lower positivity of 47% to 80% with primer targeting single copy gene was observed in same study ([6]).

In the present study, after fixation of biopsy specimens and processing these within 5 to 7 days for PCR, 83% positivity was observed in multibacillary BB/BL patients and 43% to 55% in paucibacillary

(Indeterminate and BT). This is similar to the positivity rates of 50% to 70% for smear-negative and about 100% in smear-positive cases observed with fresh frozen biopsy specimens in earlier studies ([5, 7, 8]). Prior to this study it has been reported that formalin fixation of tissues for longer than 24 hrs is detrimental for PCR detection of *M. leprae*. It appears that this study provides a major improvement in the ability to detect *M. leprae* in tissues fixed in buffered formalin.

This improvement is most likely due to the soaking of the tissues for 8 hrs in 15 ml of sterile distilled water prior to homogenizing tissues, lysing and extraction of DNA. In the study of Fiallo, *et al*., tissues were soaked in HBSS solution for 30 minutes. In the present study, the tissues were collected as well as processed in buffered formalin which did not inhibit the PCR amplification, and the results are comparable to the earlier reports when PCR amplification was done in freshly biopsied samples obtained in the outpatient clinic of the Institute. It appears that the approach of collecting the specimens in buffered formalin will be very suitable for field situations and will have the added advantage of the same specimen being used for histology, probe application in solution, as well as for *in situ* applications. This, however, is a pilot study and trends need to validated by similar studies on a large number of specimens using the same method by other workers.

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