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Evaluation of Polymerase Chain Reaction (PCR) Amplification of *Mycobacterium leprae* in biopsy specimens from leprosy patients

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Eighty two skin biopsy specimens from leprosy patients attending at Central Special Skin Clinic, Yangon General Hospital were collected before taking chemotherapy. They were examined by a two-step polymerase chain reaction assay using a set of four nested oligonucleotide primers for the detection and identification of *Mycobacterium leprae*. It did not require the use of radioactively labelled hybridization probes. The nested-primer procedure amplified a 347 base-pair product from *M. leprae* genomic DNA. The PCR results were then compared with bacterial indices (BI) of slit-skin smears. PCR was positive in 8 (80%) of 10 biopsy specimens with BI of 0 determined for the slit-skin smear from the same patients. PCR also gave positive results for 71 (98.6%) of 72 BI positive cases. The agreement rate of these two tests is 89% but the false negative rate of BI for diagnosis of leprosy compared to PCR method is 10.39%. According to these results, PCR has an advantage over microscopic examination in detecting *M. leprae* in biopsy specimens negative for acid-fast bacilli, and is a useful tool for laboratory diagnosis.

INTRODUCTION

The recent development of polymerase chain reaction (PCR) has brought an un-precedented opportunity for sensitive, specific, and rapid detection of *Mycobacterium leprae* in clinical specimens. In the literature, there have been various target sequences for PCR and DNA probes specific for *M. leprae*, such as genes encoding the 36-kDa antigen [1, 2], the 18-kDa antigen [3], or the 65-kDa antigen [4] and repetitive sequences of *M. leprae* [5, 6]. Most of the reports showed that PCR with or without DNA probes seemed very sensitive, so that even 1 to 100 organisms are detectable by the method. In addition, PCR provided virtually 100% specificity in detecting the organism in clinical samples. In the study of De Wit *et al* 1991 with biopsy specimens from leprosy patients, PCR gave a positive result in about 80% of biopsies from leprosy patients negative for acid-fast bacilli (AFB), thus indicating that PCR is a useful tool for the laboratory diagnosis of leprosy [1-3]. In Myanmar, there is no information about PCR based on other target sequences and protocol was available for *M. leprae*. In this study, therefore, we attempted to evaluate PCR using primers amplifying a 347 base-pair product from *M. leprae* genomic DNA [4] in skin biopsy specimens from untreated leprosy patients, and the results were then compared with microscopic findings.

MATERIALS AND METHODS

Ethical clearance was approved by Department of Medical Research (Lower Myanmar). After taken inform consent, biopsy specimens were obtained from 82 untreated leprosy patients attended at the Central Special Skin Clinic, Yangon General Hospital. Bacterial indices (BI) were determined microscopically for skin slit smear samples before skin biopsy. For each patient, slit-skin smears from three sites depending on the clinical type of leprosy were prepared also, as WHO described [7] which consist of two

categories, paucibacillary (PB) and multibacillary (MB). PB leprosy is defined as five or fewer skin lesions with no bacilli in skin smears, and MB leprosy cases have six or more lesions and may be skin smear positive. The collected skin scrap smears were examined by Z-N stain to check Bacillary Index (BI) and Morphological Index (MI). The average BI was then calculated for each patient before analysis. Tissue samples from persons with dermatologic problems other than leprosy also were included as controls. Biopsy specimens were cut in half; one half was used for paraffin embedding, and the other half was preserved in 70% ethanol used for PCR. Preparation of *M. leprae* DNAs from biopsy specimens were done by QIAGEN, Germany kit. Amplification of *M. leprae* DNA was done by nested PCR. The primers amplifying a 347 base-pair product from *M. leprae* genomic DNAs are as follow.

L1 1236-1253 GTGGCTCAGATCCGTACC
L21813-1792(C) ATGCCACCGGTCGGGTCGCTCG
L3 1458-1476 CTACAGGCTGCTCCGGCTC
L4 1804-1782 (C) GTCGGGTCGCTCGCCGGAGCTGC

The 25 μl reaction mixture contained 2.5 μl of template solution prepared from biopsy samples, 0.1 μl of *Ex Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan), oligode oxyribonucleotide primer 0.25 μl of each (L1 and L2) 20 μM stock solution, 2.5 μl of 10x DNA PCR buffer, 0.5 μl of dNTP solution and 18.9 μl of water. As a positive control, DNA purified from Thai-53 strain of *M. leprae* was provided by Dr. M. Matsuoka, NIID, Japan, and distilled water was included as negative control in each experiment.

The amplifications were carried out in a programmable thermal Mastercycler personal eppendrof USA in a two-step cycle of 20 s at 98°C followed by 1 min at 68°C. The samples were usually amplified through 25 cycles by using the outside pair of primers (L1 and L2); 10% of the amplified mixture was then transferred to a fresh tube containing reaction mixture with the inside primers (L3 and L4). The second amplification was usually allowed to proceed through 30 cycles. Ten microliters of the reaction mixture was electrophoresed on 2% agarose gels. After electrophoresis, the gel was stained with ethidium bromide, and the 347 bp DNA band was examined under UV illumination.

RESULTS

Figure 1 shows some of the PCR results for biopsy samples from leprosy patients. As expected, biopsy specimens from patients with BI positive showed strong 347-bp bands (Fig. 1, lanes 3 and 8 to 11). Positive control (Thai-53 strain) also shows strong 347-bp bands (lane 13) and Lane 1 is negative control.

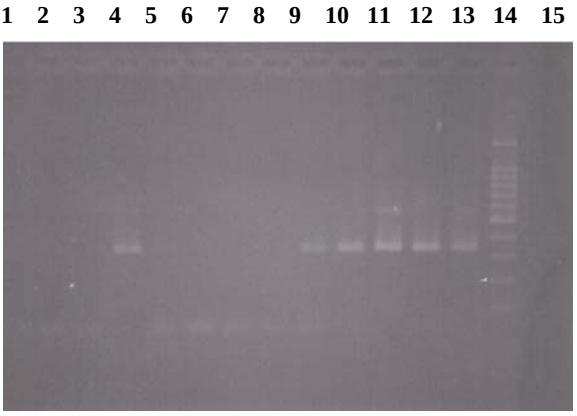

Fig.1. Nested PCR for amplification of 347bp DNA of *M. leprae* from biopsy tissue. Lane 1, negative control, lane 2, 3, 5, 6, 7, 8, 9, negative cases, lane 10, 11, 12, 13, positive cases, lane 14, positive control (Thai 53 strain) and lane 15, Molecular Wt: marker (100bp)

When the PCR results were analyzed in relation to the BI of biopsy specimens were made, 8 (80%) of 10 specimens with BI of 0 before the start of chemotherapy were PCR positive (Table 1). Therefore, PCR using primers targeting 347 base-pair product from *M. leprae* genomic DNA showed a clear advantage over microscopic examination in detecting *M. leprae* in biopsy specimens with BI of 0. Meanwhile, of 8 specimens with BI of 1, 7 (87.5%)

showed the 347-bp DNA band in the gel. The rest of the 64 specimens with BI of 2 or above were PCR positive. This study also showed that PCR was not always successful in detecting *M. leprae* in clinical biopsy samples which were microscopically confirmed to have the organisms.

Table 1 . Comparison of PCR results with BI based on microscopic examination for the detection of *M. leprae* in skin slit smears from leprosy patients

BI	n	PCR results	
		No. Positive (%)	No. Negative (%)
0	10	8 (80%)	2 (20%)
1	8	7 (87.5%)	1 (12.5%)
2-4	24	24 (100%)	0
5	18	18 (100%)	0
6	22	22 (100%)	0
Total	82	79 (96.3%)	3 (3.7%)

The overall agreement between PCR and microscopic examination in detecting *M. leprae* was 89% (Table 2), and but false negative of BI for diagnosis of leprosy compared to PCR method is 10.9%. Again, PCR performed with biopsy specimens was superior to slit-skin smear examination for the detection of *M. leprae* in leprosy patients with BI of 0.

Table 2. Comparison of PCR with microscopic examination for the detection of *M. leprae* in skin slit smears from leprosy patients

Microscopic results	PCR results		Total No.(%)
	No. Positive (%)	No. Negative (%)	
Positive	71 (98.6%)	1 (1.4%)	72 (87.8%)
Negative	8 (80%)	2 (20%)	10 (12.2%)
Total	79 (96.3%)	3 (3.7%)	82

*Agreement rate = (71+2)/82 x 100 = 89%

DISCUSSION

This study was initiated to evaluate a PCR technique using primers amplifying the 347-bp DNA[4] of genome sequence of *M. leprae* in detecting the organism in biopsy specimens of leprosy patients.

A nested-primer amplification approach was used to increase specificity and sensitivity, to avoid the use of radioactive probes, and to shorten the time required to obtain a result. Sensitivity and specificity were increased because (i) successful amplification requires the binding of four primers; (ii) fresh reagents are added after 25 cycles; and (iii) background bands were reduced, since each pair of primers was responsible for only a small number of amplification cycles. In addition, the use of radioactivity (and concerns regarding the shelf-life of a radioactive reagent) as well as the need to transfer the products to nitrocellulose and perform hybridization reactions and autoradiography is avoided. This, in turn, reduces to some extent the amount of experimental manipulation required and the overall time for analysis. The entire nested-primer assay, from preparation of the crude lysate to analysis of the gel, can be finished in less than 8 hours, in contrast to the 24 to 48 hours needed for the PCR assays involving radioactive probes [8,9].

The specificity of the assay was assessed by using 22 *Mycobacterium* species and 19 non-*Mycobacterium* species. These particular species were chosen for study because they are phylogenetically and closely related to *M. leprae* or they are species that might be found in clinical samples such as sputum, nasal secretion, or skin biopsies. None of these organisms produced the 578- or 347-bp *M. leprae* product [4]. Thus, the results reported here are quite encouraging for the potential use of PCR technology in rapid detection and definitive identification of small numbers of *M. leprae* in clinical specimens.

Since bacteriological findings for biopsy specimens were not always matched with the BI of slit-skin smears, PCR results should be correlated better with the BI of each biopsy specimen, a portion of which was used in PCR. In this study, 80% of biopsy samples with no detectable AFB by

microscopic examination showed amplification of the 347-bp DNA of *M. leprae* De Wit *et al*. [1] also reported that about 60 to 80% of specimens with no AFB detected by microscopic examination had PCR positive results, although slit skin smear information for patients whose biopsy specimens were BI negative was not available. With that report and our results together, it seemed apparent that PCR is more sensitive in detecting *M. leprae* in biopsy specimens with no or low bacterial loads than the conventional microscopic examination. In order to apply PCR technology for the diagnosis of leprosy in clinical settings, however, a more careful evaluation of PCR results compared with conventional microscopic examination seemed very important. In our study, 8 of 10 patients with BI of 0 in slit-skin smear showed PCR positive.

But biopsy specimens from 1 of 72 patients with confirmed presence of bacteria did not show amplification of the 347-bp DNA in repeated PCR runs. In this study, therefore, the advantage of PCR over conventional microscopic examination could not be fully demonstrated, partly because of a relatively small number of specimens with BI of 0, despite the fact that PCR was far more sensitive than microscopic examination in terms of absolute numbers of AFB detected and in terms of detecting *M. leprae* in slit skin samples. The results might indicate that the sensitivity achievable by PCR in the detection of *M. leprae* for the diagnosis of leprosy could be attained if maximum efforts are put to examine microscopically in slit-skin smears. Further study on equal numbers of BI-positive and BI-negative specimens will address the usefulness of PCR as a supplementary diagnostic tool for leprosy. One of the skin slit specimens that were BI positive showed no amplification of *M. leprae* DNA in repeated tests. The presence of inhibitors in sputum specimens was not uncommon with PCR amplification of *M. tuberculosis* DNA [10,11,12]. It was also noted that there were some interferences in PCR for detection of *M. leprae* in human tissue homogenates spiked with the organisms [3]. De Wit *et al.* [1] showed that several biopsy specimens with BI positivity by microscopic examination did not show an amplification of *M. leprae* DNA by PCR. This study, therefore, showed some limitations of PCR for the detection of *M. leprae* in tissue specimens from leprosy patients. In clinical practice, it may not be necessary to run PCR on BI-positive biopsy samples. Rather, the results here suggest that PCR is useful for detecting *M. leprae* in clinical samples in which no AFB are detectable by microscopic examination. Further evaluation of PCR is desirable for the diagnosis of leprosy, particularly with BI-negative specimens.

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