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Comparison of Polymerase Chain Reaction, immunohistochemistry and conventional histopathology in the diagnosis of leprosy in Myanmar

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Skin biopsy specimens were obtained from 69 leprosy patients attending at Central Special Skin Clinic, Yangon General Hospital. All biopsy samples were examined by polymerase chain reaction (PCR) using the primers amplifying the 130 base-pair fragment of the gene from the 16S ribosomal RNA of *Mycobacterium leprae*, hematoxylin and eosin (H&E) staining, modified Fite-Faraco (FF) technique for *M. leprae* and immunostaining with the antibody against the phenolic glycolipid-1 (PGL-1) and Bacille Calmette-Guerin (BCG) using ABC (Avidin Biotin Complex) method. PCR was positive for 49 (71%) of 69 specimens. In 22 (31.8%) cases, only PCR was positive for *M. leprae* and all other tests were negative. AFB was positive for 19 (27.5%) of 69, PGL-1 was positive for 20 (29%) of 69, BCG was positive for 27 (39%) of 69. Epithelioid cells granuloma was detected in 17 (24.6%) patients and peripheral nerve inflammatory infiltration in 42 (60%) of 69. Comparison of PCR with other method showed statistically significant difference ($p<0.001$). PCR has an advantage over microscopic examination in detecting *M. leprae* in biopsy specimens which are negative for acid-fast bacilli.

INTRODUCTION

Mycobacterium leprae, the etiologic agent of leprosy remains one of the few pathogens that can not be cultivated *in vitro*. The diagnosis of leprosy is still based upon principles used a century ago: clinical examination of the patient lesions, demonstration of acid-fast bacilli (AFB) in slit-skin smears, and histopathology. Leprosy is most easily diagnosed when *Mycobacterium leprae* is demonstrable in diseased tissues, but this is often difficult in the indeterminated and in the tuberculoid types of leprosy in which *M. leprae* is rarely detected. Such a histopathological diagnostic procedure is relatively insensitive and does not give a definitive identification of the infecting organism like *M. leprae*.

Several attempts have been made in recent years to improve the sensitivity and specificity of the detection of *M. leprae* with immunology, biochemistry and nucleic acid probes [1]. For example, Clark-Curtiss and Docherty have described a DNA probe that can be used in a dot blot hybridization assay to detect as little as 1pg of purified DNA, the amount in approximately 300 bacilli [2].

Recently, several investigators have studied the use of polymerase chain reaction (PCR) to detect mycobacteria. This method has been used to detect extremely low number of *M. leprae* in fresh unfixed human skin biopsy specimens, providing a powerful direct and unequivocal test for *M. leprae* infection [3,4]. Since routine biopsy specimens for histopathological analysis are prepared in standard fixative, fresh tissues

are generally not available for PCR analysis. Adaptation of PCR for detecting *M. leprae* in fixed tissue would give clinicians the option of testing biopsy specimens for the presences of *M. leprae*, potentially aiding in the diagnosis of difficult cases. Recent data have shown that PCR can be used to detect genes from eukaryotic and viral genomic DNA derived from formalin-fixed paraffin-embedded tissues [5,6,7]. *M. leprae* DNA has also been detected in extracts from paraffin-embedded skin biopsy [8]. 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 [3,8], 18-kDa antigen [4], 65-kDa antigen [9] or repetitive sequences of *M. leprae* [10,11]and 16S ribosomal RNA of *M. leprae* [12]. Most of the reports showed that PCR with DNA probes seemed very sensitive, so that even 1 to 100 organisms were detectable by the method. In addition, PCR provided virtually 100% specificity in detecting the organism in clinical samples.

However, PCR tools have not been fully evaluated for detecting *M. leprae* in clinical specimens from leprosy patients. In a study with biopsy specimens from leprosy patients, PCR gave a positive result in about 61% of biopsies from leprosy patients who were negative for AFB [8], thus indicating that PCR is a useful tool for the laboratory diagnosis of leprosy. We use the primers for 16S rRNA of *M. leprae* to amplify 130-base-pair product of *M. leprae* genomic DNA extracted from paraffin-embedded sections of biopsy specimens from untreated leprosy patients and the results were then compared with microscopic findings.

At the same time, an investigation on the demonstration of phenolic glycolipid-1 (PGL-1) as a *M. leprae*-specific antigen of *M. leprae*, Bacille Calmette-Guerin (BCG) as a common antigen in all species of Mycobacterium in skin biopsies by immunostaining using Avidin Biotin Complex (ABC) Technique was done. In this study, we attempt to correlate the clinically diagnosed or suspected cases having leprosy with the finding of *M. leprae* using immunostaining method and PCR to evaluate whether this test for demonstrating the presence of *M. leprae* will enhance our ability to diagnose early leprosy.

MATERIALS AND METHODS

Biopsy specimens

After taken informed consent, biopsy specimens were obtained from 82 untreated leprosy patients presented to the Central Special Skin Clinic (CSSC), Yangon General Hospital (YGH) from June 2004 to June 2005. 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. Three-micron thick sections were cut serially and air dried for the study.

Staining methods

The following methods were applied to serial section: hematoxylin and eosin (H&E), Fite's acid-fast method, immunohisto-chemical staining by using ABC technique [14]. The primary antibody against PGL-1 monoclonal was provided by Dr. M. Makino, NIID, Japan using dilution of 1:1000 [15] and antibody to BCG polyclonal derived from *M.tuberculosis* (DAKO-B 012402), used at a dilution of 1:2000. They were incubated for 30 minutes at room temperature. Counter stain was done with Mayer’s hematoxylin.

Immnohistochemical assessment

Direct visual assessment was done using Nikon X 100 dry objective lens. The histological localization of recognized *M. leprae* was determined by comparing the sections incubated with the antibody against PGL-1 and BCG to parallel serial section stained with H&E.

DNA extraction

Preparation of *M. leprae* DNA from biopsy specimens was done by QIAGEN (Germany) kit and DNA extraction from paraffin-embedded skin biopsy samples by DEXPART (TAKARA, Japan).

Specificity of the primers

In order to examine the specificity of primers amplifying the 130bp of a sequence of 16SrRNA of *M. leprae*, genomic DNA purified from *M. leprae*, 16 other *Mycobacterium* species (*M. scrofulaceum, M. kansasii, M. nonchromogenicum, M. malmoense, M. microti, M. intracellulare, M. triviale, M. tuberculosis, M. fortuitum, M. africanum, M. simiae, M. gastri, M. terrae, M. chelonae subsp. chelonae, M. marinum, M. ulcerans*), and DNA extracted from healthy human skin were used for PCR. The 130bp DNA was amplified by PCR only in DNA from *M . leprae* and not in DNA from human and other *mycobacterium* species which were examined in the study [12]. This indicated that the 130-bp DNA amplified with primes ML16S and ML16SA is specific to *M. leprae*.

Amplification of M. leprae DNA by PCR

The primers corresponding to portions of the sequence of 16SrRNA of *M. leprae* and generate a130-bp fragment. The sequences of primers were:

ML16S:
5' AAAAAATCTTTTTTAGAGAT 3'(Forward)
ML16SA:
5′TTCAAGGCGCATGTCTTG 3′(Reverse)

The 50 µl reaction mixture contained 10 µl of template solution, 0.2 µl of *Ex Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan), 0. 5 µl 1 µM of each primer, 5µl of 10x DNA PCR buffer, 8 µl of dNTP solution and 25.8 µl of water. The reaction was performed with a Mastercycler personal Eppendof AG, Hamburg, Germany as follows:
First, the reaction mixture was heated to 94°C for 1 min. One round of amplification consisted of a 30 second denaturation step at 94°C, a 2 min annealing step at 44°C and a 3 min elongation step at 72°C and run for 45 cycles. Then 10µl of the PCR product was electrophoresed on 2% agarose gels for half an hour. After electrophoresis, the gel was stained with ethidium bromide, and visualized under UV illumination.

RESULTS

Histopathological finding

AFB was detected in 19 (27.5%) of 69 located mainly in the superficial dermis. Most of the bacilli showed solid or nonsolid, lymphohistiocytic infiltrate in skin adnexa in 12 (17.4%) of 69 and early granuloma formation with a small number of epithelioid cells surrounded by collection of lymphocytes in 17(24.6%) patients. PGL-1 antigen was positive in 20 (29%) of 69, BCG antigen was positive in 27 (39%) of 69. PGL-1 antigens and BCG were positive in 17 (25%) of 69. Both AFB and PGL-1 were positive in 18 (26.1%) of 69. Peripheral nerve inflammatory infiltration was seen in 42 (60%) of 69.

PCR studies

Detection of the *M. leprae*-specific 130-bp fragment by PCR , indicating the presence of *M. leprae*, was obtained from 49(71%) of 69 specimens. In 22 (31.8%) among them, the only PCR were positive for *M. leprae* and all other tests were negative. Twenty-nine (42.02%) of 69 AFB negative specimens were PCR positive. Therefore, PCR using primers targeting the 130bp fragment of *M. leprae* showed a clear advantage over microscopic examination in detecting *M. leprae* in biopsy specimens with AFB negative. All 21 AFB positive cases by Fite's method were PCR positive. This study also showed that PCR was always successful in detecting *M. leprae* in clinical biopsy samples which were

microscopically confirmed to have the organisms. The overall agreement between PCR and microscopic examination in detecting *M. leprae* was 57.9%. There was highly significant difference between PCR and microscopic examination for the detection of *M. leprae* in biopsy specimens (P =0.0001,<0.01, t-test ,Table 1).

Table 1. Comparison of PCR results with AFB results for the detection of *M. leprae* in biopsy specimens from leprosy patients

AFB result (%)	PCR results (%)		Total No. (%)
	No. Positive (%)	No. Negative (%)	
Positive	21 (100%)	0	21 (30.4%)
Negative	29 (60.4%)	19 (39.6%)	48 (69.6%)
Total	50 (72.5%)	19 (27.5%)	69

*Agreement rate = (21+19)/69 x 100 = 57.9%

All of 20 PGL-1 antigen positive samples were also PCR positive. Among 49 PGL-1 antigen negative samples, 28 (57.1%) were PCR positive and 21 (42.9%) were PCR negative. There was a significant difference between PCR and PGL-1 antigen (P = 0.001, <0.01, t-test) (Table 2) for the detection of *M. leprae*.

Table 2. Comparison of PCR results with PGL-1results for the detection of *M. leprae* in biopsy specimens from leprosy patients

PGL - 1 result (%)	PCR results (%)		Total No. (%)
	No. Positive (%)	No. Negative (%)	
Positive	20 (100%)	0	20 (29%)
Negative	28 (57.1%)	21 (42.9%)	49 (71%)
Total	48 (69.6%)	21 (30.4%)	69

*Agreement rate = (20+21)/69 x 100 = 59.4%

All of 24 BCG antigens positive samples were PCR positive. Among 45 BCG antigens negative samples, 24 (53.3%) were PCR positive and 21 (46.7%) were PCR negative. There was also a significant difference between PCR and BCG antigens (P = 0.0001 <0.001, t-test) (Table 3) for the detection of *M. leprae*.

Table 3. Comparison of PCR results with BCG PGL-1 results for the detection of *M. leprae* in biopsy specimens from leprosy patients

BCG result (%)	PCR results (%)		Total No. (%)
	No. Positive (%)	No. Negative (%)	
Positive	24 (100%)	0	24 (34.8%)
Negative	24 (53.3%)	21 (46.7%)	45 (65.2%)
Total	48 (69.6%)	21 (30.4%)	69

*Agreement rate = (24+21)/69 x 100 = 65.2%

DISCUSSION

All of the 69 patients included in the study had a similar or different clinical picture; having one or more hypopigmented macules mostly on the extremities with minimal or no impairment of sensation, or having slight facial infiltration with impairment of sensation. The skin adnexal involvement was shown in 26 cases, selective intraneural or perineural inflammation in 45 and epithelioid cell granuloma with lymphocytic infiltration in 35 cases. So, H&E staining still plays an important role in the histopathological examination and is the basic method we should never rule out from the routine work. *M. leprae* was found in the subepithelial connective tissue in 15 cases, AFB were also found in the macrophage granuloma in the dermis in 13 cases, and dermis nerves in 15 cases. Further, dermal nerves also showed destruction of the entire perineurium with much of the endoneurium preserved. In a case of early leprosy with a single skin lesion, proliferation of perineurial cells with AFB in one of them and no significant endoneural changes has

been reported. This study also confirms Ridley's contention that in all biopsies of patients with early leprosy, AFB could be detected if fairly large numbers of serial section stained properly for *M. leprae* were carefully examined under oil immersion lens and searched for in the appropriate sites. Opinion is also expressed that early leprosy lesion may gradually or abruptly evolved into either tuberculoid or lepromatous leprosy [16,17,18].

PGL-1 is a species-specific antigen of *M. leprae* [19,20]. In contrast to it, BCG is revealed as a common antigen in all species of the *Mycobacterium* [21]. The specificity of the immunohistochemical staining using the antiserum against NTP conjugated with KLH to identify the PGL-1 antigen on formalin fixed and paraffin embedded leprosy skin biopsy specimens has been proved by Goto *et al* [22]. The specificity of the immunohistochemical staining using the antibody against BCG is just similar to that of acid-fast staining. As for the sensitivity, the result indicated that the sensitivity of the demonstration of the PGL-1 antigens (positive in 20) was more than that of the demonstration of the BCG antigen (positive in 17) [22]. It is probably due to the amount of BCG and the amount of PGL-1 accumulated in the skin lesion. Both PGL-1 antigen and BCG antigen was demonstrated in some nerve bundles, erector pili muscles, epithelioid cells and endothelial cell of blood vessels where AFB was absent. The conventional acid fast staining such as Fite's method can demonstrate *M. leprae* with acid-fast staining features only. Some modified procedure are able to display *M. leprae* with or without acid-fast staining features which is so called chromophobic bacilli after the pretreatment with periodic acid. But the above procedures all fail to demonstrate the soluble antigen released from *M. leprae* [20]. However, immuno-histochemical staining can identify both the bacilli and released soluble antigens in the vacuolar pattern [21]. It is useful and very important for the demonstration of *M. leprae* in early lesion and regressive lesions particularly in paucibacillary cases. In this study, the PGL-1 was positive in 20, but AFB was detected by microscopic examination only in 19 cases. BCG was positive in 27 cases. So PGL-1 and BCG showed higher positive rate than AFB by microscopic examination.

In this study, the PCR showed the highest positive rate (71%). The *M. leprae* specific 130bp pair fragment was amplified by using the primers specific for the sequence from 81 to 210 which include the V1 region of *M. leprae* 16SrRNA gene. Cox *et al.* (1991) have found the sequence of this region consisting of 12 extra nucleotides (6As and 6Ts) which represents a unique *M. leprae* sequence compared to the other mycobacterial sequences [12]. It can be used to design PCR primers that provide a rapid, unequivocal and non-radioactive test for the presence of *M. leprae*. A primer based on this sequence to develop a PCR test was used for the detection and the product showed appropriate size with cloned *M. leprae* DNA, with purified genomic DNA and with various *M. leprae*-infected tissues, but not with other mycobacterial species. The group also showed that there are significant differences between the *M. leprae* 16SrRNA sequences and other mycobacterial sequences [12]. These differences can be exploited to detect and identify *M. leprae* infected tissue. In this study, the 130-bp DNA product amplified by PCR using primers ML16S and ML16SA was obtained only from *M. leprae* and not from other 16 mycobacterial species. The finding supported that PCR procedure provides an advantage of not requiring hybridization using a DNA probe or any other step to confirm that the 130-bp DNA product is specific to *M. leprae*. In this study, 42.02% of specimens with no detectable AFB by microscopic examination showed PCR positive. De Wit *et al.* (1991) reported that about 60 to 80% of microscopically negative specimens are PCR positive for

AFB [8]. The results from this study showed a slightly lower positive rate, but higher than that of Cox *et al.* (1991). In the latter, only 11% of paucibacillary disease showed the positive results. It was 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 summary, PCR using primers of 16SrRNA of *M. leprae*, to amplify the 130 fragment of *M. leprae* DNA showed a clear advantage over microscopic examination and immunostaining in detecting *M. leprae* in tissue with negative AFB. It is recommended that PCR studies to detect *M. leprae* should be done wherever possible in conjunction with histopathological examination and immunohistochemical staining in order to obtain the precise diagnosis of early leprosy.

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