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The role of immunohistopathology and polymerase chain reaction (PCR) in the diagnosis of leprosy

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The aim of the study was to determine the role of immunohistopathology and polymerase chain reaction (PCR) in the diagnosis of leprosy. A total of 112 skin biopsy specimens from leprosy patients attending the Central Special Skin Clinic, Yangon General Hospital have been examined by hematoxylin and eosin (H&E) staining, modified Fite-Faraco (F-F) technique for *M. leprae*, immunostaining with the antibody against the phenolic glycolipid-1 (PGL-1) and Bacille Calmette-Guerin (BCG) using Avidin Biotin Complex (ABC) method and polymerase chain reaction (PCR) using the primers amplifying the 130 base-pair fragment of the gene from the 16S ribosomal RNA of *Mycobacterium leprae.* Based on the clinical diagnosis and histological (Ridley-Jopling) classification, indeterminate (ID) and tuberculoid (TT) types were the most common in paucibacillary (PB) cases (64.9%) and borderline lepromatous (BL) and lepromatous (LL) types were most common in multibacillary (MB) cases (58.7%). So, H&E stain still plays an important role in the histo-pathological examination and is still the basic method for diagnosis and categorization of leprosy. Fite-Faraco stain was negative in all PB cases and half of the MB cases were positive. The finding was consistent with the clinical diagnosis of leprosy. According to the histological classification, detection of *M. leprae* by Fite stain was 100% negative in ID and TT types. Fite stain positivity was lowest in ID type (0%) and highest in LL type (94%). So F-F stain positivity correlated with the histological types of leprosy. F-F, BCG, and PGL-1 staining compared to PCR provided virtually 100% specificity, but only 40%, 50% and 46% sensitivity in detecting the organisms in clinical samples respectively. Application of histopathology, histochemistry, immunohistopathology and molecular techniques (PCR) for identification of *M. leprae* was proved to be useful to diagnosis of early leprosy, which is important to maintain the achievements of Leprosy Elimination Program in Myanmar.

INTRODUCTION

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae*, an acid-fast, rod-shaped bacillus. The disease mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract and also the eyes, apart from some other structures. Currently, global situation of leprosy prevails mainly in a limited number of countries in Africa, Asia and South America. The strategy to eliminate leprosy as a public health problem (defined as a registered prevalence rate of less than 1 case per 10,000 population) has helped countries to further reduce the burden of disease. WHO will continue its support to the remaining highly endemic countries, to ensure that the goal of elimination is achieved. In addition, it will provide essential support to countries that have achieved elimination, to reduce the disease

burden further and to ensure that the leprosy control services are sustained, by providing technical support, monitoring the leprosy situation, providing MDT drugs free of charge, improving drug supply logistics and promoting advocacy to maintain political commitment at all levels [1].

In Myanmar, leprosy stands at the 8th position of our national health priority diseases (2001) [2]. Currently, leprosy elimination has been achieved at the national level at the end of January 2003. The prevalence rate (PR) at the end of June 2003 was lower than 1 per 10,000 population in all states and divisions [3].

After complete elimination of leprosy in the year 2003, there should be no full-blown cases of leprosy developing. Therefore, early diagnosis and prompt treatment is very important to prevent nerve damage and development of deformities. The diagnosis of early leprosy, especially single-lesion leprosy, based upon clinical and skin smear examination is often difficult under field conditions as well as in leprosy clinics. Therefore, in clinically doubtful cases, confirmation of diagnosis is essential by performing a skin biopsy to detect early histopathological lesions, supplemented by special histochemical, immunohisto-chemical and molecular techniques.

In Myanmar, a large number of research for dissertations and theses have been done on leprosy problem in many fields of study such as Microbiology, Pharmacology, Paediatrics, Internal Medicine, Public Health, Preventive and Tropical medicine, Ophthalmology and Dermatology. However, early tissue changes in leprosy, immuno-histopathological techniques and molecular studies have not been conducted.

In this study, routine conventional histo-pathology using H & E stain, special stain using F-F, IHC using BCG and PGL-1 antibodies and PCR methods were performed on skin biopsies with the hope of getting an early diagnosis of leprosy.

MATERIALS AND METHODS

This study is a descriptive, cross-sectional, hospital and laboratory-based study and was carried out from year 2003 December to 2006 June at Immunology Research Division, Department of Medical Research (LM) and Central Special Skin Clinic, Yangon General Hospital, Yangon. One hundred and twenty cases of leprosy patients attending the CSSC, YGH were included. Diagnosis and classification of leprosy was done according to WHO criteria [4].

Ethical clearance was taken form Ethical Review Committee, DMR (LM). The research procedures were explained thoro-ughly to the patients before taking the inform consent. A thorough history taking and physical examination were done and the findings were recorded in the proforma. Slit skin smear from skin lesions and a skin biopsy were taken. 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.

The following methods were applied to serial sections: hematoxylin and eosin (H&E), Fite's acid-fast method and immunohistochemical staining by using ABC technique [5]. The primary antibody against PGL-1 monoclonal was provided by Dr. M. Makino, NIID, Japan using dilution of 1:1000 [6] and antibody to BCG polyclonal derived from *M. tuberculosis* (DAKO), used at a dilution of 1:2000. They were applied for 30 minutes at room temperature. Counter stain was done with Mayer’s hematoxylin. Preparation of *M. leprae* DNA from biopsy specimens was done using QIAGEN (Germany) kit. Amplification of *M. leprae* DNA by PCR using 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′

ML16SA: 5′TTCAAGGCGCATGTCTTG 3′

The 50 µl reaction mixture contained 10 µl of template solution, 0.2 µl of *Ex Taq* DNA polymerase (Takara Shuzo Co., Shiga, Japan), 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 eppendorf AG, Hamburg, Germany. The reaction mixture was heated to 94°C for 1 min, rounds 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 for 45 cycles. Ten microliters of the reaction mixture were electrophoresed on 2% agarose gels. After electrophoresis, the gel was stained with ethidium bromide, and the 130 bp DNA band was examined under UV illumination.

Reporting of results

1. Histopathology results

Histopathology results were reported as indeterminate (ID), tuberculoid (TT), borderline tuberculoid (BT), mid borderline (BB), borderline lepromatous (BL) and lepromatous (LL) types according to Ridley-Jopling classification**.**

2. Histochemical results

F-F stain results were reported as positive and negative. *Mycobacterium leprae* are pink rod-shaped organisms measuring approximately 3 to 8 µ in length. The acid-fast organisms were seen against a blue background lying singly, as cigar shaped bundles, or as globi in positive cases.

3. Immunohistochemical results

Immunoexpression of BCG antigen and PGL-1 antigen were reported as positive and negative, the degenerated bacilli can be seen as brown color inside the foamy macrophages in positive results.

4. PCR results

Identification of *M. leprae* DNA by PCR method was reported as positive and negative. The samples were analyzed by electrophoresis on 2% (wt/vol) agarose gels for the presence of DNA bands. The positive cases for *M. leprae* DNA were seen as DNA bands at the 130bp level of molecular weight markers and at the same site as positive control. There were no DNA bands at the 130bp of molecular weight markers for the negative results (Fig.1).

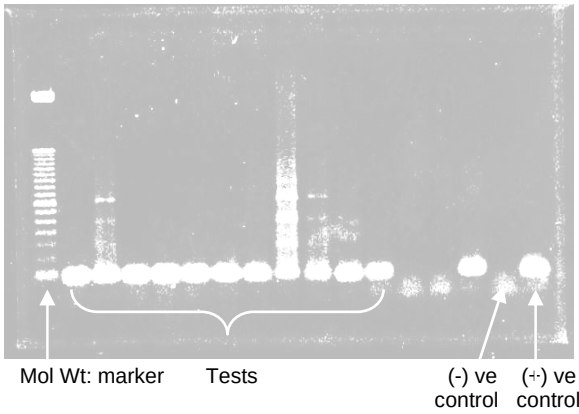

Fig. 1. Gel electrophoresis of PCR product for amplification of 130bp DNA of *M. leprae* from biopsy tissue. Lane 1, Molecular wt: marker (100bp), lane 2 to 12 and 15 positive cases, lane 13 and 14 negative cases, lane 17 positive control (Thai 53 strain) and lane 16 negative control.

RESULTS

A total of 112 skin biopsy specimens from leprosy patients attending the CSSC, YGH were studied during 18 months period from June 2004 to December 2005.

Age

Minimum age was 5 years and maximal age was 78. Mean was 47 with standard deviation of 15.75. Mode was 50.

Clinical types of leprosy by WHO classification

The patients were clinically classified by WHO criteria as PB and MB, which constituted 33 % and 67% respectively.

WHO and Ridley-Jopling classifications

Based on both clinical (WHO) and histological (Ridley - Jopling) classification of leprosy, ID type was most common in PB

cases and LL type was most common in MB cases (Table 1).

Table 1.WHO and Ridley-Jopling classifications

WHO classi-fication	Ridley-Jopling classification									Total
	LL	BL	BB	BT	TT	ID	Normal	NSD	Healed	
PB	0	0	1	6	6	18	6	0	0	37
MB	17	13	5	9	11	13	2	2	3	75
Total	17	13	6	15	17	31	8	2	3	112

Positivity of lepra bacilli by F-F stain in clinical types of leprosy

All PB cases were F-F stain negative (100%) and 57.3% of MB cases were F-F positive. AFB was negative in slit skin smears of PB cases.

Histopathological types and detection of M. leprae by F-F, BCG, PGL-1 and PCR

Fig. 2 shows histopathological types and positive detection of *M.leprae* by F-F, BCG, PGL-1 and PCR. Positive results of F-F were lowest in ID type and positive results of PCR were highest in LL type.

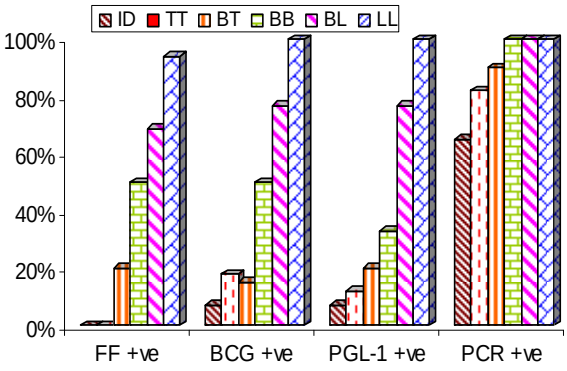

Fig. 2. Histopathological types and positive detection of *M.. leprae* by FF, BCG, PGL-1 and PCR

Comparison of agreement rates between PCR and F-F, BCG, PGL-1

For comparison of agreement rates between PCR and F-F, BCG, PGL-1, agreement rate of F-F was 53.6%, PGL-1 was 60.71% and BCG was 65.17%. BCG was the best test among 3 tests compared with PCR by agreement rates.

Identification of M. leprae in skin biopsy by F-F and PCR

All 30 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* using F-F was 53.6%. 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).

Identification of M. leprae in skin biopsy by PGL-1 and PCR

All of 37 PGL-1 antigen positive samples were also PCR positive. Among 75 PGL-1 antigen negative samples, 44 (59%) were PCR positive and 31 (41.3%) were PCR negative. There was significant difference between PCR and PGL-1 antigen (P= 0.001, <0.01, t-test) for the detection of *M. leprae*.

Identification of M. leprae in skin biopsy by BCG and PCR

All of 40 BCG antigens positive samples were PCR positive. Among 72 BCG antigens negative samples, 39 (54.2%) were PCR positive, 33 (45.83%) were PCR negative. There was significant difference between PCR and BCG antigens (P= 0.0001 <0.001, t-test) for the detection of *M. leprae*.

Table 2. Sensitivity, specificity and predictive values (PV) of F-F, BCG and PGL-1 stain compared to PCR

Tests compared to PCR	PCR				
	Sensi-tivity 95% CI	Speci-ficity 95% CI	Positive PV 95% CI	Negative PV 95% CI	Kappa 95% CI
F-F	0.398 (0.299-0.505)	1 (0.901-1.000)	1 (0.896-1.000)	0.412 (0.13-0.518)	0.281 (0.13-0.433)
BCG	0.506 (0.398-0.614)	1 (0.896-1.000)	1 (0.912-1.000)	0.458 (0.348-0.573)	0.345 (0.192-0.498)
PGL-1	0.457 (0.533-0.565)	1 (0.89-1.00)	1 (0.908-1.000)	0.423 (0.309-0.526)	0.292 (0.14-0.433)

Sensitivity, specificity and predictive values (PV) of F-F, BCG and PGL-1 stain compared to PCR

Table 2 shows sensitivity, specificity and predictive values (PV) of F-F, BCG and PGL-1 stain compared to PCR.

DISCUSSION

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, a clinical examination of the patient's lesions, demonstration of acid-fast bacilli (AFB) in slit-skin smears, and histopathology. Leprosy is most easily diagnosed when *Mycobacterium leprae* are demonstrable in diseased tissues, but this is often difficult in the indeterminate and tuberculoid types of leprosy where *M. leprae* is rarely detected. Histopathological diagnostic procedures allow histological categorization of different types of leprosy but it is relatively insensitive as a definitive identification of the infecting organism when *M. leprae* is not positive with routine H&E staining. Several attempts have been made in recent years to improve the sensitivity and specificity for the detection of *M. leprae* with Immunology, Biochemistry and nuclei acid probes [7]. For example, Clark-Curtiss and Docherty [8] 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.

Recently, many scientists have studied the use of polymerase chain reactions (PCR) to detect mycobacteria. This method has been used to detect extremely low numbers of *M. leprae* in fresh unfixed human skin biopsy specimens, providing a powerful direct and unequivocal test for *M. leprae* infection [9]. In the literatures, there have been various target sequences for PCR and DNA probes specific for *M. leprae,* such as genes encoding the 36-kDa antigen [9], 18-kDa antigen [10] or repetitive sequences of *M. leprae* [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 [13].

At the same time, the demonstrations of phenolic glycolipid-1 (PGL-1) as a *M. leprae*-specific antigen and of Bacille Calmette-Guerin (BCG) as a common antigen in all species of Mycobacterium in skin biopsies were done by immunostaining using Avidin Biotin Complex (ABC) technique. This study has attempted to compare the diagnostic accuracy between immunostaining method and PCR-based molecular method in *M. leprae* infection of various clinical settings and then to evaluate the role of these tests in the early diagnosis of leprosy.

All of the 112 patients included in the study had a similar clinical picture, having one or more hypopigmented macules mostly on the extremities with minimal or no impairment of sensation, or having skin lesions with impairment of sensation. Based on clinical classification (WHO), 75 cases (67%) were MB and 37 cases (33%) were PB. Regarding the histological (Ridley-Jopling) classification, ID type was the most common in PB (18 cases) and LL type was most common in MB (17 cases). So H&E staining still plays very important role in the histopathological examination and is the basic method which should always be done in the routine work on leprosy diagnosis. Liu, *et al.* [14] reported that in the study of histology in indeterminate leprosy, involvement of the dermal nerves, with perineural infiltration being the most common finding. Porichha *et al.* [15] has stated that the presence of AFB and nerve involvement were taken as definite features for a diagnosis of leprosy, and infiltration of the dermal appendages, neurovascular bundles and dermis by epitheloid cells and

lymphocytes were regarded as suggestive signs of leprosy.

By using Fite-Faraco stain, *M. leprae* were found in the subepithelial connective tissue, in the macrophage of granuloma in the dermis, and in dermal nerves. Further, dermal nerves also showed destruction of the entire perineurium with much of the endoneurium being 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 [16]. This study also confirmed Ridley's contention that in all biopsies of patients with early leprosy, AFB could be detected if fairly large numbers of serial sections stained properly for *M. leprae* were carefully examined under oil immersion lens and searched for in the appropriate sites. Fig.2 shows Fite stain positivity was lowest in ID type and highest in LL type. So F-F stain positivity was correlated with histological types of leprosy. Fite stain for *M. leprae* has confirmed the clinical diagnosis of leprosy.

Lejbkowicz *et al.* [17] reported that leprosy is rare and non-endemic in Israel, cases of leprosy were invariably imported by immigrants or foreign workers and pathologists are not always alert to the possibility of leprosy or recognize potential symptoms. Therefore it is recommended that confirmation of leprosy should be done in all cases by histopathology of suspected lesions supplemented with staining for acid-fast bacilli by modified Fite-Faraco staining. PGL-1 is a species-specific antigen of *M. leprae* [18].

In contrast to it, BCG is revealed as a common antigen in all species of the *Mycobacterium* [19]. The specificity of the immunohisochemical staining using the antiserum against PGL-1 antigen on formalin fixed and paraffin embedded leprosy skin biopsy specimens has been proved by Goto *et al*. [20]. 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 was more than that of the demonstration of the BCG antigen [20]. 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 were demonstrated in some nerve bundles, erector pili muscles, epithelioid cells and endothelial cells of blood vessels where AFB were absent. The conventional acid-fast staining such as Fite's method can demonstrate *M. leprae* with acid-fast staining features only. But the above procedures all fail to demonstrate the soluble antigen released from *M. leprae* [19].

However immunohistochemical staining can identify both the bacilli and released soluble antigens in the vacuolar pattern [19]. 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 BCG was positive in 2.7% and PGL-1 was positive in 8.1% among PB cases. Among MB cases, the BCG was positive in 50.7% and PGL-1 was positive in 45.3%. Fig. 2 shows higher positive rates of PGL-1 and BCG than F-F stain in all types of Ridley-Jopling histological classification ie., ID, TT, BT, BB, BL and LL by microscopic examination. The detection of bacillary antigens, together with the clear demonstration of nerve bundles enhanced the capacity to fulfill morphologic criteria for the diagnosis of early leprosy. These observations indicated that immunohistochemical methods represented useful tool for the early diagnosis of leprosy.

In this study, the positive rate of BCG was higher than that of PGL-1 in both PB and MB cases as well as in TT, BT and BB types. Deps *et al.* [21] reported that false positive reaction of the immunohistochemistry technique using anti-BCG polyclonal antibodies to identify *M. leprae* in wild nine-banded armadillos was identified. In our study, positive and negative controls were tested simultaneously

with patients' specimens. Results were always compatible with controls. However, basal cells of skin, fibroblast and endothelium cells were showed BCG positive. The positive rate with BCG was found to be higher than with PGL-1. This may be due to false positive reaction as reported by other study [21]. Therefore, the diagnosis of leprosy should be done with histopathological study supplemented by other IHC test like PGL-1 and AFB stain with Fite-Faraco. PCR was 100% positive in BB, BL and LL types of leprosy and 64.5%, 82.4% and 60% positive in ID, TT and BT types. These findings confirm the previous reports of others that PCR has high sensitivity and near perfect specificity to detect *M. leprae* [10, 22, 23, 24].

Agreement rates between PCR and F-F, BCG and PGL-1 stain were also 53.6%, 65.17% and 60.7% respectively. BCG stain showed higher sensitivity compared to the other two tests, F-F and PGL-1 stain. Table 2 shows sensitivity, specificity and positive and negative predictive values of F-F, BCG, and PGL-1 stain with PCR, 100% specificity in all tests and 40%, 50% and 46% sensitivity in FF, BCG and PGL-1 stain. Scollard, *et al.* [25], Qinxue *et al.* [26] and Haque *et al.* [27] have also reported the specificity of the PCR was 100% in their clinical referral material. Sensitivity ranged from 50% to 83%. The assay is highly informative when acid-fast bacilli are not detectable by light microscopy.

Job CK *et al*. [28] has reported that even in endemic countries the profile of leprosy was changing, and detection of leprosy lesions in their early stages has become increasingly important. Since the finding of *M. leprae* is crucial in the confirmatory diagnosis of early leprosy, it is suggested that PCR studies to detect *M. leprae* should be done wherever possible in conjunction with histopathologic examination. It is also recommended that the feasibility and the cost-effectiveness of both of these methods to find *M. leprae* be evaluated.

In this study, the PCR showed highest positive rate in all histological types. The *M. leprae* specific 130 bp pair fragment was amplified by using the primers specific for the nucleotide sequence from No. 81 to 210 which include the V1 region of *M. leprae* 16 SrRNA gene. Cox *et al.* [12] 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 species sequences. 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 was used in a PCR protocol for the detection of *M. leprae* and the PCR 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 were significant differences between the *M. leprae* 16 SrRNA sequences and other mycobacterial sequences [12].

These differences could 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*.

With the availability of numerous effective anti-leprosy drugs, leprosy is being rapidly controlled throughout the world. The profile of leprosy patients presenting in an out patient clinic is also radically changing, and more and more early lesions are encountered. Therefore, any additional tool for use in detection will be a great advantage in the field of early diagnosis. Detection of *M. leprae* using PCR methodology has recently emerged as a diagnostic tool. It is, at present, too elaborate and

expensive to be used in routine diagnostic work. Improving the technique of PCR study and making it cheaper is imperative.

Conclusion & recommendation

The PCR assay was also useful as a tool for detection and follow-up of possible leprosy cases. It could be used to monitor high-risk populations and thereby maintain the achievements of leprosy elimination programs in countries where the disease's prevalence has been significantly reduced. Early diagnosis and prompt treatment is very important for any infection including leprosy to prevent deformity and stigmatizations of patients. So PCR technique needs to be established at reference laboratory as National Health Laboratory and special stain (Fite-Faraco) and introduction of immunohistochemical stain facilities in the laboratory referred center as CSSC, YGH. It could be used to monitor high-risk populations and also to maintain the achievements of leprosy elimination programs in Myanmar, where the disease prevalence has been significantly reduced.

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