Detection of *Mycobacterium leprae* in Tissue and Blood by Polymerase Chain Reaction

Kyu Suk Lee, M.D., Kwang Youl Oh, M.D., Young Wook Ryoo, M.D.,
Min Ho Suh, M.D.*, Joon Young Song, M.D.

Department of Dermatology, and Microbiology, Keimyung University School of Medicine, Taegu, Korea*

***Background*: Methods to detect and quanitify *Mycobacterium leprae*(M. *leprae*)are needed for studies involving the epidemiology, pathogenesis, and chemotherapy of leprosy. Serological assays and skin tests lack the sensitivity and specificity to serve as diagnostic tools for M. leprae infection. The polymerase chain reaction(PCR) based on the selective amplification of an 530-bp frangment of the gene encoding the proline-rich antigen of M. *leprae* was performed with sections of fixed or frozen biopsy samples from leprosy patients.**

***Objective*: This study was done to investigate the applicability of PCR for the detection of low numbers of M. *leprae* in tissues and peripheral blood.**

***Methods*: The PCR was used to amplify a 530-base-pair M. *leprae* DNA with the thermoxtable Taq DNA polymerase.**

***Results*: The In frozen skin tissues and peripheral blood of leprosy patients. relatively high detection rates of PCR products was achieved by using direct gel analysis as well as Southern blot hybridization.**

***Conclusion*: These results suggest that PCR amplification for the detection of M. *leprae* may be useful for the epidemiologic study of large papulations as well as coinical astudies on the individual patients. (Ann Dermatol 6:(2)130-135, 1994)**

Key Words: Blood, Leprosy, PCR

Leprosy is a chronic, systemic infectious disease caused by *Mycobacterium leprae*(M. *leprue*). Serologic assays and skin tests lack the sensitivity and specificity to serve as diagnostic tools for M. *leprae* infection [1,2]. Even when satisfactory immunodetection is achieved, it may be a reflection of a past infection, giving no information on the current bacteriological status[3]. Monoclonal antibodies offer quick and precise indentification of M. leprae[4] and DNA probes offer the same, rapid and reliable detection of bacteria[5]. A new diagnostic method which is more sensitive and specific for the detection of M. *leprae* is required. Polymerase chainreaction(PCR) has been shown to be very sensitive and, using appropriate primers, specific, and has already been applied successfully to the diagnosis of genetic disorders and to the detection of viruses [6-8]. Recently there were some reports about the use of PCR for the detection of M. *leprae*[9-13], but they used only skin biopsy samples. In this study, we applied the PCR to detect M. *leprae* DNA on skin and blood samples from leprosy patients which may have potential use as a tool in the epidemiology and control of leprosy.

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Reprint request to: Kyu Suk Lee, M.D., Department of Dermatology, Keimyung University School of Medicine, Taegu, Korea
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MATERIALS AND METHODS

1. Clinical samples

Leprosy patients were classified clinically and histopathologically according to the Ridley-Jopling scale[15] as lepromatous, borderline lepromatous, borderline, borderline tuberculoid, tuberculoid, tuberculoid or indeterminate. Punch skin biopsy samples were taken from skin lesions of 31 leprosy patients(17 males and 14 females, between 14 and 73 years of age). Biopsy samples were either quick-frozen(7 samples) in liquid nitrogen or formalin fixed(22 samples). Peripheral blood samples were also taken from 11 leprosy patients and 2 normal individuals.

M. *leprae* was isolated from the foot-pad tissue of an experimentally infected nude mouse(kindly provided by Dr. Yong-Ma Ha, Institute for Leprosy Research).

2. Preparation of chromosomal DNA

M. leprae DNA was purified by a modification of the method described by Okanishi & Manome [16]. Isolated DNA was used as a positive control.

Frozen sections(6mm thickness) were incubated with 50μl of 100 mM Tris-HCl pH 8.5, containing 0.05% Tween 20 and 60 μg/ml proteinase K for 18 hours at 60°C. Fixed paraffin-embedded tissue sections(6 μm thickness) were deparaffinized with 400μl of xylene and washed twice with 800μl of aceton, after which the tissue pellet was allowed to dry. This pellet were treated identically as the frozen sections.

Peripheral blood was centrifuged for 10 min at 13,000 r.p.m. and mixed with 500μl TE (10mM Tris-HCl, 1 μM EDTA). the above procedures were done twice and then the pellet was resuspended with 50 mM KCl, 2.5 μM $MgCl_2$, 0.5% Tween 20, 100 μg/ml protease K with incubation at 56°C for 45 min.

3. Polymerase Chain Reaction

A set of primers, S13(5'-CTCCACCTGGACCGGCGAT-3') and S62(5'-GACTAGCCTGCCAAGTCG-3'), was selected from the gene encoding the 36 K antigen of M. *leprae* to amplify a 530-base-pair(bp) DNA fragment. The 100μl PCR mixture contained 50 mM KCl, 10 mM Tris-HCl(pH 8.8), 1.5 mM MgCl2, 0.1%(wt/vol) gelatin, 1 mM each of dATP, dGTP, dTTP and dCTP, 200ng of dach primer, 2.5 U of Taq polymerase(Perkin-Elmer Cetus, Norwalk, USA) and 2μl of sample DNA. Rounds of amplification consisted of a 2 min denaturation step at 94°C, a 2 min annealing step at 55°C, and a 3 min extension step at 72°C. After the 32nd cycle, the samples(10μl) were analyzed by electrophoresis and by subsequent Southern blot hybridization.

4. Southern blot hybridization

For direct gel analysis 10μl of the reaction mixture was subjected to electrophoresis on an 1.2% agarose gel for 45 min. at 50 volts and DNA was visualized by UV fluorescence(320 nm) after staining with ethidium bromide.After electrophoresis of PCR products, DNA fragements were tranferred to a nitrocelllulose paper by capillary transfer. A 1.0 kb EcoRI fragments were tranferred to a nitrocelllulose paper by de Wit et al[3], that was labelled with digoxigenin-11-dUTP with a DNA labeling and detection kit(Boehringer Mannheim, Germany). Pre-hybridization and hybridization were performed at 42°C for 2 hr and 16 hr, respectively. After hybridization the hybrids are detected by enzyme-linked immunoassay using anti-digoxigenin-alkiline phosphatase conjugate and subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indotyphosphate and nitro blue tetrazolium salt. The final washing of the membrane was performed in solution containing 0.1 X SSC and 01.% SDS at 68°C for 30 min.

RESULTS

1. Detection of M. leprae in tissues and blood

In frozen and paraffin embedded tissues of leprosy patients, relatively high detection rates of amplified PCR products were achieved by using direct gel analysis as well as Southern blot hybridization(Table 1-3, Fig. 1). In the peripheral blood of 5 untreated leprosy patients and 2 patients of ENL, positive amplified PCR products were detected and so we knew that M. *leprae* bacilli would exist for a relatively long period in the peripheral blood of active cases (Table 4, Fig. 2).

2. Sensitivity and specificity of the PCR

For the determination of sensitivity of the PCR in

Table 1. Detection of M. *leprae* by PCR in frozen sections from leprosy patients

Sample	Type	No. of biopsy sections showing PCR amplification		Total
		+	–	
Frozen	TT	0	0	0
	BT	0	0	0
	BL	3	0	3
	LL	4(3)	0	4(3)
	Control	0	3	3

() : Untreated patients
TT : Tuberculoid leprosy, Bt : Borderline tuberculoid leprosy
BL : Borderline lepromatous leprosy, LL : Lepromatous leprosy

Table 2. Detection of M. *leprae* by PCR in paraffin-fixed tissue sections from leprosy patients

Sample	Type	No. of biopsy sections showing PCR amplification		Total
		+	–	
Paraffin	TT	o	2	2
	BT	2(1)	2	4(1)
	BL	2	1	3
	LL	5(3)	8	13(3)
	control	0	2	2

() : Untreated patients.
TT : Tuberculoid leprosy, BT : Borderline tuberculoid leprosy
BL : Borderline lepromatous leprosy, LL : Lepromatous leprosy

Table 3. Detection of M. *leprae* by PCR in biopsy sample from leprosy patients

Patients	Sample	No. of sections showing PCR amplication		Total
		+	–	
BI-positive	Fr	7	0	7
	P	8	9	17
BI-negative	Fr	o	o	o
P	1	4	5	

Fr : Frozen section
P : Paraffin-fixed section

this study, 5μl M. *leprae* bacilli suspension(8×10^1 badteria/ l) was taken and serially diluted, each diluted sample was amplified by using PCR. In direct gel analysis, PCR products could be detected 6.25×10^{-1} bacteria/μl (Fig. 3) and 3.13×10^{-1} bacteria/μl in Southern blot hybridization. The specificity of PCR was evaluated by using M. *tuberculosis*, bacteriophage DNA. No amplification was detected by direct gel or Southern blot analysis. Only in M. *leprae*, 530 bp DNA band was shown(Fig. 4).

DISCUSSION

The diagnosis of leprosy is often based solely on the clinical manifestation and observation of acid-fast bacilli in a lesion displaying characteristic

Table 4. Detection of M. *leprae* by PCR in peripheral blood samples from leprosy patients

Sample	Type	No. of blood samples showing PCR amplification		Total
		+	−	
Peripheral blood	TT	1	1	1
	BT	0	1	3(1)
	BL	3(1)	0	6(4)
	LL	4(4)	2*	2
	Control	0	2	

() : Untreated patients, * : Patients under therapy.
TT : Tuberculoid leprosy, BT : Borderline tuberculoid leprosy
BL : Borderline lepromatous leprosy, LL : Lepromatous leprosy

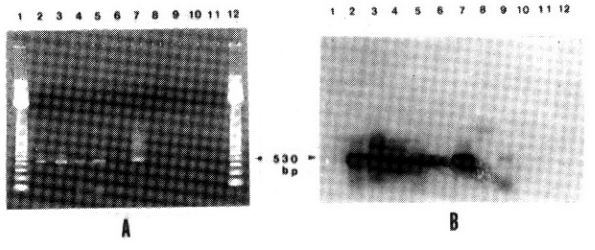

Fig. 1. PCR detection of M. *leprae* DNA in biopsy samples from leprosy patients. Agarose gel analysis of PCR products of samples which contained DNA extracted from purified M. *peprae* and from sections of frozen and paraffin-fixed biopsy samples. PCR was performed with target DNA from : purified M. *leprae* (lane 2), frozen sections from LL (lane 3 - 5) and BL (lane 2), patients, paraffin-fixed sections from LL (lane 7, 8), BT (lane 9) and TT (lane 10) patients, and no DNA (negative control, lane 11). Lane 1 and 12 contained molecular size markers.

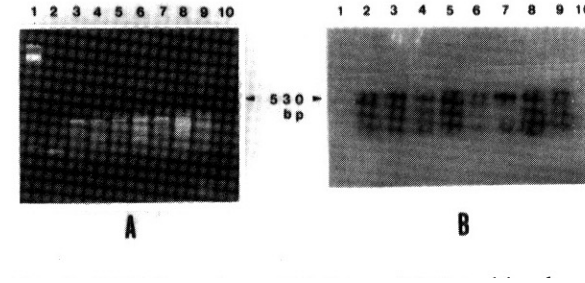

Fig. 2. PCR detection of M. *leprae* DNA in blood samples from leprosy patients. (A) Agarose gel analysis of PCR products of samples which contained DNA extracted from purified M. *leprae* (lane 2) and from periperal blood samples (lane 3-9). Lane 1 contained molecular size markers and lane 10 is negative control. (B) Southern blot analysis of A. Digoxigenin-labeled M. *leprae* DNA probe was used.

histopathologic features. This is due mainly to the inability to cultivate M. *leprae* in vitro.

This study assessed the appicability of PCR, coupled with DNA hybridization analysis, for the detection of low numbers of M. *leprae*. In this study, a set of primers, S13 ad S62 were selected from the gene encoding the 36 K antigen of M. leprae to amplify a 530-base-pair(bp) DNA fragment.

When the PCR was done essentially as recommended by Hartskeerl[9] on paraffin-embedded and fozen biopsy tissues from leprosy patients, the results on the frozen sections were far better. In all the frozen samples from untreated or treated leprosy patient, M. *lepare* DNA could be amplified. But in paraffin-fixed skin biopsy, about half of the PCR positives was found. When biopsy sections from untreated patients were examined, 100% was PCR positive, both in frozen and paraffin-fixed sections.

One of the untreated BI-negative was PCR positive. A similar result has been reported by Wit et al[3]. It has been shown before that low densities of acid-fact bacilli in skin are underestimated or missed entirely by conventional microscopy, so that cases are missed as well as misclassified[17]. In this respect, apparent identification of M. *leprae* DNA in tissue by PCR holds promise as an additional tool for the diagnosis of suspected cases of early leprosy.

When we applied PCR on peripheral blood of 5

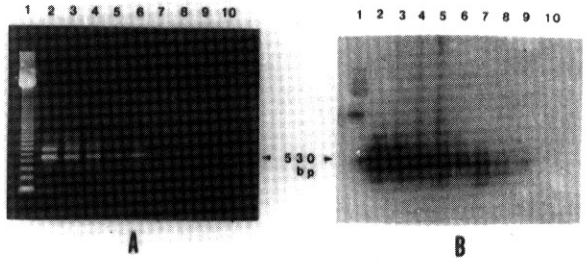

Fig. 3. Sensitivity of the PCR detection of M. *leprae* DNA. Agarose gel analysis of PCR products of samples which contained DNA extracted from 20μl of 2-fold sequential dilutions of a suspension of M. *leprae* containing8 X 10^1 bacteria/μl. the numbers of bacteria per sample were as follows : 4 X 10 1 (lane 2), 2 X 10^1 (lane 3), 1 X 10^1 (lane 4), 5 X 10^0 (lane 5), 2.5 X 10^0(lane 6), 1.25 X 10^0 (lane 7), 6.25 X 10^{-1} (lane 8), 3.13 X 10^{-1} (lane 9), 1.56 X 10^{-1} (lane 10). Lane 1 contained molecular size markers.

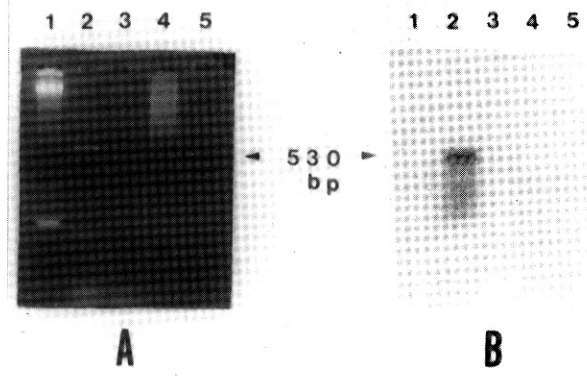

Fig. 4. Specificity of the PCR detection of M. *leprae* DNA. (A) Agarose gel analysis of PCR products of samples which contained DNA extractde from purified M. *leprae* (lane 2), purfied M. tuberculosis (lane 3), bacteriophage DNA (lane 4), and no DNA (negative control, lane 5). Lane 1 contained molecular size markers. (B) Southern blot analysis of A. Digoxigenin-labled M. *leprae* DNA probe was used.

untreated leprosy patients and 2 patients of ENL. It suggests that M. *leprae* bacilli exists for a relatively long period in the peripheral blood of active cases.

In order to investigate the sensitivity of the PCR, reaction mixtures DNA extracted from samples were serially diluted. The addition of 3.13 × 10^{-1} bacteria/μl resulted in a detectable band on Southern blot analysis. A similar detection limit with PCR has been reported by Hartskeerl[9] and P-likaytis[10]. These results indicate that the detection limit of the PCR as performed here approximates 1 to 10 bacilli[9]. This implies that the PCR described here is much more sensitive than other methods for the direct detection of M. *leprae*, such as microscopic visualization[14] and DNA hybridization[5].

The use of primers S13 and S62 resulted in the specific amplification of M. *leprae* DNA. No detectable amplification occured with DNA form purified M. *tuberculosis* or bacteriophage. A band at the 530 bp position was visible only in the samples containing M. *leprae* DNA as reported by De Wit[3] and Hartskeerl[9].

Our study suggests that PCR amplification for the detection of M. *leprae* may be useful for the epidemiologic study of large populations as well as clinical studies on individual patients.

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