

## Monitoring of the Efficacy of Multidrug Therapy (MDT) by Polymerase Chain Reaction (PCR) and *Mycobacterium leprae* Particle Agglutination (MLPA) Test in Leprosy Patients

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### Abstrak

Pada penelitian ini diamati keberhasilan multidrug therapy (MDT) pada penderita lepra dengan menggunakan teknik polymerase chain reaction (PCR) untuk mendeteksi DNA *M. leprae* dari hapusan hidung dan tes *Mycobacterium leprae* particle agglutination (MLPA) untuk menentukan titer antibodi IgM terhadap phenolic glycolipid-I (PGL-I). Dilakukan pengamatan lanjutan setiap 3 bulan pada penderita pausibasiler (tipe TT dan BT) selama 12 bulan dan penderita multibasiler (tipe BL dan LL) selama 27 bulan. Pada penelitian ini diperiksa 52 penderita lepra yang belum diobati terdiri dari 31 tipe TT, 5 BT, 11 BL and 5 LL. Dengan menggunakan teknik PCR, ditemukan secara berturut-turut pada tipe TT, BT, BL dan LL sebanyak 7/31 (22.6%), 4/5 (80.0%), 11/11 (100.0%) dan 5/5 (100.0%) PCR positif sebelum pengobatan. Semua penderita pausibasiler dan multibasiler menjadi PCR negatif pada bulan kesembilan dan dua puluh tujuh setelah pengobatan. Selanjutnya dengan tes MLPA, ditemukan secara berturut-turut pada tipe TT, BT, BL dan LL sebanyak 14/31 (45.1%), 4/5 (80.0%), 11/11 (100.0%) and 5/5 (100.0%) MLPA positif sebelum pengobatan. Semua penderita pausibasiler dan 13 dari 16 penderita multibasiler menjadi MLPA negatif pada bulan kedua belas dan delapan belas setelah pengobatan. Dan 3 dari 16 penderita multibasiler tetap MLPA positif sampai bulan kedua puluh tujuh setelah pengobatan. Sebagai kesimpulan, PCR dan tes MLPA dapat berguna untuk pemantauan keberhasilan MDT pada penderita lepra.

### Abstract

This study was undertaken to monitor the efficacy of multidrug therapy (MDT) in leprosy patients using polymerase chain reaction (PCR) to detect the *M. leprae* DNA from nasal swab specimens and *Mycobacterium leprae* particle agglutination (MLPA) test to determine the titer of IgM anti-phenolic glycolipid-I (PGL-I) antibodies. The follow up was performed every 3 months in paucibacillary (TT and BT types) patients for a period of 12 months and in multibacillary (BL and LL types) patients for 27 months. Fifty-two previously untreated leprosy patients (31 TT, 5 BT, 11 BL and 5 LL type) were included in this study. Before treatment, using the PCR technique, the TT, BT, BL and LL types respectively showed 7/31 (22.6%), 4/5 (80.0%), 11/11 (100.0%) and 5/5 (100.0%) PCR positive. All of the paucibacillary and all of the multibacillary patients became PCR negative respectively at 9 and 27 months after MDT treatment. Furthermore, using the MLPA test, the TT, BT, BL and LL types respectively showed 14/31 (45.1%), 4/5 (80.0%), 11/11 (100.0%) and 5/5 (100.0%) MLPA positive before treatment. All the paucibacillary and 13 of 16 multibacillary patients became MLPA negative at 12 and 18 months after treatment. And 3 of 16 multibacillary patients remained MLPA positive till 7 months observation. In conclusion, the PCR and MLPA test might be of benefit to monitor the efficacy of MDT in leprosy patients.

**Keywords :** PCR, *Mycobacterium leprae* particle agglutination test, Multidrug Therapy, Leprosy

In recent years there has been considerable interest in the application of molecular techniques to detect and identify microorganisms in the nasal cavity.<sup>1-4</sup> In infections such as leprosy, the causative organism cannot be grown *in vivo*<sup>5</sup> and the long incubation period together with the wide spectrum of clinical manifestations of leprosy have prevented reliable and rapid

diagnosis of infections, especially in the tuberculoid and indeterminate forms of the disease.<sup>6</sup> New molecular methods have been developed as reliable and sensitive diagnostic tools for identification of leprosy bacilli. The most significant advance of useful molecular methods, applicable to diagnosis, has been the polymerase chain reaction (PCR).<sup>7-8</sup>

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In this study, PCR was performed to detect specific *M. leprae* DNA in nasal swab specimens from untreated leprosy patients by a set of primers with a detection

limit of approximately one bacterium during MDT treatment.

Phenolic glycolipid-I (PGL-I) antigen has been used as a serological tool for leprosy. The *Mycobacterium leprae* particle agglutination (MLPA) test is a new test using sensitized gelatin particle to detect anti-PGL-I IgM.<sup>9</sup>

Furthermore, a serological test to detect anti PGL-I antibody using MLPA test as a tool in the epidemiology of leprosy in high prevalence villages have been reported.<sup>10-12</sup> These studies suggest that the MLPA test can only reliably detect anti-PGL-I antibodies in the community and may be useful for follow up study of sub-clinical infection of leprosy among individuals living in an endemic area.

Up to 1993, more than 4 million leprosy patients in the world have completed their treatment with multidrug therapy (MDT) recommended by a WHO Study Group.<sup>13</sup> Data of routine leprosy control programs have indicated that, after completion of MDT, the relapse rates are below 1%.<sup>14</sup> However, the low relapse rates must be interpreted with great caution, because the duration of follow up in the majority of patients were relatively short and the relapses after treatment with any rifampicin-containing regimens occur late, at least 5 years after starting treatment.

In this study, follow up has been performed on the various types of leprosy patients during MDT treatment using PCR to detect *Mycobacterium leprae* DNA from their nasal cavities and MLPA test to detect the titer of IgM anti-PGL-I antibodies.

## METHODS

**Leprosy patients.** Study subjects were enrolled from those patients presenting for initial diagnosis at the clinics of leprosy of Ujung Pandang and at the Primary Health Centres in South Sulawesi, Indonesia. Patients were classified according to the Ridley and Jopling method.<sup>15</sup> The bacterial index (BI) and morphology index (MI) were determined from slit-skin smears taken from all patients.

Enrollment began in mid 1993 and continued until the end of 1995. Pernal swabs (Medical Wire and Equipment Co, UK) were used to collect nasal specimens from untreated patients. The specimens were collected by gently rubbing the swab several times over the inferior lateral nasal conchae.

Sera were collected by finger prick from untreated patients. The samples collected were coded. PCR and MLPA tests were performed without prior knowledge of the classification of the samples. All samples were stored at -20°C pending their analysis.

**Preparation of specimens and PCR.** Treatment of the swab specimens with lysis buffer and PCR was done as reported previously.<sup>1</sup> Briefly, the specimens were subjected to PCR for the species-specific amplification of a fragment of the *pra* gene of *M. leprae* using set primers with sequences of 5'CTCCACCTGGACCGGCGAT3' and 5'GACTAGCCTGCCAAGTCG3' and subsequently were analysed for the presence of a 531-bp amplification product by 2% agarose gel electrophoresis, followed by hybridization with a 286 bp fragment of T3T4c PCR product comprising the *pra* gene of *M. leprae* as a DNA probe. To determine if the sample contained inhibiting components that could have caused a negative result, samples in which 531-bp fragments were not detected, were submitted to a second round PCR in which 25 copies of 531-bp modified template was added to the reaction mixture.

DNA was purified using Glass Max DNA Isolation (Gibco BRL, USA) from the samples which were found to inhibit amplification of the modified template. These samples were run again in PCR. PCR reaction mixtures contained dUTP and uracil-DNA-glycosylase to prevent false-positive reactions due to cross-contamination with amplified DNA. In each run, from 25 pg to 2.5 fg positive controls of chromosomal *M. leprae* DNA were included, as well as two negative controls without target DNA (i.e., distilled water, Baker analyzed HPLC, J.T Baker B.V., Deventer, Holland).

A specimen was considered positive when it revealed, with or without purification of DNA, a 531-bp fragment by both agarose gel electrophoresis and subsequent hybridization. A sample was considered negative when it did not show amplification in the first round PCR and did not inhibit the amplification of the modified template, even after purification, it could not be determined whether it contained *M. leprae* DNA or not. No samples were excluded from analysis as being uninterpretable.

**Blood collection and MLPA test.** The blood samples were collected by finger prick. Samples were centrifuged at 3000 rpm for 5 minutes to separate the

serum and transferred to 500 µl vials (Sarstedt, Assist Trading Co., Ltd, Japan) on the same day.

MPLA test was performed using serodia leprae microtitre particle agglutination test kit (Fujirebio Inc, Japan).

Serum samples were diluted to 1:16 and 1:32 in 96 wells U-bottom microplates. Twenty five µl of unsensitized gelatin particles and 25 µl of the particle sensitized with synthetic trisaccharide of PGL-I (NT-P-BSA) were mixed with 25 µl of 1:16 and 25 µl of 1:32 diluted samples, respectively. After being incubated for 2 hours at room temperature, the plate was read for agglutination using a mirror reader. Serum samples showing agglutination at a titre of  $\geq$  1:32 were considered positive. When samples were found to agglutinate at 1:32, they were serially diluted to a concentration of 1:1024 for quantitative testing.

**Data analysis.** All data were entered on a personal computer and analyzed using EPI-Info version 6.04. Analysis of variance methods were applied as indicated in the text. All probabilities presented are two-tailed.

## RESULTS

### Leprosy Patients

The patients studied resided in South Sulawesi, Indonesia. Leprosy patients were classified clinically and histopathologically according to the Ridley-Jopling scale as 31 TT, 5 BT, 11 BL and 5 LL respectively (Table 1).

Table 1. Number of untreated leprosy patients according to sex and Ridley and Jopling classification

Type	Male	%	Female	%
TT	22	70.97	9	29.03
BT	5	100.00	0	0.00
BL	6	54.55	5	45.45
LL	2	40.00	3	60.00
Total	35	67.31	17	32.69

The bacterial index (BI) and morphology index (MI) in all the patients of the TT and BT types were 0. The BI in patients of the BL type was 1 with (BI=1), 3 (BI=2), 4 (BI=3) and 3 (BI=4) respectively. While the MI in patients of the BL type was 1 with (MI=1), 5 (MI=2), 4 (MI=3) and 1 (MI=4) respectively. In all the five patients of the LL type the BI was 4, while the MI was 3 in 2 patients and 4 in 3 patients respectively (Table 2).

### PCR and MLPA test results

Table 3 shows the results of PCR and MLPA test applied on nasal swabs and sera from untreated patients (n=52). Twenty two percent of TT type from BI and MI negative untreated patients were PCR positive, while 45% of those from BI and MI negative untreated patients were antibodies positive to phenolic glycolipid-I (PGL-I) by MLPA test.

Among BT type from untreated patients in which no acid-fast bacilli were detected by microscopy, 80% were PCR positive and MLPA test positive.

Table 2. Number of untreated leprosy patients according to bacterial index and morphology index and Ridley and Jopling classification

Type	Bacterial Index					Morphology Index				
	0	1	2	3	4	0	1	2	3	4
TT	31	0	0	0	0	31	0	0	0	0
BT	5	0	0	0	0	5	0	0	0	0
BL	0	1	3	4	3	0	1	5	4	1
LL	0	0	0	0	5	0	0	0	2	3
Total	36	1	3	4	8	36	1	5	6	4

### MLPA titer of untreated patients

All of the BL and LL types of untreated patients with BI and MI- positive cases were PCR positive.

Table 4 shows the results of MLPA test to determine the titer of antibodies in various types of untreated patients. The cut off value for MLPA positive is 32.

The titer of antibodies in 31 TT type of untreated patients was 17 cases negative, 11 cases 32, 2 cases 64 and 1 case 128, while among the 5 BT type of untreated patients 1 case negative, 2 cases 32 and 2 cases 64.

Among the 11 BL type of untreated patients, there were 2 cases 64, 6 cases 128, 2 cases 256 and 1 case 1024, while among the 5 LL type of untreated patients the results were 4 cases 128 and 1 case 246 (Table 4).

Table 3. Number of untreated leprosy patients according to PCR and MLPA test results

Type	PCR				MLPA			
	Positive *)	%	Negative	%	Positive **)	%	Negative	%
TT	7	22.58	24	77.42	14	45.16	17	54.84
BT	4	80.00	1	20.00	4	80.00	1	20.00
BL	11	100.00	0	0.00	11	100.00	0	0.00
LL	5	100.00	0	0.00	5	100.00	0	0.00
Total	27	51.92	25	48.08	34	65.38	18	35.62

\*) PCR positive after second round amplification and hybridization

\*\*) Cut off value for MPLA positive is 32

Table 4. Number of untreated leprosy patients according to the titer of MLPA test and the Ridley and Jopling classification

Type	Titer						
	Negative	32 *)	64	128	256	514	1024
TT	17	11	2	1	0	0	0
BT	1	2	2	0	0	0	0
BL	0	0	2	6	2	0	1
LL	0	0	0	4	1	0	0
Total	18	12	6	11	3	0	52

\*) Cut off value for MLPA positive is 32

### Monitoring of MDT treatment in paucibacillary leprosy patients by PCR

In order to further investigate the usefulness of PCR for monitoring of MDT treatment, the paucibacillary (TT and BT types) of untreated patients were followed up every 3 months during one year. As shown in Figure 1, 11 of 36 paucibacillary untreated patients were PCR positive before treatment (month 0) and 4 of those patients remained PCR positive until 3 months treat-

ment (month 3). Two of the 36 paucibacillary untreated patients were PCR positive until 6 months treatment (month 6), but all of those patients became PCR negative at 9 months treatment (month 9).

The number of PCR positive patients were significantly decreased after 6 months treatment compared with the number of PCR positive patients before treatment (Fischer exact test  $P < 0.05$ ).

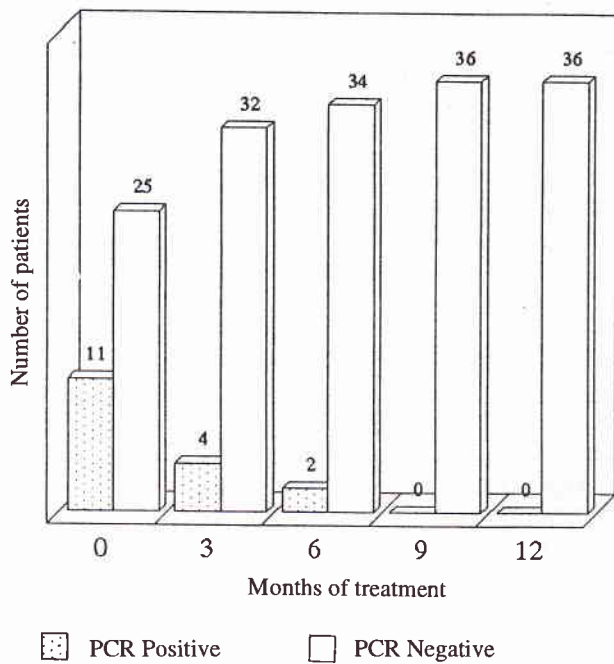


Figure 1. PCR results during MDT treatment in paucibacillary leprosy patients

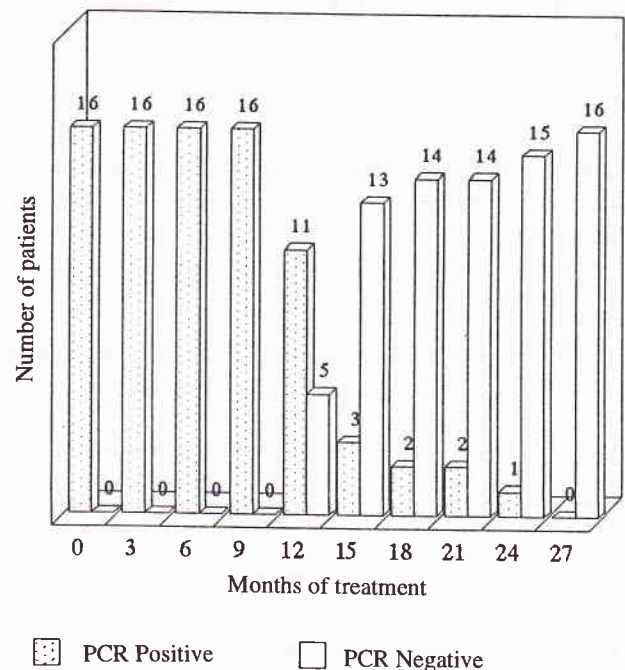


Figure 2. PCR results during MDT treatment in multibacillary leprosy patients.

### Monitoring of MDT treatment in multibacillary leprosy patients by PCR

As shown in Figure 2 to monitor the efficacy of MDT in multibacillary leprosy patients using PCR to detect the *M. leprae* DNA from nasal swab specimens, follow up of 16 untreated multibacillary (BL and LL) patients every 3 months during 27 months treatment was performed.

All of the untreated patients were persistent PCR positive until 9 months treatment (month 9) and 11 of those patients remained PCR positive until 12 months treatment.

The number of PCR positive patients were significantly decreased after 12 months treatment compared with the number of PCR positive patients before treatment (Fischer exact test  $P < 0.05$ ).

Thereafter the number of PCR positive patients gradually decreased until 24 months treatment and all of those patients became PCR negative at 27 months after treatment (month 27).

### Monitoring of MDT treatment in paucibacillary leprosy patients by MLPA test

The MLPA test is to determine the titer of anti-phenolic glycolipid-I (PGL-I) IgM antibodies. As shown in Figure 3, no MLPA positive patients were found at 12 months after treatment. The number of MLPA positive patients gradually decreased from 3 months treatment (month 3) until 9 months after treatment (month 9).

The number of MLPA positive patients were significantly decreased after 6 months treatment (month 6) compared with the number of MLPA positive patients before treatment (month 0) (Fischer exact test  $P < 0.05$ ).

### Monitoring of MDT treatment in multibacillary leprosy patients by MLPA test

Follow up of the multibacillary (BL and LL type) untreated patients were performed every 3 months during 27 months. As shown in Figure 4, all these patients were MLPA positive until 9 months treatment (month 9) and 1 patient became MLPA negative after 12 months (month 12) treatment, while 4 of those

patients were MLPA positive until 15 months treatment (month 15).

The number of MLPA positive patients were significantly decreased after 15 months treatment (month 15) compared with the number of MLPA positive patients before treatment (month 0) (Fischer exact test  $P < 0.05$ ).

Thereafter 3 of those patients remained MLPA positive until 27 months after treatment (month 27).

### DISCUSSION

Because *M. leprae* has not yielded to cultivation, the application of PCR for selective amplification of *M. leprae* DNA may be a breakthrough in leprosy research. Wide-scale application of this technology in routine diagnostic laboratories requires that the procedure should be simple and reproducible.<sup>16</sup> This need is addressed by PCR to monitor the efficacy of MDT from untreated leprosy patients described in this report.

The utility of the PCR using nasal swab specimens from untreated leprosy patients and follow up every 3 months during MDT treatment until 12 months for PB patients and 27 months for MB patients was demonstrated.

In the group of untreated PB patients only 11 of 26 patients were PCR positive, while all of those were bacterial negative by microscopic observation. PCR positivity in specimens in which no acid-fast bacilli can be detected by microscopy has been reported.<sup>17</sup> The number of PCR positive patients were significantly decreased after 6 months of treatment compared with the number of PCR positive patients before treatment (Fischer exact test  $P < 0.05$ ).

In the group of untreated MB patients all samples were PCR positive and persisted until 9 months of treatment and the number of PCR positive patients were significantly decreased after 12 months treatment compared with the number of PCR positive patients before treatment (Fischer exact test  $P < 0.05$ ).

Thus, the results reported here are quite encouraging for the potential use of PCR technology for the rapid detection and definitive identification of small numbers of *M. leprae* bacilli in nasal swab specimens during MDT treatment both in PB and MB patients.

The discovery of PGL-I and the development of a serodiagnostic technique employing PGL-related semisynthetic antigens provide us a new powerful tool for monitoring of chemotherapeutic effects in leprosy patients. With the introduction of reproducible serological tests it is hoped that relapse in leprosy patients, after discontinuing treatment, could be detected before damaging reactions occur and before the patients become infectious.

This study also included the MLPA test for monitoring of the efficacy of MDT from untreated leprosy patients.

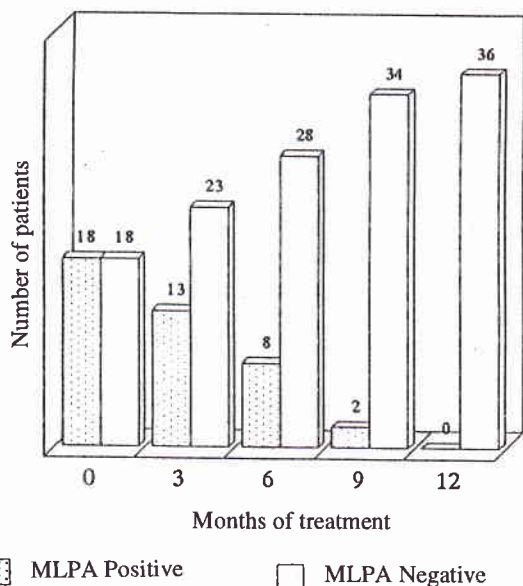


Figure 3. MLPA results during MDT treatment in paucibacillary leprosy patients.

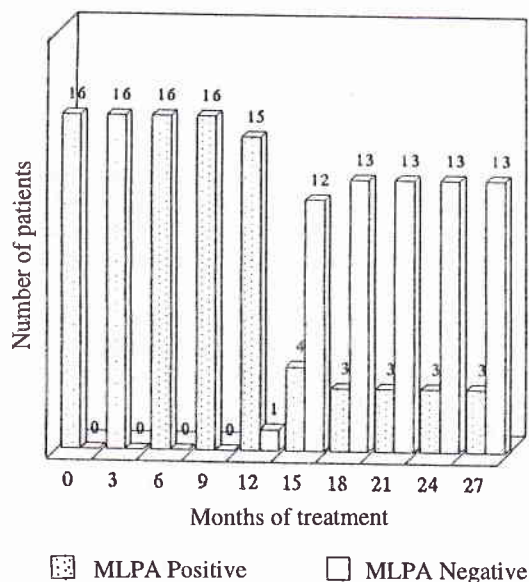


Figure 4. MLPA results during MDT treatment in multibacillary leprosy patients.

No MLPA positive in PB patients was found at 12 months after treatment. The number of MLPA positive patients gradually decreased from 3 months treatment (month 3) until 9 months after treatment (month 9).

The number of MLPA positive in PB patients were significantly decreased after 6 months treatment (month 6) compared with the number of MLPA positive patients before treatment (month 0) (Fischer exact test  $P < 0.05$ ).

Above-mentioned data suggest that the levels of antibody in PB patients before treatment were low and only 1 of those patients had a MLPA titer of 128 (table 4).

Follow up of the MB (BL and LL types) untreated patients every 3 months during 27 months was performed. All of those patients were MLPA positive until 9 months treatment (month 9) and 1 patient became MLPA negative after 12 months (month 12) treatment, while 4 of those patients were MLPA positive until 15 months treatment (month 15).

The number of MLPA positive in MB patients were significantly decreased after 15 months treatment (month 15) compared with the number of MLPA positive patients before treatment (month 0) (Fischer exact test  $P < 0.05$ ).

Thereafter 3 of those patients remained MLPA positive until 27 months after treatment (month 27).

In contrast, a rise in the level of antibody after the start of treatment in relapse patients has been observed.<sup>18</sup> In this study no relapse patients were found during treatment.

In conclusion, the PCR and MLPA test described here are suitable methods for monitoring the efficacy of MDT in untreated leprosy patients and these methods were limited for untreated leprosy patients without relapse during treatment.

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