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Effects of Fixation on Polymerase Chain Reaction Detection of *Mycobacterium leprae*

PAOLO FIALLO,[1] DIANA L. WILLIAMS,[2] GERTRUDE P. CHAN,[3] AND THOMAS P. GILLIS[2]*

CIR LEP, University of Genoa, Genoa, Italy[1]; Gillis W. Long Hansen's Disease Center, P.O. Box 25072, Baton Rouge, Louisiana 70894[2]; and Research Institute of Tropical Medicine, Alabang, Metro Manila, Philippines[3]

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The effects of standard fixatives (10% neutral buffered formalin, ethanol and mercury based) on the detection of *Mycobacterium leprae* DNA by the polymerase chain reaction (PCR) were studied. Mercury-based fixatives (Zenker's and Carnoy-Lebrun's fluids) strongly inhibited PCR amplification of *M. leprae* DNA. Ten percent neutral buffered formalin was inhibitory, but significant inhibition was observed only when fixation times exceeded 24 h. Ethanol-based fixatives provided the best medium for holding specimens for subsequent PCR with both free bacilli and skin biopsy specimens containing *M. leprae*. The *M. leprae*-specific, 360-bp region of the 18-kDa protein gene could be amplified from paraffin-embedded sections of formalin-fixed skin biopsy specimens from patients with either multibacillary or paucibacillary infections when proper fixation conditions were used. Results of the study demonstrate that tissues properly fixed with two standard fixatives (10% neutral buffered formalin and 50 or 70% ethanol) can be analyzed by PCR for the presence of *M. leprae* with no loss in specificity and only minimal diminution in sensitivity compared with the specificities and sensitivities obtained by use of freshly prepared, unfixed specimens.

The use of polymerase chain reaction (PCR) amplification has become an increasingly common means of detecting pathogenic microorganisms in biological specimens (9, 10, 15). Recently, this method has been used to detect extremely low numbers of *Mycobacterium leprae* in fresh, unfixed human skin biopsy specimens, providing a powerful direct and unequivocal test for *M. leprae* infection (6, 16). Most routine procedures for diagnosing and monitoring leprosy include, among other tests, histopathologic analysis of skin lesions to determine disease category. Since routine biopsy specimens for histopathologic analysis are prepared in standard fixatives, fresh tissues are generally not available for PCR analysis. Adaptation of PCR for detecting *M. leprae* in fixed tissues would give clinicians the option of testing biopsy specimens for the presence of *M. leprae*, potentially aiding in the diagnosis of difficult cases. In addition, PCR could be used to provide confirmatory data on *M. leprae* infection in fixed tissues obtained from subjects enrolled in large-scale field studies, often undertaken in leprosy vaccine trials and for epidemiologic purposes.

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 (2, 5, 14). *M. leprae* DNA has also been detected in extracts from paraffin-embedded skin (1); however, a thorough examination of fixation procedures for preserving *M. leprae* DNA for PCR has not been evaluated. In the present study we analyzed the effects of different fixatives and fixation times on PCR detection of *M. leprae* DNA and provide recommendations for fixing skin biopsy specimens to be used in PCR tests for detecting *M. leprae* DNA.

MATERIALS AND METHODS

Fixation of *M. leprae*. *M. leprae* was purified from four hind footpads of athymic nude mice and then pooled as described previously (4). A stock suspension of *M. leprae* was prepared and counted by the method of Shepard (13), and aliquots (10^6 bacilli) were held at 25°C in 0.5 ml of the following fixatives prepared as described previously (12): Zenker's fluid, Carnoy-Lebrun's fluid (Lillie), 10% neutral buffered formalin, and 50 and 70% ethanol in water. Lyophilized *M. leprae* was prepared by freeze-drying 10^6 bacilli suspended in 0.5 ml of Hanks balanced salt solution (HBSS). Autoclaved suspensions of *M. leprae* were prepared and used as a negative control.

Following fixation, HBSS (5 ml) was added to each sample and the bacterial suspensions were held for 30 min with gentle mixing on a rocking platform. The bacteria were recovered by centrifugation at 10,000 × *g* for 15 min, and the washing procedure was repeated once. Washed *M. leprae* (3×10^5) was resuspended in 100 μl of sterile deionized water. Each sample was subjected to three cycles of freezing and thawing (−160°C for 5 min and 65°C for 1 min) and heated at 95°C for 10 min, and then 10 μl was added to the PCR reagents for amplification as described below.

Fixation of human skin biopsy specimens. Diagnosis and classification of leprosy was based on clinical symptoms and histopathology of skin biopsy specimens by using the classification of Ridley and Jopling (11). Skin biopsy specimens were obtained in the Philippines from patients with leprosy who had received no previous antileprosy drug treatment. Each skin specimen was obtained aseptically by using a 4-mm disposable punch biopsy following local anesthesia. The biopsy specimens were placed in individual vials fitted with perforated closures for lyophilization and were freeze-dried in individual lyophilization flasks. Dried samples were individually capped and shipped to the Gillis W. Long Hansen's Disease Center. Lyophilization has previously been shown to preserve skin biopsy specimens for PCR analysis with no demonstrable change in reactivity compared with that of fresh biopsy material (17). At the time of testing, individual biopsy specimens were rehydrated in 2 ml of HBSS at 4°C for 2 h. Rehydrated biopsy specimens (approximately 50 mg) were placed in 10 ml of 10% neutral formalin, 50% ethanol, or 70% ethanol and were held for 2

* Corresponding author.

weeks at 25°C. Three separate biopsy specimens were treated with each fixative. Following fixation, biopsy specimens were washed in 50 ml of HBSS with gentle rocking for 30 min. The HBSS was decanted, and the tissue samples were washed again and then minced with scissors and homogenized with glass beads in 2 ml of HBSS by using a Mickle homogenizer (16). *M. leprae* (3×10^5) was resuspended in 100 μl of sterile deionized water and subjected to freezing and thawing as described above. Ten microliters was then added to PCR reagents and amplified for 45 cycles.

Paraffin-embedded skin biopsy specimens. Skin biopsy specimens from three patients with leprosy (two lepromatous and one borderline tuberculoid) and three uninfected individuals were fixed in 10% neutral formalin for 48 h and embedded in paraffin by using an automated tissue processor and standard paraffin-embedding procedures. From each embedded tissue sample, six 10-μm sections were cut, collected, and placed in a microcentrifuge tube. Samples were deparaffinized twice by adding 1 ml of xylene and holding the samples at 25°C for 30 min with gentle rocking. Samples were centrifuged at 10,000 × *g* for 15 min, and the supernatant was decanted. Residual xylene was removed from the samples with two washes of 0.5 ml each of 100% ethanol and centrifugation at 10,000 × *g* for 15 min. Pellets were vacuum dried at 80°C for 15 min to remove residual ethanol, resuspended in 50 μl of lysis buffer (100 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 250 μg of proteinase K), and incubated at 60°C for 2 h. Samples were heated at 95°C for 10 min and cooled to 25°C, and 10 μl was added to PCR reagents and amplified as described below. Also, paraffin-embedded sections were stained with hematoxylin-eosin or carbol-fuchsin to confirm the diagnosis of leprosy and to determine the bacterial index for each biopsy specimen.

PCR amplification of *M. leprae*-specific DNA sequence. Conditions for the *M. leprae*-specific PCR test were performed as described previously (16). Ten-microliter samples were added to 90 μl of PCR reagents which contained the 360-bp *M. leprae*-specific primer set and were amplified for 45 cycles. The PCR products were analyzed by slot blot hybridization by using a 212-bp DNA probe which binds to an internal segment of the 360-bp region of the *M. leprae* 18-kDa protein gene (16).

Slot blot hybridization. PCR products were analyzed by slot blot hybridization (16) with the following modifications. Aliquots (85 μl) of each PCR product were denatured in 415 μl of 0.4 N NaOH–0.001 mM EDTA for 10 min at 25°C and applied to individual wells of a BioDot SF microfiltration apparatus (Bio-Rad Laboratories, Richmond, Calif.) containing an Immobilon-P membrane (Millipore, Bedford, Mass.) preequilibrated in 0.2 M NaOH. DNA was immobilized onto the membrane by vacuum filtration; this was followed by air drying and baking at 80°C for 30 min under vacuum. Membranes were treated in 15 ml of hybridization solution (Sigma Chemical Co., St. Louis, Mo.) containing 50% formamide at 65°C and were then hybridized in 4 ml of the same buffer containing a 212-bp, ^{32}P-labeled probe (5×10^6 cpm/ml) for 1 h at 65°C. Membranes were washed briefly in 200 ml of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate and were then washed again for 1 h at 65°C, air dried, and exposed to X-ray film for 18 h at −70°C. Quantitative data on each sample were determined by cutting out the regions of the slot blots containing the DNA target probe hybrid and placing them into vials containing 10 ml of H_2O. Samples were counted by using an LS6000IC scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Samples were run in triplicate, and differences between variables were assessed by a two-tailed unpaired *t* test.

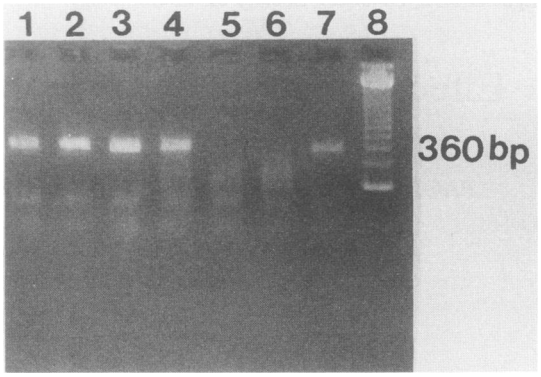

FIG. 1. Agarose gel electrophoresis analysis of PCR amplification products from 3×10^5 *M. leprae* after fixation for 24 h at 25°C under the following conditions: 10% neutral formalin (lane 1), lyophilized (lane 2), 50% ethanol (lane 3), 70% ethanol (lane 4), Carnoy-Lebrun's fluid (lane 5), and Zenker's fluid (lane 6). Lane 7, the 360-bp PCR product generated from 100 fg of purified *M. leprae* DNA; lane 8, 123-bp ladder for size reference of DNA fragments.

RESULTS

Three standard types of fixatives, mercury-based (Zenker's and Carnoy-Lebrun's fluids), 10% buffered formalin, and alcohol-based fixatives (50 and 70% ethanol in water), were tested for their effects on the *M. leprae*-specific PCR. Only *M. leprae* DNA extracted from ethanol-fixed (50 or 70%) or neutral formalin-fixed bacteria was capable of supporting primer-directed amplification of the 360-bp *M. leprae*-specific PCR product (Fig. 1). On the basis of these results, we focused our studies on fixation of *M. leprae* and *M. leprae*-infected tissues in neutral buffered formalin and 50 and 70% ethanol.

Quantitative slot blot data were highly reproducible, as judged by the coefficient of variation for replicate samples in unfixed samples (Table 1) or samples fixed in ethanol (Table 1) or formalin (Tables 1 and 2) for 48 h or less. In no instance did the coefficient of variation exceed 14%, unless the sample was autoclaved or had been held in neutral buffered formalin for more than 48 h.

Table 1 summarizes the results of the effect of prolonged fixation (2 weeks) of *M. leprae* on PCR reactivity. The PCR reactivities of formalin-fixed or ethanol-fixed *M. leprae* (murine) in suspension were compared with that of lyophilized *M. leprae*, which has previously been shown to yield PCR reactivity comparable to that of fresh, unfixed *M. leprae* (17). Fixation in 50 and 70% ethanol resulted in only minimal inhibition of the PCR reactivity of *M. leprae* DNA. The greatest effect seen was a 20% reduction in reactivity when murine-derived *M. leprae* was fixed in 70% ethanol. In contrast, ethanol fixation of *M. leprae*-infected human skin showed no diminution of PCR reactivity compared with those of the matched lyophilized specimens. By comparison, fixation of nude mouse-derived *M. leprae* and *M. leprae* fixed in situ (human skin biopsy specimens) in 10% neutral buffered formalin for 2 weeks reduced the PCR reactivity of *M. leprae* DNA by 85 and 79%, respectively. While a significant reduction in PCR reactivity was observed following formalin fixation of *M. leprae* in either suspension or tissue, the PCR reactivities of these samples (2,276 and 2,732

TABLE 1. Effects of fixation on PCR reactivity of *M. leprae* from nude mouse footpads or human skin[a]

Sample and fixation condition	PCR slot blot hybridization (cpm [mean ± SD])[b]
M. leprae (murine)	
Lyophilization	15,639 ± 640
10% Formalin (pH 7.0)	2,276 ± 1,196
50% Ethanol	14,969 ± 1,376
70% Ethanol	12,494 ± 566
Autoclaved	203 ± 59
M. leprae (human)	
Lyophilization	12,866 ± 450
10% Formalin (pH 7.0)	2,732 ± 2,459
50% Ethanol	12,904 ± 1,529
70% Ethanol	13,361 ± 1,510

[a] *M. leprae* (10^6) from nude mouse footpads was held in various fixatives for 2 weeks and washed free of fixatives. An aliquot (3×10^5 *M. leprae*) was added to 100 μl of deionized water and disrupted by three cycles of freezing and thawing, and 10 μl of this suspension was analyzed by PCR. Human skin biopsy specimens from untreated patients with lepromatous leprosy were held in various fixatives for 2 weeks. Biopsy specimens were washed free of fixatives and homogenized; and 3×10^5 *M. leprae* was added to 100 μl of deionized water and disrupted by three cycles of freezing and thawing; 10 μl of this suspension was analyzed by PCR.

[b] Values are mean counts per minute from triplicate samples for each fixative. In the case of human biopsy specimens, the mean and standard deviation was calculated from results on three separate biopsy specimens for each fixative.

cpm, respectively) were approximately 10 times greater than that of the autoclaved negative control (203 cpm).

To determine the temporal nature of the effect of formalin fixation on PCR, equal numbers (10^6) of nude mouse-derived *M. leprae* were fixed in 10% buffered formalin for 1, 2, 3, 4, 7, and 14 days. *M. leprae* DNA was extracted from samples at each time point, and the resultant PCR reactivity was compared with that of DNA derived from the same number of unfixed *M. leprae*. A minor reduction in PCR reactivity was observed as early as 24 h postfixation (Table 2). A statistically significant difference between the PCR reactivities of formalin-fixed and unfixed tissues was reached at 48 h of fixation ($P = 0.026$). PCR reactivity continued to decrease at each time point, reaching approximately 50% of prefixation values at 3 days.

Using standard fixation and embedding procedures for histopathologic analysis of skin biopsy specimens, but limiting the fixation period in formalin to 48 h, we tested paraffin-embedded sections of biopsy specimens from one

TABLE 2. Temporal effects of fixation with 10% neutral buffered formalin on PCR reactivity of *M. leprae* derived from nude mice[a]

Fixation time (days)	PCR slot blot hybridization (cpm [mean ± SD])[b]
0	15,327 ± 792
1	13,616 ± 1,622
2	11,788 ± 1,597
3	6,946 ± 1,867
4	4,987 ± 1,089
7	5,323 ± 2,095
14	2,276 ± 1,196

[a] *M. leprae* (10^6) was held in neutral buffered formalin for various lengths of time. The bacteria were processed for PCR as described in footnote [a] of Table 1.

[b] Values are mean counts per minute from triplicate ($n = 3$) samples for each time period.

TABLE 3. Detection by PCR of *M. leprae* from formalin-fixed, paraffin-embedded tissues of patients with leprosy

Sample[a]	Bacteriologic index	PCR slot blot hybridization cpm (SD)
LL	5+	7,648
LL	5+	5,812
BT	0	1,441
Normal skin	0	113 (28)
PCR buffer	–	52 (18)
100 fg of *M. leprae* DNA	–	1,763 (549)

[a] Six 10-μm sections were cut from each specimen block and treated as described in the text. LL, lepromatous leprosy; BT, borderline tuberculoid leprosy.

borderline and two lepromatous patients with leprosy for the presence of *M. leprae* DNA by PCR. The biopsy specimens from the lepromatous patients had bacteriologic indices equal to 5+ and produced strong PCR reactivities (Table 3). The biopsy specimen from the patient with borderline tuberculoid leprosy showed no bacilli in the tissues but produced a PCR signal 10 times that shown with normal skin. The slot blot hybridization value for the PCR product from the patient with borderline tuberculoid leprosy (1,441 cpm) fell between the value obtained for normal skin (113 cpm) and 100 fg of *M. leprae* DNA (1763 cpm), indicating that the theoretical amount of DNA present in this biopsy specimen was between 1 and 30 *M. leprae* genome equivalents.

DISCUSSION

In order to determine the effects of routine histopathologic fixatives on PCR analysis of *M. leprae* and *M. leprae*-infected tissues, we developed a simple means of quantifying hybridization of the PCR product with a radiolabeled DNA probe in slot blot hybridizations. Quantitation of the PCR product permitted the estimation of the theoretical concentration of *M. leprae* present in a given specimen; this was done by comparing the amount of PCR product generated from a known quantity of purified *M. leprae* DNA with that obtained from a given specimen. Quantitation of PCR results and the relationship of the absolute numbers of *M. leprae* in samples is an important first step toward using PCR as a tool for studying the bacteriologic aspects of the pathogenesis of leprosy, the spread of infection within a given population, and definition of the bacteriological relationships between patients suffering from reversal reactions and those presenting with authentic relapsed disease. While quantitative PCR may help define disease-related research problems, the use of such a sophisticated and expensive test as a tool for routine leprosy control programs is unlikely.

Our results rule out the use of Carnoy-Lebrun's and Zenker's (mercuric chloride) fluids as fixatives for subsequent PCR of *M. leprae* DNA. Similar conclusions on mercuric chloride were reached by deWit et al. (1), who showed a significant reduction in sensitivity and specificity of PCR for *M. leprae* DNA with this fixative. Carnoy-Lebrun's fluid, on the other hand, is recommended for fixation of tissues for optimal staining of glycogen and RNA. Unfortunately, both fixatives have the potential for chemical modification of DNA, resulting in degradation of the DNA or excessive depurination of the DNA because of the acidic nature of the fixatives (3, 7).

Neutral buffered formalin fixation of *M. leprae* resulted in diminished PCR reactivity at all fixation times tested. Precipitous losses in PCR reactivity were observed at 48 h and

beyond, with fixation times of 4 and 7 days showing 65 to 70% loss of reactivity. These results underscore earlier observations of the deleterious effects of formalin on DNA (2, 8) and suggest that formalin fixation of specimens to be used for both histopathologic analysis as well as PCR for *M. leprae* DNA should be held to between 24 and 48 h.

Following a 48-h fixation in neutral buffered formalin and paraffin embedding, we were able to demonstrate the presence of *M. leprae* DNA by PCR in skin biopsy specimens from two patients with lepromatous leprosy and one patient with borderline tuberculoid leprosy. For each embedded skin specimen, the PCR test was performed on DNA extracted from six 10-μm sections. In the case of the patient with borderline tuberculoid leprosy, no bacilli were seen on the acid-fast stain of a serial section. While the diseases in these three patients do not represent the entire spectrum of the disease, they do represent two important categories, namely, multibacillary and paucibacillary leprosy.

In a previous study on fixed and embedded skin biopsy specimens, deWit et al. (1) showed that with short-term fixation (2 h) in neutral buffered formalin, 11 of 12 untreated patients with a positive bacterial index tested positive by PCR. In contrast, only 11 of 18 or 61% of the bacterial index-negative, untreated patients tested positive by PCR. Taken together, our results and those of deWit et al. (1) suggest that neutral buffered formalin is a suitable fixative for detecting *M. leprae* in paraffin-embedded sections of skin biopsy specimens, provided that the fixation time is held to less than 48 h. This period of time is not incompatible with the abilities of most routine histology laboratories and should be strictly adhered to when anticipating the use of PCR as a confirmatory test for the presence of *M. leprae* in skin biopsy specimens.

Although ethanol was a superior fixative for PCR analysis in our study, histologic analysis of ethanol-fixed tissues is not recommended because of excessive shrinkage that results in poor reproduction of tissue architecture. Therefore, if a single biopsy specimen is to be taken for diagnostic purposes, our data suggest that skin biopsy specimens should be fixed in neutral buffered formalin for 24 to 48 h, embedded in paraffin, and sectioned for histopathologic and PCR analyses, if necessary. If, on the other hand, PCR analysis is anticipated, we suggest that each biopsy specimen be split into two pieces. One half would be fixed in neutral buffered formalin for routine histopathology, while the other half would be stored in 50 or 70% ethanol for subsequent PCR analysis. Skin biopsy specimens held for 6 weeks in 70% ethanol showed no reduction in PCR reactivity compared with the reactivities of matched lyophilized specimens (data not shown). An advantage that is gained by using ethanol fixation rests in the ability to extract DNA from the entire biopsy specimen, which allows for a more thorough analysis of the DNA contained within the biopsy specimen. Although we did not compare the efficiency of PCR detection of *M. leprae* DNA from formalin-fixed, paraffin-embedded tissue sections with extraction of the whole biopsy specimen following ethanol fixation, we predict that analysis of the entire biopsy specimen would be superior, particularly when analyzing biopsy specimens from patients with paucibacillary leprosy.

In summary, the development of a sensitive and specific DNA amplification test for *M. leprae* has provided a new way of detecting and potentially tracing this noncultivable human pathogen. Unfixed, and now fixed, skin biopsy specimens can be screened for the presence of *M. leprae*, creating an opportunity for epidemiologic studies on newly acquired or archived samples fixed and embedded in paraffin under standard conditions.

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