INTERNATIONAL JOURNAL OF LEPROSY Volume 60, Number 2
Printed in the U.S.A.

Polymerase Chain Reaction Amplifying DNA Coding for Species-specific rRNA of *Mycobacterium leprae*[1]

Stefaan R. Pattyn, Dominique Ursi, Margareta Ieven,
Veerle Raes, and Pierre Jamet[2]

Cox, *et al.* ([4]) recently sequenced the DNA coding for the variable, species-specific fragment of rRNA of *Mycobacterium leprae,* thus providing a specific tool for the detection of *M. leprae* with the polymerase chain reaction (PCR). We have tested the sensitivity of this application of the PCR on mouse foot pad harvests (MFPH) and human biopsies maintained in different conditions.

MATERIALS AND METHODS

MFPH, in phosphate-buffered saline (PBS) with 0.5% bovine albumin fraction V, of several strains of *M. leprae* maintained in the laboratory were stored at −20°C for several weeks before being processed for PCR.

A group of human skin biopsies from active multibacillary (MB) leprosy patients—never treated before or relapsing after treatment—taken in Bamako, Mali, were put into dry tubes and transported on wet ice to Antwerp, Belgium, where they arrived 30 hr later. Suspensions were prepared and kept at −20°C until further processing. Biopsies were eventually divided into two fragments and processed (see Results).

The bacteria were counted by standard procedures ([12]). The limit for bacterial counts was 10^4 ml. Suspensions with $< 10^4$/ml bacilli, but showing acid-fast bacilli (AFB) in a thick smear, are indicated as $< 10^4$/ml thick. Smear negative suspensions are presented as "negative."

The instruments used to handle tissues possibly infected with *M. leprae* were sterilized by boiling in water for 20 min, scrubbed with soap under tap water, cleaned with 1 N HCl, and boiled in a second waterbath for 15 min before the next use.

For the PCR, tissue suspensions were either submitted to five cycles of freezing and boiling (FB) or treated with proteinase K (PK) followed by DNA extraction.

In the FB procedure, samples in 100-μl volumes were immersed alternately for 2 min in liquid nitrogen and 2 min in a boiling waterbath, 10 μl being used in the PCR.

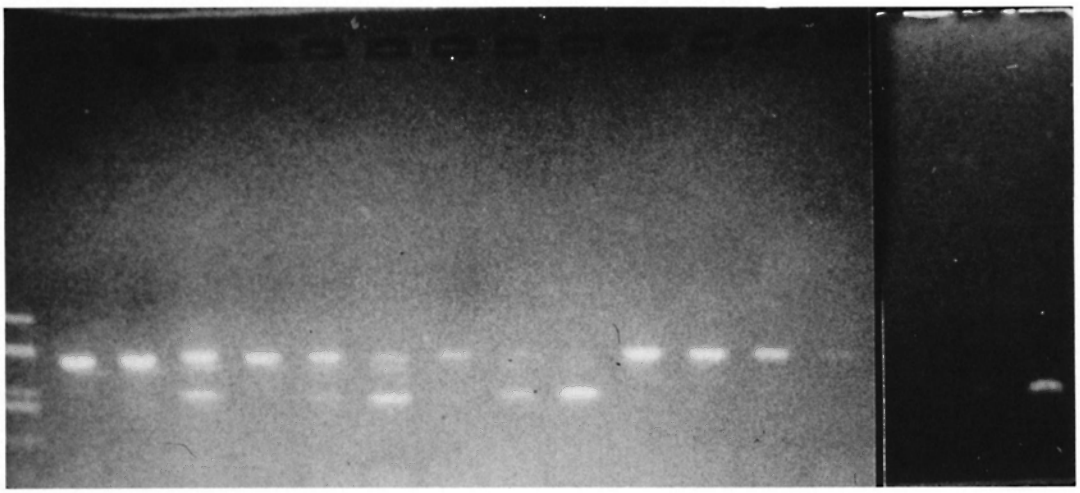
For the PK procedure, a 100-μl sample was incubated for 3 hr at 37°C with 40 μg of proteinase K in 0.01 M Tris HCl, pH 7.8, 5 mM EDTA, 0.5% (w/v) sodium dodecylsulfate. After two successive extractions with phenol : chloroform : isoamyl alcohol (25/24/1) followed by two extractions with chloroform : isoamyl alcohol (24/1), nucleic acids were precipitated twice with ethanol, washed with 80% (v/v) ethanol, dried, and dissolved in 6 μl of distilled water; 5 μl was used in the PCR and 1 μl for making 10-fold dilutions.

PCR reactions were performed in 50-μl volumes containing 10 μl of the FB sample or 5 μl of the PK-treated sample; 10 pmoles of each primer; 0.2 mM each of dATP, dCTP, dGPT, dTTP; 1 unit of Taq polymerase (The Cetus Corporation, Emoryville, California, U.S.A.); 50 mM of KCl; 10 mM Tris HCl, pH 8.3; 2.5 mM $MgCl_2$ and 100 μg/ml of bovine serum albumin. In every PCR run external negative (distilled water) and positive controls (a *M. leprae*-positive mouse foot pad harvest) were included. The amplification (Hybaid; Hybaid Ltd, Teddington, Middlesex, U.K.) cycle was as follows: denaturation for 2 min at 94°C, hybridization at 55°C for 2 min,

[1] Received for publication on 28 October 1991; accepted for publication in revised form on 10 February 1992.

[2] S. R. Pattyn, M.D., Professor, Medical Microbiology, Institute of Tropical Medicine and University of Antwerp, Antwerp, Belgium. V. Raes, Laboratory Technician, Institute of Tropical Medicine, Antwerp, Belgium. D. Ursi, Sci.D.; M. Ieven, Ph.D., University Hospital, Antwerp, Belgium. P. Jamet, M.D., Institut Marchoux, Bamako, Mali.

Reprint requests to Prof. Pattyn, Institute for Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium.



THE FIGURE. Effect of the IC on the sensitivity of the PCR for an *M. leprae* suspension from a freeze-boiled human biopsy. *M. leprae* 3.6×10^3: lanes A–C and J; Ml 3.6×10^2: lanes D–F and K; Ml 36: lanes G–I and L; Ml 3.6: lanes M and N–P, with an IC preparation diluted 10^{-3} added in lanes A, D, N; IC diluted 10^{-2} in lanes B, E, H, O; IC diluted 10^{-1} in lanes C, F, I and P (no IC added in lanes J–M); unmarked lane: HaeIII digest of plasmid PUC19.

extension at 72°C for 2 min (35 cycles), and a final extension step at 72°C for 10 min.

The primers used were those described by Cox, *et al.* ([4]) except that the sequence of primer cv 3′ was based on the 16S rRNA sequence of *M. leprae* instead of *M. bovis*.

primer 1:
5′ AAACCCAGACCTTCGTCGATG 3′
primer 2:
5′ CGGAAAGGTCTCTAAAAAATCTT 3′

The aplicon produced consists of 405 base pairs (bp).

Ten μl of the reaction mixture was electrophoresed in 1.5% agarose gels at 5 V/cm in TBE buffer (0.09 M Tris-borate, 0.002 M EDTA), and the reaction products were visualized by ethidium bromide fluorescence.

Internal control (IC). An *M. leprae* amplicon obtained from a suspension of armadillo liver, inoculated with *M. leprae* from human origin, was eluted from an agarose gel and ligated into the Eco RV site of plasmid pBR322, cleaving the tetracycline R gene. The ligation mixtures were transformed into *Escherichia coli* JM 83 and transformants selected as ampicillin R, tetracycline S colonies. The cloned amplicons, containing unique ApaI and XhI sites, were cut with these enzymes and purified by agarose gel electrophoresis. The cut ends were made blunt with T4 DNA polymerase followed by ligation. About 10% of the transformants contained the right construct with the shortened amplicon of 268 bp. Plasmids were extracted from a positive clone and linearized by treatment with ECO RI, for which the plasmids have a unique site. This improves the detection limit 1000-fold ([13]).

RESULTS

Influence of the presence of IC on the sensitivity of the reaction for detection of *M. leprae*. Since the presence of the internal control (IC) might affect the sensitivity of the assay due to competition between two templates for the primers, a titration experiment was made in which dilutions of a FB suspension of *M. leprae* were mixed with dilutions of an IC preparation. The Figure shows the results. With high amounts of *M. leprae* DNA there is inhibition of the IC (lanes A, B, D, G). With the lowest amount of *M. leprae* DNA tested, there is inhibition of the target DNA (lane I). Therefore in all further experiments a dilution of 10^{-2} of this preparation of IC was used.

PCR on mouse foot pad harvests. As shown in Table 1, three MFPH negative for *M. leprae* were negative in the PCR, both after FB and PK. The five MFPH with $< 10^4$ *M. leprae* were positive after FB; three

TABLE 1. *Comparison of bacterial counts and PCR titrations on mouse foot pad harvests treated by freeze-boiling (FB) or proteinase K (PK).*

Harvest no.	Bacterial count/ml	PCR titer after FB	Lowest no. bacilli detected	PCR titer after PK	Lowest no. bacilli detected
22	N[a]	N		N	
32	N	N, N[b]		N	
39	N	N		N	
2	$< 10^4$	UD[c]		UD	
6	$< 10^4$	10^{-1}		UD	
8	$< 10^4$	10^{-2}		UD	
9	$< 10^4$	10^{-1}, 10^{-1}		NT[d]	
35	$< 10^4$	10^{-2}, 10^{-3}		NT	
1	2.1×10^4	10^{-1}, 10^{-1}	21, 21	NT	
14	2.1×10^4	10^{-1}, 10^{-2}	21, 2.1	NT	
96	5.9×10^4	10^{-1}, 10^{-1}	59, 59	NT	
47	9.6×10^4	10^{-2}	9.6	NT	
4	1.2×10^5	10^{-2}	12	UD	10^4
98	1.5×10^5	10^{-3}	1.5	NT	
7	2.0×10^5	10^{-2}	20	UD	1.6×10^4
13	2.1×10^5	10^{-1}	210	10^{-2}	175
91	6.0×10^5	10^{-2}	60	NT	
40	7.3×10^5	10^{-3}	7.3	10^{-2}	583
31	8.7×10^5	10^{-3}	8.7	NT	
90	1.0×10^6	10^{-1}	10^3	NT	
49	1.3×10^6	10^{-1}, 10^{-1}	1.3×10^3	10^{-2}	10^3
30	2.2×10^6	10^{-1}, 10^{-1}, UD	2.2×10^3	10^{-2}	1.8×10^3

[a] N = Negative.
[b] Results from repeated tests.
[c] UD = Undiluted.
[d] NT = Not tested.

of these tested after PK were also positive. All positive PCR results after PK were on undiluted samples only; whereas after FB, all but one sample gave a positive result in dilutions of 10^{-1}, 10^{-2} and even 10^{-3}. The next series of MFPH in Table 1, with countable numbers of *M. leprae,* allow the calculation of the lower limit of detectability of this PCR. For the FB procedure, this varies from 1.5 to 2.2×10^3 bacilli. However, all of the lower sensitivities were observed in harvests with at least 10^6 *M. leprae* per ml. The mean value for the 14 lowest figures is 36.5 bacilli detected. The lowest limit of detectability after PK is 210 and goes up to 10^3 and 10^4. Clearly, the PK procedure is much less sensitive than FB.

Some PCRs were repeated involving FB of an aliquot of the original suspension. As shown in Table 1 there may be a variation of one dilution between two successive tests. The use of the IC did not reveal much interference with inhibitors. In some cases however, the control signal obtained with the undiluted specimen was less intense than with the diluted specimens.

Influence of fixatives or Dubos OAA broth on PCR results on MFPH. These influences were studied in two experiments on MFPH from two strains of *M. leprae.* From each group of harvests, one MFPH (experiment 2) or two MFPH (experiment 1) were kept at −20°C until tested in PCR; while others were kept for 2 weeks at room temperature, either in formol 10%, alcohol 70%, Lowy fixative, or Dubos OAA broth. All suspensions were PCR tested after FB. Table 2 shows the results.

In experiment 1, the frozen samples were positive at dilutions of 10^{-1} and 10^{-2}, respectively. In both cases the signal of the IC was weaker in the undiluted sample, pointing to some inhibition in the test.

Fixation of MFPH in both formol 10% and Lowy fixative increased very considerably the inhibitory effect in the reactions. This was not the case after preservation in alcohol, but the *M. leprae* amplicon failed nevertheless.

In experiment 2, formol 10% enhanced inhibition of the reaction less drastically than in the first experiment; Dubos OAA broth

provoked a considerable inhibitory effect although the *M. leprae* amplicon was produced in the undiluted sample. Alcohol enhanced the inhibition only in the undiluted sample, but once more the *M. leprae* amplicon failed.

The conclusion from these experiments is that from the different preservation methods tested, alcohol 70% has the least effect on the inhibitors, but still reduces the sensitivity of the test.

PCR results on fresh human biopsies. Fresh human biopsies were divided into two halves; one was examined as a fresh specimen (after some time at −20°C) while the other was transferred into either alcohol (70%) or Dubos OAA broth and kept at room temperature for 1 week. They were then minced and treated by FB or PK for PCR. The results appear in Table 3. Once more there may be a difference of one dilution upon repeated testing.

The results of the first five fresh biopsies show that after FB the lower limit of detectability varies between 20 (L980-) and 6300 bacilli (L982-). The result of 6.3×10^3 obtained once on sample L982-FB should be regarded as an outlier and could be disregarded in the interpretation of the results, the more so since in that particular test inhibitors were detected up to a dilution 10^{-3}. On this basis, the mean lower limit of sensitivity for the FB procedure is 122.2 bacilli compared with 194.6 bacilli after the PK treatment, a nonsignificant difference. However, if the results on sample L982 are not considered, the results after FB are much more uniform and vary between 20 and 70 bacilli, with a mean of 37.6 bacilli, and the FB procedure is 5 times more sensitive. On this basis, the number of *M. leprae* in biopsies L983, L994, L999 (with counts below 10^4 bacilli/ml) was estimated at 3.1×10^4 ml, not significantly different from 10^4, with L1001 as an unexplained exception since it should have contained 3.1×10^5 *M. leprae* per ml.

Influence of fixatives or Dubos OAA broth on PCR on human biopsies. After preservation in alcohol, the sensitivity in the case of sample L981 is 100 to 1000 times lower than on fresh material. A much smaller difference was noted for L982, but for this particular biopsy the sensitivity for the fresh specimen was very low. The sensitivity of the PCR was also considerably lower after preservation of specimens L997 and L1003 in Dubos OAA broth.

TABLE 2. *PCR results on mouse foot pad harvests kept at −20°C in PBS or at room temperature in Dubos OAA broth, formol 10%, alcohol 70% or Lowy fixative (all tests done after FB).*

		UD[a]	10^{-1}	10^{-2}	10^{-3}
	Experiment 1				
−20° PBS	MI[b]	+	±[d]	−	−
	IC[c]	±	+	+	+
−20° PBS	MI	+	+	±	−
	IC	±	+	+	+
RT[e], formol 10%	MI	−	−	−	−
	IC	−	−	+	+
RT, alcohol 70%	MI	−	−	−	
	IC	+	+	+	
Rt, Lowy	MI	−	−	−	−
	IC	−	−	−	+
	Experiment 2				
−20°C PBS	MI	+	+	−	
	IC	±	+	+	
RT, formol 10%	MI	−	−	−	
	IC	−	+	+	
RT, alcohol 70%	MI	−	−	−	
	IC	−	+	+	
RT, Dubos OAA	MI	±	−	−	−
	IC	−	−	±	±

[a] UD = Undiluted.
[b] MI = Results for *M. leprae* amplicon.
[c] IC = Results for internal control.
[d] ± = Faint signal compared with +.
[e] RT = Room temperature.

For suspensions with $< 10^4$ bacilli/ml calculations on the effect of the preservation media were not possible. However, as can be seen in the second part of Table 3, compared with the results on fresh tissues the titers after preservation in alcohol are 10 times lower, and after preservation in Dubos OAA broth they are 10 to 1000 times lower.

Table 3 also shows that in many instances inhibitory factors are distributed irregularly and are more frequent than in MFPH. They are not necessarily eliminated by PK treatment. A strong specific signal after PCR on an undiluted specimen may "overcome" inhibitors responsible for a weaker or even negative IC signal.

Effect of preservation of skin biopsies at different temperatures. Two skin biopsies were taken out of the freezer and divided into two parts: one was kept at room temperature for 3 weeks, the other was put back

TABLE 3. *Comparison of bacterial counts and PCR titrations on human biopsies examined fresh, and after conservation in alcohol or Dubos OAA broth and pretreated by FB or PK.*

Specimen no. and bacterial count/ml	Preservation			PCR result					Lowest no. bacilli detected
				u	−1	−2	−3	−4[a]	
L980	—	FB	Ml	+	+	+	+	−	20
2.0×10^6			IC	−	−	+	+	+	
		PK	Ml	++	++	+	+	−	166
			IC	±	±	−	+	+	
L981	—	FB	Ml	++	++	+	+	−	36
3.6×10^6			IC	−	−	+	+	+	
			Ml	++	++	+	+	−	36
			IC	−	−	−	+	+	
	—	PK	Ml	++	++	+	+	−	300
			IC	−	−	±	+	+	
			Ml	++	++	++	+	−	300
			IC	−	−	±	+	+	
	Alcohol	FB	Ml	+	+	−	−	−	3.6×10^3
			IC	+	+	+	+	+	
			Ml	+	−	−	−	−	3.6×10^4
			IC	+	+	+	+	−	
		PK	Ml	+	+	±	−	−	3×10^3
			IC	+	+	+	+	−	
			Ml	+	+	−	−	−	3×10^3
			IC	+	+	+	+	+	
L 982	—	FB	Ml	++	+	−	−	−	6.3×10^3
6.3×10^6			IC	−	−	−	−	+	
			Ml	++	++	+	−	−	630
			IC	+	+	±	±	+	
		PK	Ml	++	++	++	+	−	525
			IC	+	+	+	+	+	
			Ml	+	+	+	+	±	52.5
			IC	−	−	−	+	+	
	Alcohol	FB	Ml	+	+	−	−	−	6.3×10^3
			IC	+	+	+	+	+	
			Ml	+	+	±	−	−	630
			IC	−	+	+	+	+	
		PK	Ml	+	+	+	−	−	5.2×10^3
			IC	+	+	+	+	+	
			Ml	+	+	±	±	−	525
			IC	+	+	+	+	+	
L997	—	FB	Ml	++	++	+	+	−	32
3.2×10^6			IC	−	±	+	+	−	
			Ml	+	+	+	+	−	32
			IC	+	+	+	+	+	
		PK	Ml	++	++	+	+	±	26.6
			IC	−	−	±	+	+	
			Ml	+	+	+	+	−	266
			IC	+	+	+	+	+	
	Dubos	FB	Ml	+	−	−	−	−	3.2×10^4
			IC	+	−	−	−	−	
			Ml	−	−	−	−	−	—
			IC	+	+	+	+	+	
		PK	Ml	+	+	+	−	−	2.6×10^3
			IC	+	+	+	+	+	
			Ml	+	+	+	−	−	2.6×10^3
			IC	+	+	+	+	+	
L1003	—	FB	Ml	++	++	+	−	−	70
7×10^5			IC	−	−	±	+	+	
		PK	Ml	++	++	++	+	−	58
			IC	−	−	−	±	+	

TABLE 3. *Continued.*

Specimen no. and bacterial count/ml	Preservation			PCR result					Lowest no. bacilli detected
				u	−1	−2	−3	−4[a]	
			Ml	++	++	+	+	−	58
			IC	−	+	+	+	+	
	Dubos	FB	Ml	+	−	−	−	−	7×10^3
			IC	±	+	+	+	+	
			Ml	−	−	−	−	−	−
			IC	+	+	+	+	+	
		PK	Ml	+	+	±	−	−	583
			IC	+	+	+	+	+	
			Ml	+	+	+	−	−	583
			IC	+	+	+	+	+	
983	−	FB	Ml	+	+	−	−		
$\leq 10^4$			IC	+	+	+	+		
		PK	Ml	++	++	+	+		
			IC	+	+	+	+		
	Alcohol	FB	Ml	+	−				
			IC	+	+				
		PK	Ml	−	−				
			IC	−	−				
994	−	FB	Ml	+	+	−			
$\leq 10^4$			IC	+	+	−			
		PK	Ml	+	−				
			IC	−	−				
	Dubos	FB	Ml	−	−				
			IC	+	+				
		PK	Ml	−	−				
			IC	−	−				
999	−	FB	Ml	+	+	−			
$\leq 10^4$			IC	+	+	+			
			Ml	−	−				
			IC	+	+				
		PK	Ml	+	+	−			
			IC	+	+	+			
			Ml	+	±				
			IC	+	+				
	Dubos	FB	Ml	−	−				
			IC	+	+				
		PK	Ml	−	−				
			IC	−	−				
L1001	−	FB	Ml	+	+	+	−	−	
$\leq 10^4$			IC	+	+	+	+	+	
		PK	Ml	+	+	+	−		
			IC	+	+	+	+		
	Dubos	FB	Ml	−	−				
			IC	−	−				
		PK	Ml	−	−				
			IC	−	−				
L1082	−20°C	FB	Ml	+	+	+	+	±	63
6.3×10^7			IC	+	±	+	−	+	
= 982		PK	Ml	+	+	+	+	±	525
			IC	−	−	±	+	+	
	RT	FB	Ml	+	+	+	+	−	630
	3 weeks		IC	±	+	+	+	+	
		PK	Ml	++	+	+	+	−	5.2×10^3
			IC	±	+	+	+	+	
L1087	−20°C	FB	Ml	++	++	+	−	−	320
3.2×10^6			IC	−	−	+	+	+	
= 987		PK	Ml	++	++	+	+	−	266
			IC	−	−	+	+	+	

TABLE 3. *Continued.*

Specimen no. and bacterial count/ml	Preservation			PCR result					Lowest no. bacilli detected
				u	−1	−2	−3	−4[a]	
	RT	FB	MI	++	+	−	−	−	3.2×10^3
	3 weeks		IC	−	−	−	+	+	
		PK	MI	++	++	+	−	−	2.6×10^3
			IC	−	−	−	+	+	
L1081	−20°C	FB	MI	+	+	+	−	−	360
3.6×10^6			IC	−	−	+	+	+	
= 981		PK	MI	+	+	+	−	−	3×10^3
			IC	−	−	−	−	−	
	Boil 6′ + RT	FB	MI	+	+	−			3.6×10^3
			IC	+	+	+			
	3 weeks	PK	MI	+	+	−			3×10^4
			IC	+	+	+			
L1085	−20°C	FB	MI	+	+	+	−	−	320
3.2×10^6			IC	−	+	−	−	−	
= 985		PK	MI	+	+	+	±	−	266
			IC	−	−	−	+	+	
	Biol 6′ + RT	FB	MI	+	+	−			3.2×10^3
			IC	+	+	+			
	3 weeks	PK	MI	+	+	±			3.2×10^3
			IC	+	+	+			
L1086	RT	FB	MI	+	+	−	−	−	4.2×10^3
4.2×10^6	3 weeks		IC	+	+	+	+	+	
= 986		PK	MI	+	+	+	−	−	3.5×10^3
			IC	+	+	+	+	+	
	Boil 6′ + RT	FB	MI	+	+	+	−	−	4.2×10^3
		PK	IC	+	+	+	+	+	
	3 weeks		MI	+	+	+	−	−	3.5×10^3
			IC	+	+	+	+	+	

[a] u = undiluted, −1 to −4 logarithmic dilutions, i.e., 1 to 10 = −1, 1 to 100 = −2, etc.

at −20°C for 3 weeks when the suspensions were prepared and PCR performed after FB and PK treatment. Two other skin biopsies were thawed and divided in halves: one was put back at −20°C; to the other, 1 ml PBS was added and the tube held in a boiling waterbath for 6 min. It was then kept at room temperature for 3 weeks. PCR was performed 3 weeks later on the 6 specimens.

A last biopsy was divided into two parts: one half was kept fresh at room temperature for 3 weeks, the other half was boiled for 6 min and kept at room temperature for 3 weeks when PCR was performed.

Table 4 shows the results. Again, in 5 out of 10 cases the PK procedure was less sensitive than FB. Preservation of biopsies at room temperature after boiling for 6 min or not, reduced the sensitivity of the test 10-fold. In the last experiment, a higher titer was obtained when the biopsy was boiled rather than kept fresh for 3 weeks at room temperature.

DISCUSSION

For PCR on *M. leprae,* genes coding for proteins have been amplified, such as those coding for the 18-kDa ([2, 14]) or the 36-kDa proteins ([6]). Since at least some of these proteins may be heat-shock proteins the amplification of genes coding for the species-specific rRNA, existing in all living cells, should by definition be more specific. This region has recently been identified by Cox, *et al.* ([4]). We used the primers they proposed in the present work, the aim of which was to investigate the sensitivity not the specificity of the test.

Since PCR is difficult to quantify, we performed the reaction on decimal dilutions of the specimens with the result that there frequently was a variation of one dilution upon repeated testing. This could probably be overcome by performing the PCR on 4–6 tubes per dilution, as done in other instances of biological measurements, allow-

ing calculations of an end point and statistical evaluation ([10]). This was not done at present because of the workload and cost of reagents involved.

As found previously ([5, 15]), on the whole, FB was superior to PK treatment for sample preparation.

The addition of an internal control (IC) as used in the present investigations does inhibit the production of amplicon from the target DNA if the latter is present in amounts near the limit of detectability. Therefore, the IC should be added in a carefully defined concentration to minimize this inhibitory effect. This shortcoming could be circumvented by performing two reactions in two separate tubes: one with and one without an IC.

In contrast with MFPH, inhibitors of the PCR were frequently present in human skin biopsies. This may be due to the presence of traces of hemoglobin known to be a potent inhibitor of Taq polymerase ([7]). The IC was therefore particularly useful in tests on human skin biopsies. In some cases, when in the undiluted specimen inhibitors are present together with a large amount of target DNA, a specific *M. leprae* amplicon may be produced in the absence of, or with a weak IC amplicon; in subsequent dilutions the specific amplicon then disappears, while the IC amplicon appears (Tables 2 and 3).

In the presence of inhibitors both IC and specific amplicon are present in dilutions. However, a small amount of target DNA may be missed if the reaction is inhibited in the undiluted sample and the target DNA is diluted out in the dilutions. For all these reasons the use of an IC is superior to the procedure of blind dilution of the sample as done previously ([5]).

The quantitative aspects of the present work were based on microscopic counts of bacilli without determination of their viability. Theoretically, the PCR can be positive for dead organisms if the portion of DNA to be amplified is intact, which is most probably a question of chance. The results with MFPH show an important difference between harvests containing more or fewer than 10^6 bacilli per ml. For the 14 tests performed on 11 harvests with counts between 2.1×10^4 and 8.7×10^5, the mean sensitivity was 36.5 bacilli, range 1.5 to 210; whereas for 6 tests on 3 harvests with counts above 10^6 bacilli per ml the sensitivity was 10^3 to 2.2×10^3. Thus, the sensitivity decreases 10 to 1000-fold with increasing numbers of bacilli in the MFPH. This may reasonably be ascribed to an increasing proportion of dead bacilli during the late logarithmic and plateau phases of the growth curve ([11]).

The sensitivity of the PCR on suspensions of fresh, human multibacillary leprosy biopsies, kept frozen at −20°C for 4 to 6 weeks, varied widely from 3 to 3×10^3 bacilli; the mean for the 6 lowest values after FB treatment was 37.6. The great variation in sensitivity may, in this case, also be due to variable proportions of viable bacilli in the suspensions. Unfortunately, the suspensions were not titrated in mice, although it is possible again that there is not necessarily a parallelism between viability as measured by multiplication in the mouse foot pad and the presence of the undamaged fragment of the PCR target DNA.

Paucibacillary leprosy lesions with a bacterial index (BI) of 0 contain a maximum of 10^5 bacilli per g of tissue ([9]). A suspension of a biopsy weighing 20 mg in 0.5 ml PBS would contain a maximum of 2×10^3 organisms, and 10 μl of this suspension used in the PCR would contain 40 bacilli, very close to the mean of the lowest level of detection in the present experiments. With a sensitivity 10 times higher, which can exceptionally be reached with the present technique or more regularly by using a probe to detect the amplicon, the minimum number of bacilli that could be detected would be 10^4 organisms per g of tissue, provided the DNA is not degraded and there are no inhibitors of the reaction in the sample. Thus, it is very unlikely that the PCR will be able to solve the problems of the diagnosis of indeterminate or minimal tuberculoid leprosy lesions.

The genes coding for ribosomal RNA are present in all bacteria in multiple copies. Unfortunately, in slow-growing mycobacteria and *M. leprae* only one copy is present ([1]). Thus, although this DNA is by definition highly specific, a PCR based on it may be somewhat less sensitive than in the case of other bacteria. Whether the sensitivity of the PCR may be increased by amplification of repetitive sequences present in the genome, such as the ones described by Clark-

Curtiss and Docherty ([3]) and used by Woods and Cole ([15]), remains to be investigated.

For the PCR to be useful in practical circumstances, preservation and transportation of specimens at ambient temperature is desirable. However, preservation of biopsies at room temperature resulted in a loss of sensitivity of 1 log. As shown with MFPH, fixation of suspensions in formol 10% or in Lowy fixative was very harmful, and Dubos OAA broth was also deleterious.

Biopsies kept at room temperature after boiling for 6 or 10 min brought no solution. On the whole, alcohol 70° gave the most satisfactory results.

Hsu, *et al.* compared the effect of formol, buffered formol, and alcohol either at room temperature or with heating for the detection of the hepatitis-B virus in liver tissue ([8]). They found heating of tissues in alcohol the best procedure. The loss of sensitivity of the PCR was thought to result from nucleases degrading the DNA, and they concluded that for preservation of specimens a procedure is required that rapidly inactivates these enzymes. This seems to be the reason why heating in a microwave oven was the most efficient procedure. Clearly, a comparably rapid-acting fixation technique for leprosy biopsies has to be found.

In previous work with PCR, amplifying the gene for the 36-kDa protein, the harmful effect of unbuffered formalin had been ascribed to the interaction of the formalin with nucleic acids ([5]). The present work shows that the fixatives act by increasing inhibition. It is possible that in some cases, particularly with the FB procedure, the fixatives themselves, remaining in the specimens, may inhibit the PCR. In the present study, the inhibitors were also not related to antileprosy treatment, since all the human biopsies were from untreated patients.

SUMMARY

The sensitivity of the polymerase chain reaction (PCR) on the DNA coding for the species-specific fragment of 16S rRNA of *Mycobacterium leprae* studied on mouse foot pad harvests and human skin biopsies varies widely between 1 and 3×10^4 organisms. This is probably the result of variations in the proportions of organisms with sufficiently intact DNA suitable for PCR. Preserving human skin biopsies for 3 weeks at an ambient temperature even after boiling for 6 minutes gives rise to a 10-fold decrease in sensitivity. Fixation of tissues in formol 10% or Lowy fixative or preserving in Dubos OAA broth is very harmful to the PCR, mainly due to the enhancement of an inhibitory effect on the PCR reaction. For preservation, the best choice at the moment seems to be alcohol 70%. Sample preparation of five cycles of freeze-boiling is simple and generally more efficient than proteinase K treatment and DNA extraction.

RESUMEN

La sensibilidad de la reacción en cadena de la RNA polimerasa (PCR) sobre el DNA que codifica para el fragmento de RNA 16S específico del *Mycobacterium leprae* (aislado de la almohadilla plantar del ratón y de biopsias de piel humana), varía ampliamente entre 1 y 3×10^4 organismos. Esto probablemente se debe a la variación en la proporción de organismos con DNA lo suficientemente intácto como para permitir el análisis por PCR. Si las biopsias de piel humana se mantienen durante 3 semanas a temperatura ambiente, la sensibilidad disminuye unas 10 veces aún despues de hervir el material durante 6 min. La fijación de los tejidos en formol al 10% o en fijador de Lowy, o su preservación en caldo Dubos con OAA, también resultan muy perjudiciales para el PCR porque se aumenta el efecto inhibitorio sobre la reacción. Hasta el momento, la mejor forma de preservar el tejido parece ser su inclusión en alcohol al 70%. La preparación del material por cinco ciclos de congelación y ebullición, es simple y generalmente más eficiente que el tratamiento con proteinasa K y extracción del DNA.

RÉSUMÉ

La sensibilité de la réaction de polymérase en chaine (PCR) sur le décodage de l'ADN pour le fragment spécifique d'espèce de 16S rRNA de *Mycobacterium leprae*, étudiée sur des prélèvements du coussinet plantaire de souris et des biopsies cutanées humaines, varie largement entre 1 et 3×10^4 organismes. Ceci est probablement le résultat de variations dans les proportions d'organismes avec un ADN suffisamment intact pour la PCR. La conservation des biopsies cutanées humaines pendant 3 semaines à température ambiante, même après ébullition pendant 6 minutes, diminue la sensibilité d'un facteur de 10. La fixation des tissus dans du formol à 10% ou le fixateur de Lowy ou leur conservation dans le liquide de Dubos OAA est très mauvaise pour la PCR, principalement par la stimulation de l'effet inhibiteur sur la réaction de PCR. Pour la conservation, le meilleur choix pour le moment paraît être l'alcool à 70%. La préparation de l'echantillon en cinq cycles d'ébullition-réfrigération est simple et généralement plus efficiente que le traitement à la protéinase K et l'extraction de l'ADN.

REFERENCES

1. Bercovier, H., Kapri, O. and Sela, S. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem. Biophys. Res. Comm. **136** (1986) 1136–1141.
2. Booth, R. J., Harris, D. P., Love, J. M. and Watson, J. D. Antigenic proteins of *Mycobacterium leprae*: complete sequence of the gene for the 18 kDa protein. J. Immunol. **140** (1988) 597–601.
3. Clark-Curtiss, J. E. and Docherty, M. A. A species-specific repetitive sequence in *Mycobacterium leprae* DNA. J. Infect. Dis. **159** (1989) 7–15.
4. Cox, R. A., Kempsell, K., Fairclough, L. and Colston, M. J. The 16S ribosomal RNA of *Mycobacterium leprae* contains a unique sequence which can be used for identification by the polymerase chain reaction. J. Med. Microbiol. (in press).
5. de Wit, M. Y. L., Faber, W. R., Krieg, S. R., Douglas, J. T., Lucas, S. B., Montreewasuwat, N., Pattyn, S. R., Hussain, R., Ponnighaus, J. M., Hartskeerl, R. A. and Klatser, P. R. Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. J. Clin. Microbiol. **29** (1991) 906–910.
6. Hartskeerl, K. D., de Wit, M. Y. L. and Klatser, P. R. Polymerase chain reaction for the detection of *Mycobacterium leprae.* J. Gen. Microbiol. **135** (1989) 2357–2364.
7. Higuchi, R. Rapid, efficient DNA extraction for PCR from cells or blood. Amplifications **2** (1989) 1–3.
8. Hsu, H.-S., Peng, S.-Y. and Shun, C.-T. High quality of DNA retrieved for Southern blot hybridization from microwave-fixed, paraffin-embedded liver tissues. J. Virol. Methods **31** (1991) 251–261.
9. Shepard, C. C. Recent developments in the chemotherapy of leprosy. Leprologia **19** (1974) 230–234.
10. Shepard, C. C. Statistical analysis of results obtained by two methods for testing drug activity of drugs against *Mycobacterium leprae.* Int. J. Lepr. **50** (1982) 96–101.
11. Shepard, C. C. and McRae, D. H. *Mycobacterium leprae* in mice: minimal infectious dose, relationship between staining quality and infectivity and effect of cortisone. J. Bacteriol. **89** (1965) 365–372.
12. Shepard, C. C. and McRae, D. H. A method for counting acid-fast bacteria. Int. J. Lepr. **36** (1968) 78–82.
13. Triglia, T., Peterson, M. G. and Kemp, D. J. A procedure for *in vitro* amplification of DNA segments that lie outside the boundaries of known sequences. Nucleic Acids Res. **16** (1988) 8186.
14. Williams, D. L., Gillis, T. P., Booth, R. J., Looker, D. and Watson, J. D. The use of a specific DNA probe and polymerase chain reaction for the detection of *Mycobacterium leprae.* J. Infect. Dis. **162** (1990) 193–200.
15. Woods, S. A. and Cole, S. T. A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol. Lett. **65** (1989) 305–310.