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Simple Colorimetric Microtiter Plate Hybridization Assay for Detection of Amplified *Mycobacterium leprae* DNA

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The detection of amplified products resulting from polymerase chain reactions (PCRs) remains a complicated process. To simplify the detection procedures, we developed a colorimetric microtiter plate hybridization assay for the specific detection of 5′-biotinylated PCR fragments of *Mycobacterium leprae* DNA. For this assay, an *M. leprae* DNA capture probe was made and immobilized on the wells of a microtiter plate. Hybridization of the biotin-labeled PCR fragments was detected through enzymatic color development. The resulting optical densities showed a logarithm-linear relationship with the amount of template DNA and corresponded to the intensity of the bands obtained through gel analysis and Southern blotting of the PCR products. The sensitivity of the assay was found to be 125 fg of genomic *M. leprae* DNA, or 20 lysed bacilli, revealing a detection limit similar to that of agarose gel analysis. The efficient coamplification of human DNA was used as a positive control for the presence of inhibitory substances in clinical material. For detection of human PCR products, a human DNA capture probe was also constructed for the colorimetric assay. This dual setup for hybridization, which thus detected both *M. leprae* and human DNA PCR products, was useful for ascertaining the presence of inhibiting substances in clinical specimens. All biopsy specimens (n = 10) from untreated patients with leprosy were positive. Apparently, this assay is more sensitive than microscopy, because biopsy specimens from half of the patients were negative upon histopathological examination. Biopsy specimens from three treated patients were negative, as were those from the three patients who did not have leprosy. We conclude that this colorimetric assay can replace agarose gel analysis and Southern hybridization, because it is as sensitive as those methods. Its advantages over conventional gel analysis and Southern hybridization are that it is less cumbersome and more rapid.

Leprosy is still a major health problem in developing countries. *Mycobacterium leprae*, the causative organism of leprosy, cannot be grown in vitro and detection of the organism depends on the microscopic observation of acid-fast bacilli, a technique which is not specific for *M. leprae*. Advances in new molecular techniques have offered the potential of a more sensitive and specific means of identifying pathogenic organisms and may enable detailed epidemiological studies (11, 16). In particular, the polymerase chain reaction (PCR) promises to have a great impact on the improvement of the diagnosis of leprosy (3, 5, 18, 20). Recently, we reported the development of a PCR for the specific detection of *M. leprae*; the PCR was based on the selective amplification of a 531-bp fragment of the proline-rich antigen (*pra*) gene (5). That PCR was further developed for the detection of *M. leprae* in various biological specimens and was shown to be more sensitive than microscopy (3).

For that PCR technique to be implemented as a tool in large studies, it is necessary that the hybridization procedure be easy to perform to ensure the specificity of the amplified DNA. We therefore decided to simplify the detection of amplified fragments, to circumvent the cumbersome procedure of gel electrophoresis and then Southern blotting with an *M. leprae*-specific probe (3, 5). We chose to adapt a previously described colorimetric microtiter plate hybridization assay (9) for the simple detection of the 531-bp DNA fragments resulting from the amplification of *M. leprae* DNA. The advantage of the technique is that it requires equipment that is already used for enzyme-linked immunosorbent assays and that is readily available in many routine diagnostic laboratories. Furthermore, the technique has been shown to be comparable in sensitivity to Southern hybridization, but it is less labor-intensive and more rapid (7–9).

In the study described here, we investigated the usefulness of a colorimetric microtiter plate hybridization assay in comparison with agarose gel analysis and Southern hybridization for the detection of *M. leprae* and human DNA PCR fragments formed in a multiplex PCR by using biopsy specimens from patients with leprosy.

MATERIALS AND METHODS

Clinical specimens. Skin biopsy specimens from 10 untreated patients with leprosy, 3 patients at the end of their 24 months of multiple-drug treatment (MDT), and 3 patients with skin diseases other than leprosy were collected and quickly frozen in liquid nitrogen. Classification of leprosy was based on the enumeration of acid-fast bacilli in skin biopsy specimens expressed as the bacterial index of the granuloma on sections stained by the modified Fite method (15). Multibacillary (MB) patients were defined as having a bacterial index of >0; patients with a bacterial index of 0 were defined as paucibacillary (PB), meaning that by histopathological examination of the sections no acid-fast bacilli were detected.

Sample preparation. Three frozen 6-μm sections from each biopsy specimen were incubated with 100 μl of lysis buffer (1 mg of proteinase K per ml and 0.05% Tween 20 in 10 mM Tris-HCl [pH 8.6]) for 18 h at 60°C and 15 min at 97°C

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as described before (3). Five microliters of the lysate was tested in the PCR.

M. leprae DNA was kindly supplied by M. J. Colston as part of the U.N. Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. The DNA concentration was determined by measuring the optical density (OD) at 260 nm, and 10-fold dilutions were made; 1,250, 125, or 12.5 pg or 1,250 or 125 fg of purified *M. leprae* DNA was added as a template to the PCR mixtures, unless stated otherwise.

Human placental DNA was purchased from Pharmacia. Tenfold serial dilutions were made; 2.5 μg, 250 ng, 25 ng, 2.5 ng, or 250 pg was added as a template to the PCR mixtures.

M. leprae bacilli were extracted from armadillo tissue as recommended by the World Health Organization (21), and the concentration of bacilli was measured by determining the OD at 420 nm (17). Tenfold serial dilutions of intact bacilli were made; the bacilli were then lysed as described previously for the frozen sections (3). The numbers of lysed bacilli added to the PCR mixture ranged from 2 to 2×10^5.

Multiplex PCR amplification. Inhibition of the PCR by substances present in biological material is a major concern when applying PCR to clinical specimens (3). A possible solution to this problem is the coamplification of an alternative target that is always present in human specimens, to serve as a positive control for the PCR. We chose to amplify a region of the human β-globin gene for simultaneous amplification with the 531-bp PCR fragment of *M. leprae* in a multiplex PCR (9, 10). A fragment of the β-globin gene of human DNA was coamplified in the PCR mixture with the primer set PC04 (80 ng) and 5′-biotinylated GH20 (80 ng), resulting in a 268-bp PCR fragment (1).

A 531-bp *M. leprae*-specific fragment of the *pra* gene was amplified under the same standardized conditions as described previously (5), except for the use of the 5′-biotinylated S62 primer and the addition of 10% (vol/vol) dimethyl sulfoxide. The mixtures were subjected to 35 cycles, each of which consisted of 2 min at 94°C, 2 min at 60°C, and 3 min at 72°C. Although the conditions used in the present study were established for optimal amplification of the *M. leprae* DNA, the human DNA was also efficiently amplified.

For agarose gel analysis, 25 μl of the amplification reaction mixture was electrophoretically separated on a 2% (wt/vol) agarose gel (12). For identification of amplified DNA in Southern blots (12), a sequence internal to the 531-bp *M. leprae* PCR fragment was amplified by using the primer set T3 and T4c (for sequences, see below), resulting in a 286-bp fragment which was then labeled with digoxigenin (5). Blots were scanned transmissively by using a BioImage System (Millipore Corp.). The intensities of the bands were determined and were expressed as the integrated OD; the background signals were subtracted from the scan data.

Construction of the *M. leprae* and human DNA capture probe. An *M. leprae* capture probe was constructed by amplifying *M. leprae* DNA with the nested primer set T3 (5′-CTC AAG CTT CTA CAC ATT CTG GGT CAC C-3′) and T4c (5′-CTC CTG CAG GAA CTT CAT TAC CGT CTT GCC-3′) (as chosen by N. G. Stoker [16a]). Because these primers contained tail sequences with restriction sites, amplification with the primer set resulted in a 286-bp fragment with restriction sites for *Hin*dIII and *Pst*I at the 5′ and 3′ ends, respectively. This fragment was digested with the enzymes *Pst*I and *Hin*dIII and was cloned into the corresponding sites of the M13BM21 replicative form (RF) (Boehringer Mannheim). This construct was then used to transfect *Escherichia coli* JM105 to obtain large quantities of M13 phages (12). Single-stranded DNA was isolated from the recombinant M13 phages and was used as a capture probe for identification of *M. leprae* PCR fragments. A human capture probe was constructed in a manner similar to that used to construct the *M. leprae* capture probe, using primer set PC03 (5′-CTC AAG CTT GGT AGT GAA CAC AGT TGT GT-3′) and KM29 (5′-CTC CTG CAG TTG GCC AAT CTA CTC CCA GG-3′), resulting in a 113-bp fragment with *Pst*I and *Hin*dIII ends. The single-stranded DNA of M13BM21, the construct without the insert, was isolated in the same way and was used to exclude the possibility of cross-hybridization of the PCR products to this vector.

Immobilization of the capture probe. We used the method of noncovalent attachment of the capture probe to microtiter wells essentially as described by Nagata et al. (14). The *M. leprae* and human DNA capture probes were dissolved in water to make a stock solution (500 μg/ml). For coating of the capture probe onto the wells, the stock solution was diluted in phosphate-buffered saline (PBS)–0.1 M $MgCl_2$ to a final concentration of 5 μg of DNA per ml. Microtiter plates (Greiner; gamma irradiated) were coated with 100 μl (500 ng of DNA) per well overnight at room temperature. The wells were emptied and irradiated with UV light (480 kJ/m^2) for 5 min and were subsequently washed with PBS. The wells were emptied and used immediately or were sealed and stored at room temperature.

Colorimetric microtiter hybridization assay. To block nonspecific binding, 200 μl of hybridization solution (5× SSC [0.75 M NaCl and 0.075 M sodium citrate], 0.5% [wt/vol] blocking reagent [Boehringer Mannheim], 0.1% [wt/vol] *N*-lauryl sarcosine, Na salt, 0.02% [wt/vol] sodium dodecyl sulfate [SDS]) was then added to the wells and incubated for at least 1 h at 37°C. To denature the amplified DNA, 10 μl of each amplified PCR product was boiled and then quenched on ice for 10 min. Ninety microliters of ice-cold hybridization solution was added to the boiled PCR mixtures. This mixture was added to the empty wells. Plates were covered with a plastic seal and were incubated at 37°C overnight with gentle shaking on a flatbed rotator. After hybridization, the wells were emptied and washed twice with 200 μl of 2× SSC and twice with 200 μl of 0.5× SSC–0.1% (wt/vol) SDS at 60°C. The plates were washed with prewarmed solutions, and the washing solutions were left in the wells for 5 min, with the plates floating in a water bath. Two hundred microliters of blocking buffer (1× PBS, 0.1% [wt/vol] Tween 20, 5% bovine serum albumin) was added and the plates were incubated for an additional 1 h at 37°C. Following blocking of the wells, the streptavidin-conjugated horseradish peroxidase was diluted 1:10,000 in blocking buffer; 100 μl was added to each well, and incubation was continued for 1 h. The plates were washed four times with PBS–0.1% (wt/vol) Tween 20, and 100 μl of substrate solution (0.2 mM *ortho*-phenylenediamine in 20 mM citric acid–50 mM Na_2HPO_4 [pH 5.0]–0.03% H_2O_2) was added. After 20 min, the reaction was stopped with 100 μl of 2.5 M H_2SO_4. The OD of the resulting yellow color was measured at 492 nm. The values were expressed as net ODs after the OD of the buffer blank was subtracted.

RESULTS

The colorimetric microtiter plate hybridization assay for the specific detection of 531-bp *M. leprae*-specific PCR fragments and 268-bp human PCR fragments is a modification of the assay described by Keller et al. (9). The immobilized *M. leprae* and human DNA capture probes were

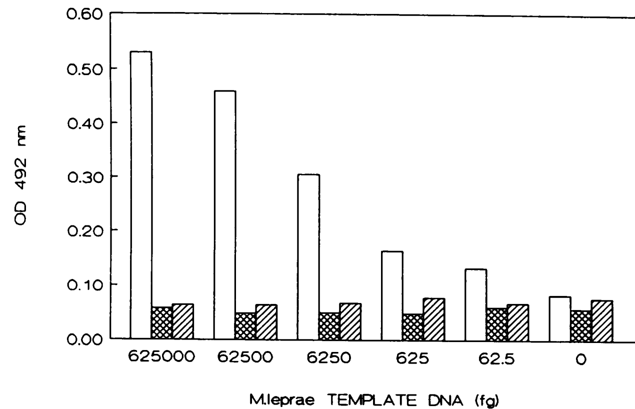

FIG. 1. Specificity of colorimetric microtiter plate hybridization assay for *M. leprae* PCR products when different capture probes were coated onto the wells of a microtiter plate. □, *M. leprae* DNA capture probe; ▩, human DNA capture probe; ▨, single-stranded DNA of M13BM21 as a capture probe.

attached to the microtiter plate wells, which allowed formation of a hybrid structure which immobilized the biotin-labeled PCR fragments in the wells. Stringent washing was necessary to remove the nonincorporated 5′-biotinylated primers and the nonhomologous hybrids. The immobilized amplified DNA was detected by using a streptavidin-horseradish peroxidase conjugate and then *ortho*-phenylenediamine as the substrate.

In order to examine the stability of the coated plates at room temperature, we performed the colorimetric assay with amplified *M. leprae* DNA originating from 625 pg of template DNA, using *M. leprae* capture probe-coated microtiter plates which were stored for 0, 1, or 2 days or 1, 2, 4, 8, or 12 weeks in the dark. The variations in the resulting ODs were never more than 4%.

The specificity of the hybridization was investigated by coating plates with the *M. leprae* DNA capture probe, the human DNA capture probe, and as a control, single-stranded DNA of M13BM21. The colorimetric assay was performed by using the biotin-labeled PCR products of 10-fold serial dilutions of *M. leprae* and human template DNAs. The results are shown in Fig. 1. ODs were shown to be related to the amounts of the PCR products from template *M. leprae* DNA applied to the *M. leprae* DNA capture probe-coated wells. By using the same amplified products, the ODs of the wells coated with the single-stranded DNA of M13BM21 or the human DNA probe illustrated the background signals. These were 0.060 and 0.079, respectively, at the most. When the human PCR fragments were added to the *M. leprae* capture probe-coated wells, the resulting ODs were 0.087 at the most (data not shown). In the following experiments, an OD of 0.100 was taken as the cutoff value for a positive test result.

To determine the detection limit of the colorimetric microtiter plate hybridization assay, the ODs were plotted against the amount of purified *M. leprae* template DNA or bacilli used in the amplification. The results were compared with those obtained by agarose gel analysis of the same samples (Fig. 2). As illustrated in Fig. 2A, the OD was still above the background signal when we used 125 fg of purified *M. leprae* template DNA (corresponding to 25 bacilli), which is in accordance with the results obtained with the same samples by gel agarose analysis (Fig. 2B). As shown in Fig. 2C, the detection limit of the assay was reached when 20 lysed bacilli were used as the source of template DNA; agarose gel analysis revealed the same detection limit (Fig. 2D).

The results obtained by the colorimetric assay were compared with those obtained by the traditional Southern hybridization technique. For that comparison, the amplified material of a serial dilution of purified *M. leprae* DNA was subjected to PCR and the resulting products were used in both assays. The resulting ODs and integrated ODs were significantly correlated (Pearson correlation coefficient r = 0.987; $P < 0.01$).

The reproducibility of the colorimetric assay was determined by assessing both between-run and within-run variations. The between-run variation was examined by testing independently amplified samples containing the same amount of *M. leprae* template DNA (6.25 pg) and adding each resulting PCR product to a different well in the colorimetric microtiter plate hybridization assay. The mean OD thus obtained was 0.275 ± 0.030 (n = 20), giving a between-run variation of 10.9%. The within-run variation was determined by adding the PCR product from one mixture, derived from 6.25 pg of template DNA, to different wells in the colorimetric assay. The mean OD was 0.267 ± 0.017 (n = 20), giving a within-run variation of 6.4%. The between-run and within-run variations of the microtiter assay with the human DNA capture probe and PCR products derived from human template DNA (25 ng) were assessed in the same way. The between-run and within-run variations were 8.3 and 2.6%, respectively.

To assess the usefulness of the assay in laboratory practice, it is a prerequisite to know the influence of the presence of human DNA on the amplification efficiency of *M. leprae* DNA when testing human specimens. Therefore, human placental DNA was coamplified in the same tube as purified *M. leprae* DNA under the standardized *M. leprae* PCR conditions. Tenfold serial dilutions of *M. leprae* DNA (625 pg to 62.5 fg) were amplified in the absence and presence of 25 ng of human placental DNA (corresponding to 3,750 diploid cells). The resulting PCR products were analyzed in the colorimetric assay and by gel electrophoresis (Fig. 3). In addition, *M. leprae* DNA (6.25 pg) was amplified in the presence of various amounts of human placental DNA ranging from 250 pg to 2.5 μg, which is equivalent to 38 to 375,000 diploid cells (Fig. 3B). Agarose gel analysis showed that there was no cross-reactivity when using both primer sets in one PCR mixture (Fig. 3B). Furthermore, both experiments showed that the detection limit of the *M. leprae* PCR products was not influenced by the presence of human DNA. Although the presence of human DNA in the *M. leprae* PCR caused a slight decrease in the overall intensity of the signals obtained by both agarose gel and colorimetric analyses (Fig. 3), this observation was independent of the amount of human DNA present in the PCR mixture (Fig. 3C and D).

Thirteen skin biopsy specimens from 10 untreated patients with leprosy and 3 patients with leprosy at the end of their MDT and biopsy specimens from 3 patients with skin diseases other than leprosy were examined by the multiplex PCR. The resulting PCR products were tested in the colorimetric microtiter plate hybridization assay described above; the results were compared with those obtained in the gel analysis (Table 1). *M. leprae* DNA was detectable in the biopsy specimens from all the untreated MB and PB patients, as determined by the presence of the 531-bp PCR fragment on the agarose gel. The biopsy specimens from the treated patients with leprosy were PCR negative, as con-

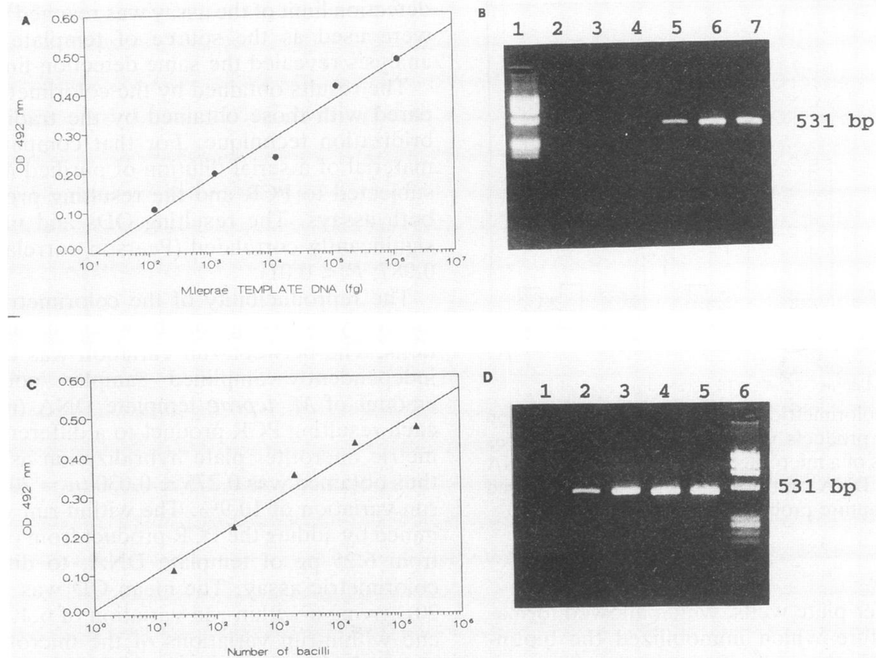

FIG. 2. Sensitivity of the colorimetric microtiter plate hybridization assay compared with that of agarose gel analysis. Tenfold serial dilutions of *M. leprae* DNA were subjected to PCR; the resulting products were analyzed by the colorimetric assay (A) (plot of OD versus amount of template DNA) and agarose gel electrophoresis (B). PCR products were derived from 125 fg (lane 3), 1,250 fg (lane 4), 12.5 pg (lane 5), 125 pg (lane 6), and 1,250 pg (lane 7) of template DNA. The no-template control is shown in lane 2, and the DNA size marker (*Hae*III digest of ϕX174 RF DNA) is shown in lane 1. Tenfold serial dilutions of *M. leprae* bacilli were subjected to PCR, and the products were analyzed by the colorimetric assay (C) and agarose gel analysis (D). PCR products were derived from 20 (lane 1), 200 (lane 2), 2×10^3 (lane 3), 2×10^4 (lane 4), and 2×10^5 (lane 5) bacilli. Lane 6 contains a DNA size marker (*Hae*III digest of ϕX174 RF DNA). The negative control samples in the colorimetric assay were products from amplification reactions which contained no *M. leprae* template DNA or lysed *M. leprae* bacilli. These were added to the wells coated with the *M. leprae* DNA capture probe. The resulting ODs were always lower than the cutoff value.

firmed by agarose gel analysis. In addition, in the samples derived from the patients who did not have leprosy, no *M. leprae* PCR fragment could be detected after agarose gel analysis. These negative PCR results could not be explained by inhibitory factors in the PCR mixtures, because the human internal control PCR fragment could be visualized in the gel when any of these specimens was used.

The ODs obtained by the colorimetric assay were in agreement with agarose gel analysis results (Table 1). Higher ODs were found when biopsy specimens from untreated patients were used than when those from treated patients were used. The ODs obtained for control biopsy specimens from patients who did not have leprosy were all negative. By using the colorimetric assay with the human DNA capture probe, 268-bp PCR products could be detected in all specimens, which was in agreement with the results of agarose gel analysis.

DISCUSSION

PCR holds promise as a sensitive and specific system for the detection of *M. leprae* in a variety of specimens (3). Wide-scale application of this technology in routine diagnostic laboratories requires that the procedures be simple and reproducible (16). This need is addressed by the colorimetric microtiter plate hybridization assay for the detection of amplified *M. leprae* DNA described in this report.

The hybridization step in the assay allows specific detection of amplified *M. leprae* DNA and is not dependent on the use of radioisotopes. The specificity of the hybridization reaction was illustrated by the fact that amplified *M. leprae* DNA hybridized only to the *M. leprae* DNA capture probe but not to the human DNA capture probe or to the single-stranded DNA of M13BM21. Similar findings have been reported by others using the M13 capture probe system for the detection of amplified DNA of viral origin (9, 10).

The sensitivity of the colorimetric assay was shown to correspond to that of agarose gel analysis. Both had a detection limit of 125 fg of amplified DNA derived from purified *M. leprae* template DNA, which is equivalent to 25 bacilli, given that the size of the *M. leprae* genome is 2.2×10^9 Da (2). This corresponded to the detection limit of the colorimetric assay when we used template DNA derived from lysed bacilli. Other investigators (6, 13), who used similar detection assays, have reported that they could detect 10 to 13 copies of target DNA, which is the same range that we found. The addition of various amounts of human placental DNA slightly decreased the sensitivity of the assay in detecting *M. leprae*. Thus, the coamplification of human DNA had only a minimal effect on the detection of small amounts of template DNA, as was the case in a PCR for viral DNA (10). These findings suggest that reproducible amplification can be measured in the presence of various amounts of human DNA.

The significant correlation between the ODs from the colorimetric assay and the integrated ODs from Southern hybridization implies that the results of the colorimetric assay truly reflect the amount of specifically amplified PCR

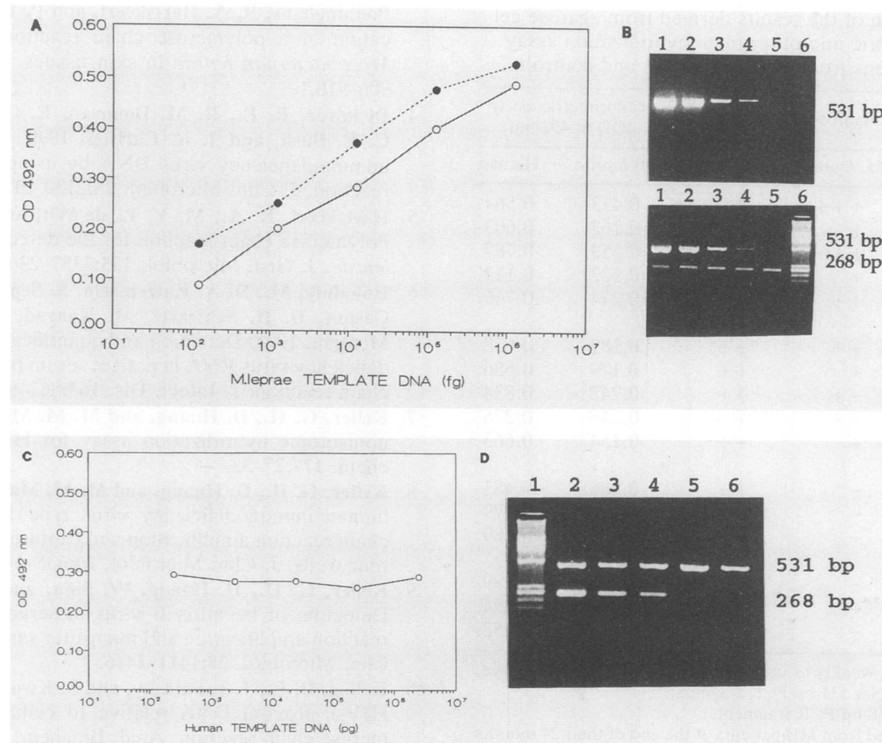

FIG. 3. Detection of amplified *M. leprae* DNA in multiplex PCR in the colorimetric microtiter plate hybridization assay compared with agarose gel analysis. (A) A plot of ODs of the PCR products resulting from a 10-fold serial dilution of *M. leprae* DNA (see legend to Fig. 2) in the absence (●) and presence (○) of 25 ng of human placental DNA. (B) Agarose gel analysis of the samples in panel A. Upper row, amplification of purified *M. leprae* DNA; lower row, a 10-fold serial dilution of *M. leprae* DNA in the presence of 25 ng of human placental DNA. The upper row contains 1,250 pg (lane 1), 125 pg (lane 2), 12.5 pg (lane 3), 1,250 fg (lane 4), and 125 fg (lane 5) of DNA. Lane 6 contained the no-template control. The lower row shows the results of the multiplex PCR when the same *M. leprae* dilutions were amplified in the presence of 25 ng of human placental DNA. Lane 6 contains a DNA size marker (*Hae*III digest of ϕX174 RF DNA). (C) ODs of *M. leprae* PCR products resulting from digestion of 12.5 pg of *M. leprae* template DNA in the presence of various amounts of human placental DNA ranging from 2.5 μg to 250 pg. (D) The amount of *M. leprae* DNA was 12.5 pg, together with 2.5 μg (lane 2), 250 ng (lane 3), 25 ng (lane 4), 2.5 ng (lane 5), and 250 pg (lane 6) of human placental DNA. Lane 1 contains a DNA size marker (*Hae*III digest of ϕX174 RF DNA). The negative control samples used in the colorimetric assay were the PCR samples which contained no *M. leprae* DNA or human DNA. These were added to the wells coated with the *M. leprae* DNA capture probe and the human DNA capture probe, respectively. The resulting ODs were always lower than the cutoff value.

fragments. In addition, the logarithm-linear relationship between the amount of template DNA (20 to 2×10^5 bacilli) and the OD of the colorimetric assay (Fig. 2) suggests that the assay could be used semiquantitatively to assess the relative amount of *M. leprae* DNA in a sample. Other investigators (6, 13, 19) have indeed reported the use of similar colorimetric assays which were linear over a range of 1×10^2 to 5×10^4 copies of template DNA. Since logarithm-linear relationships can be observed by different assays, colorimetric detection of the PCR products might be useful for semiquantification.

When the reproducibility of the colorimetric microtiter plate hybridization assay was tested, it showed a variation of 10.9%. This is similar to the performance of other nonisotopic assays, which have been reported to show variabilities of between 10 and 14% (4, 6, 13). The within-run variation was lower than the between-run variation. This was most likely because the latter includes the variation of the PCR in addition to that of the colorimetric assay. Capture probe-coated microtiter plates were shown to be stable for at least 12 weeks at room temperature; the variation for different times of storage did not exceed the between-run variation. These results suggest that the colorimetric microtiter plate hybridization assay described here would be useful in routine diagnostic laboratories.

We demonstrated the utility of the colorimetric assay using biopsy specimens from patients with leprosy. All biopsy specimens from untreated MB and PB patients were positive in the assay, which was in concordance with the results of agarose gel analysis. One sample (sample 4, Table 1), however, revealed low ODs in the colorimetric assay when either the *M. leprae* or the human DNA capture probe was used. This suggests the presence of PCR-inhibiting substances in this sample, a phenomenon which we have previously reported in a PCR with unpurified biopsy specimens (3). PCR positivity in specimens in which no acid-fast bacilli can be detected by microscopy has been reported (3); it likely is due to the higher sensitivity of the PCR-based technology. The specimens from the patients who had received MDT were negative. These results are in accordance with previous findings (3), and considering the semiquantitative nature of the assay, these results are suggestive of the possible usefulness of nucleic acid amplification techniques for monitoring the bacillary load of patients with leprosy during MDT.

In conclusion, the colorimetric microtiter plate hybridiza-

TABLE 1. Comparison of the results derived from agarose gel analysis and colorimetric microtiter plate hybridization assay using biopsy specimens from leprosy patients and controls

Class	Sample no.	Agarose gel analysis[a]		Colorimetric assay (OD at 492 nm)	
		M. leprae[b]	Human[c]	*M. leprae*	Human
MB	1	+++	+++	0.473	0.564
	2	+	++	0.162	0.603
	3	+++	+++	0.359	0.787
	4	+	+	0.122	0.112
	5	++	++	0.274	0.749
PB	6	++	++	0.189	0.638
	7	+	++	0.179	0.686
	8	++	++	0.242	0.834
	9	++	+	0.246	0.276
	10	+	++	0.154	0.663
MB-MDT[d]	11	−	++	0.086	0.481
	12	−	+++	0.086	0.479
	13	−	++	0.099	0.189
Controls[e]	14	−	+++	0.096	0.799
	15	−	+++	0.094	0.254
	16	−	+++	0.077	0.452

[a] −, negative; + to +++, weakly to very strongly positive, judged by eye.
[b] *M. leprae*, *M. leprae* DNA 531-bp PCR fragment.
[c] Human, human DNA 268-bp PCR fragment.
[d] Biopsy specimens derived from MB patients at the end of their 24 months of MDT.
[e] Biopsy specimens derived from patients with skin diseases other than leprosy.

tion assay described here is a method for the sensitive and specific detection of *M. leprae* DNA. The results of the assay with a limited number of clinical specimens described here are encouraging and will allow us to perform larger studies, which are required to determine the actual sensitivity and specificity of the assay. The assay is simple, rapid, and reproducible. The hybridization step involved in the assay ensures that the correct PCR product is produced. An additional advantage is that it yields numerical data and thus does not depend on subjective interpretation. Furthermore, the assay does not require radioisotopes, making its implementation in routine diagnostic laboratories a feasible prospect.

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