JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1995, p. 752–754 Vol. 33, No. 3
0095-1137/95/$04.00+0

Colorimetric Microwell Plate Hybridization Assay for Detection of Amplified *Mycobacterium tuberculosis* DNA from Sputum Samples

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Received 8 August 1994/Returned for modification 20 September 1994/Accepted 5 December 1994

We developed a colorimetric microwell plate hybridization assay (CoMPHA) for the specific detection of 5′-biotinylated amplified *Mycobacterium tuberculosis* DNA. The optical densities of the CoMPHA corresponded to the initial amounts of purified template DNA. Here, we show that the CoMPHA is useful in distinguishing the PCR-positive and PCR-negative samples.

PCR has been widely used for rapid, sensitive, and specific detection of *Mycobacterium tuberculosis*. Several studies were done to evaluate the reliability of the PCR technique in clinical laboratories, and such results illustrate that PCR is a good test for detecting *M. tuberculosis* DNA even when small numbers of bacteria are present in the clinical specimens (1, 4–6, 8, 11). However, problems arose with PCR-positive but culture-negative results. The proportion of these false-positive results varied substantially among the different laboratories (11). One of the major causes of false positivity may be carryover contamination of previous amplified PCR products (11). However, this can be avoided by the use of dUTP and uracil-DNA-glycosylase (UDG) (8, 9). Difficulties may also arise by cross-contamination during specimen processing (7, 8, 11). This problem can be minimized by the introduction of good laboratory practice and inclusion of proper controls in the PCR procedure (8).

Nevertheless, it may be difficult to avoid low levels of cross-contamination during specimen processing between samples with a large bacterial load and samples with no bacteria (11). In such cases, it may be expected that only a few bacilli are introduced into the neighboring tube(s), thus resulting in barely detectable DNA bands after PCR and agarose gel analysis. An objective quantitation of the amplified products would make it possible to determine a significant level of amplified DNA which is clearly relevant for active tuberculosis. Recently, it was shown that the PCR in combination with the colorimetric microwell plate hybridization assay (CoMPHA) could be used for semiquantitative analysis of the level of *Mycobacterium leprae* template DNA in biopsy specimens (14). Here, we show that 5′-biotinylated PCR products of *M. tuberculosis* can be detected by an *M. tuberculosis* capture probe in the CoMPHA, allowing a clear distinction between PCR-positive and PCR-negative samples.

Sputum samples which had been submitted for mycobacterial culture were decontaminated with an equal volume of a 4% NaOH solution. After centrifugation, the sediment was resuspended in about 2 ml of phosphate-buffered solution, pH 7.2. About four-fifths of the suspension was used for smear examination and inoculation into two egg-based Ogawa media slants (3) including one with antibiotics. The rest of the suspension was then frozen at −20°C for later PCR analysis. The growth of mycobacteria in Ogawa media with and without antibiotics was examined once a week for 8 weeks. Identification of *M. tuberculosis* was based on colony morphology, pigmentation, and growth rate, followed by biochemical tests (13).

DNA purification from clinical samples was performed as follows. A 200-μl portion of the residual suspension (see above) was transferred to a 2-ml screw-cap conical tube and centrifuged at 12,000 × *g* for 10 min to pellet the bacteria. To the pellet, a 100-μl volume of 0.1-mm zirchonium beads (Biospec Products, Bartlesville, Okla.) in Tris-EDTA (pH 7.4) was added, and the residual supernatant fluid was removed after the beads settled. To the tube, 100 μl of Tris-EDTA-NaCl (pH 8.0) and 50 μl of phenol-chloroform-isoamyl alcohol (25:24:1) were added, and the tube was shaken vigorously for 1 min in a mechanical disrupter (MiniBead Beater model 3110; Biospec Products). After centrifugation for 5 min, the aqueous phase was collected and mixed on a vortex mixer with an equal volume of chloroform-isoamyl alcohol (24:1). After another brief centrifugation, the upper phase was collected and boiled for 10 min, and DNA was precipitated with 0.1 volume of 3 M sodium acetate and 2 volumes of cold absolute ethanol. Finally, after centrifugation at 12,000 × *g* for 20 min, the precipitated DNA was dried and resuspended in 10 μl of distilled water and used for PCR.

A 245-bp fragment of the IS*6110* repetitive sequence was amplified under the standardized conditions described previously (7, 8), with minor modifications. Here, the PCR mixtures consisted of 50 mM KCl (instead of 50 mM NaCl) and 100 pM each primer INS1 and 5′-biotinylated primer INS2 (instead of 0.4 μM INS1 and INS2). For the dUTP-UDG system, 400 μM dUTP was used instead of dTTP in the PCR mixture and 0.5 U of UDG (Gibco-BRL, Gaithersburg, Md.) was added to each tube. All the PCR mixtures were aliquoted in a final volume of 40 μl per reaction vial, to which 10 μl of the sample could be added. The PCR was carried out as follows: 10 min at 37°C to allow breakdown of amplicons by UDG, followed by 32 cycles consisting of denaturation at 94°C for 1 min, annealing at 60°C for 2 min, and primer extension at 72°C for 3 min. The denaturation time was extended to 6 min in the first cycle, and the primer extension time in the final cycle was prolonged at 72°C

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to retard UDG activity until the vials were taken out. If the PCR products were not analyzed directly after the final cycle, the vials were stored at −20°C. Tenfold serial dilutions of purified DNA from *M. tuberculosis* H37Rv were included as positive controls, and distilled water was included as a negative control in each experiment. The DNA concentration of the stock solution was determined by measuring the optical density (OD) at 260 nm; the positive control consisted of 625, 62.5, 6.25, 0.625, and 0.0625 pg of template DNA.

After amplification, 10 μl of the PCR mixture was electrophoretically separated on a 0.8% (wt/vol) agarose gel containing ethidium bromide. The 245-bp DNA band was visualized by UV illumination. Of the amplified products, 30 μl was used for the CoMPHA, performed as described below. An *M. tuberculosis* probe was constructed by amplifying *M. tuberculosis* DNA with the nested primer pair Pt3 + *Pst*I (5-CTCCTG CAGGAACGGCTGATGACCAAACT-3) and Pt6 + *Hin*dIII (5-CTCAAGCTTAGGTAGGCGAACCCTGCCCA-3). Amplification with this primer set resulted in a 188-bp fragment with restriction sites at both ends. This fragment was digested and cloned into M13BM20 and subsequently transfected in *Escherichia coli* as described previously (14). Single-stranded DNA was isolated from the recombinant M13 phages and served as a capture probe for identification of the *M. tuberculosis* PCR products. The procedure for detection and identification of amplified *M. tuberculosis* DNA using the 188-bp capture probe for *M. tuberculosis* which is located within the target sequence of the INS1 and 5′-biotinylated INS2 primers was followed as described for detection of *M. leprae* PCR products by Van der Vliet et al. (14). Each PCR mixture was tested in four wells: two wells containing the capture probe and two wells without the capture probe. In order to calculate the OD of the sample, the mean OD of the duplicate wells was calculated after subtracting the OD of the wells without the capture DNA probe. In each microwell plate, the PCR products from a serial dilution of purified *M. tuberculosis* DNA were included (see positive controls above) to standardize the OD readings.

Previously, the CoMPHA was validated for the use of the semiquantitative analysis of *M. leprae* template DNA after PCR amplification using dTTP (14). However, to prevent false-positive results due to contamination of previously amplified PCR products, the use of the dUTP-UDG system for PCR is necessary (8). In theory, such modification might influence the CoMPHA by degradation of the PCR products after amplification due to active UDG which might still be present in the sample. Therefore, the results obtained by the CoMPHA in combination with the PCR using the dUTP-UDG contamination prevention system were compared with the results of the PCR using dTTP. It can be seen from Fig. 1 that the ODs were comparable when the same amount of the template was used for amplification. Therefore, in subsequent PCRs the dUTP-UDG carryover prevention system was employed.

To determine the detection limit of the CoMPHA, the OD values were plotted against the amount of purified *M. tuberculosis* DNA used as the template in the amplification (Fig. 1B). The results were compared with those obtained by agarose gel analysis of the same samples. The OD was still above the background signal when 62.5 fg of DNA was added (corresponding to 12.5 mycobacteria), which is in accordance with the results obtained with the same samples after gel analysis (Fig. 1A). Furthermore, it can be seen from the plot that a plateau was reached when more than 62.5 pg of template DNA was added to the PCR mixture (Fig. 1B). Thus, only the absorbance values for the positive controls of 62.5, 6.25, and 0.625 pg of template DNA in a plate were used for the correction of the day-to-day variation of CoMPHA.

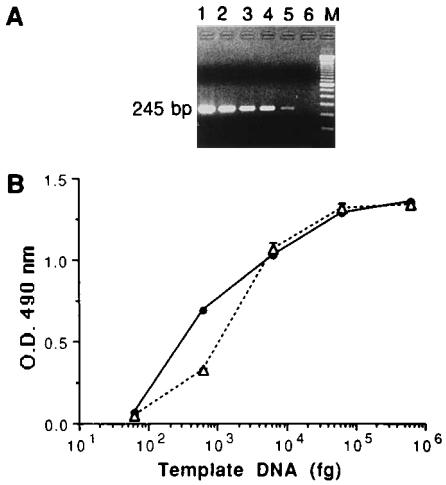

FIG. 1. Detection of amplified *M. tuberculosis* DNA by agarose gel analysis and by CoMPHA. Tenfold serial dilutions of *M. tuberculosis* DNA were amplified by PCR. (A) Lanes 1 to 5, 625, 62.5, 6.25, 0.625, and 0.0625 pg of template DNA, respectively; lane 6, distilled water. M, 100-bp DNA marker. (B) CoMPHA of PCR products using dTTP (circles) or dUTP-UDG (triangles) for carryover prevention; mean ODs and standard deviations (bars) for both assays were plotted against template DNA concentrations.

A total of 37 sputum samples were analyzed by culture, PCR, and CoMPHA. Of 37 sputum samples examined in this study, 10 were culture positive and 27 were culture negative. Of the 27 culture-negative sputum samples, one was PCR positive by agarose gel analysis and all 10 culture-positive samples were PCR positive by agarose gel analysis. The results of the CoMPHA after PCR are shown in Fig. 2. The mean OD of 26 PCR- and culture-negative samples was 0.023 ± 0.024. The cutoff value for PCR positivity by the CoMPHA was this mean plus 3 standard deviations, or 0.095. All of the 10 culture-positive specimens had ODs higher than the cutoff value, ranging from 0.153 to 0.854. One culture-negative sputum sample had a PCR-positive result. However, this "false"-positive result could readily be explained, because this sample was collected from a tuberculosis patient undergoing chemotherapy. This phenomenon is likely the result of the presence of nonviable bacteria and has been reported in other studies as well (4, 10, 12). Although there were other culture-negative specimens from tuberculosis patients, which might contain dead bacilli, their ODs were lower than the cutoff values. An insufficient amount of the probe coated in the microwell also might result in a lower sensitivity in this study.

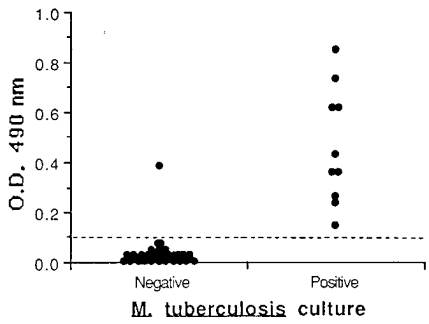

FIG. 2. Detection of PCR-amplified products of *M. tuberculosis* DNA from culture-negative and -positive sputum samples (dots) by CoMPHA. Dashed line, cutoff for PCR-CoMPHA positivity.

The wide range of ODs by CoMPHA for the culture-positive samples may reflect a wide variation of bacterial loads in sputum specimens. However, our attempt to correlate the ODs with bacterial loads failed because all of the culture-positive specimens were graded as 3+, which indicates more than 200 colonies in culture (13). But it is conceivable that the 3+ grade includes counts ranging from just over 200 colonies to more than could be counted. In a previous report using the same CoMPHA system, the ODs were in accordance with bacterial loads determined by the microscopic examination of biopsy samples from leprosy patients (14). Wilson et al. (16) also reported that ODs were closely related to the number of genome equivalents of *M. tuberculosis* DNA in a colorimetric system in which PCR products amplified with a biotinylated primer were captured by avidin coated in the microwell. There was a marked variation in ODs when PCR products from clinical samples were analyzed by the colorimetric method.

In this study, another major factor affecting ODs besides the number of organisms in clinical samples may be the copy numbers of the target sequence, IS*6110*, present in each sample, because the copy number of IS*6110* per chromosome varies from 0 to 26 (2, 15). In addition, variations in sputum contents such as minor PCR inhibitors and in the efficiency of lysis of the organisms during sample processing also may affect the CoMPHA results.

This study thus demonstrates that the OD values derived in the CoMPHA of the PCR products are related to the initial amounts of template *M. tuberculosis* DNA. In addition, the hybridization step involved in the CoMPHA ensures that the correct PCR product is detected. The CoMPHA does not require radioactive isotopes, and it is readily applicable in routine diagnostic laboratories. Furthermore, in this study the CoMPHA system clearly distinguished between PCR-negative and PCR-positive samples. Therefore, the CoMPHA may be useful in detecting PCR products of *M. tuberculosis* DNA from clinical samples.

This research was financially supported partly by the Development Project grant and faculty funds (92-3) from the Yonsei University College of Medicine and by research contract 6864/RB from the International Atomic Energy Agency, Vienna, Austria. Furthermore, this investigation received financial support from the Netherlands Leprosy Relief Association and from the Commission of European Communities Directorate General for Science, Research and Development (grant TS3-CT91-0036).

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