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Use of Polymerase Chain Reaction for Rapid Diagnosis of Tuberculosis

DEBRA V. COUSINS,[1]* STEPHEN D. WILTON,[2] BARRY R. FRANCIS,[1] AND BETH L. GOW[3]

Animal Health Laboratories, Department of Agriculture, South Perth,[1] and Biotech International, Bentley,[2] Western Australia 6151, and Mycobacteria Reference Laboratory, State Health Laboratory Services, Nedlands, Western Australia 6009,[3] Australia

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A DNA amplification assay using the polymerase chain reaction technique designed for the rapid identification of *Mycobacterium bovis* organisms was used to test 211 human mycobacterial isolates and 177 clinical specimens previously submitted for routine mycobacterial culture. The procedures described could be used by routine or specialist laboratories for identification of *M. tuberculosis* complex organisms in 4 h and/or as a rapid screening method for the direct detection of *M. tuberculosis* complex organisms in specimens.

Tuberculosis is a disease of worldwide importance, particularly in third world countries. A recent report suggests that there are 20 million cases of active tuberculosis in the world and that around 5,000 people die from tuberculosis every day (5). In Western Australia, approximately 9,000 samples are submitted annually to the Mycobacteria Reference Laboratory for culture. Culture and subsequent identification of isolates is the traditional method of confirming the diagnosis of tuberculosis. However, because the organisms are slowly growing, laboratory diagnosis by conventional methods can take as long as 10 weeks. Despite the acceptance of culture as the definitive tool for the diagnosis of tuberculosis, some microscopy-positive specimens fail to yield mycobacteria on culture. This may be due to the harsh chemical treatment which is used to decontaminate specimens, to contamination with other bacteria, or to the presence of nonviable mycobacteria. A recent report by Daniel (4) estimates that the sensitivity of culture can be as low as 50%. Radiometric methods (11) have been used in the detection of *Mycobacterium tuberculosis*, reportedly decreasing detection times. The development of DNA probes for the rapid detection of mycobacteria has been reported (14), but although they may be of use in identifying cultures of *M. tuberculosis*, they lack the sensitivity required for direct detection of the organism in specimens (10). The polymerase chain reaction (PCR) (8) can specifically amplify discrete fragments of DNA in which target material is present in only picogram quantities (9). Researchers have been quick to apply the PCR to the diagnosis of tuberculosis. Reports have centered on the amplification of DNA sequences coding for a 65-kDa antigen present in all mycobacterial species (2, 6, 10) or the MPB64 antigen thought to be specific for the *M. tuberculosis* complex (13). The PCR described here uses primers designed to target a sequence encoding the MPB70 antigen, which is considered to be species specific for *M. bovis* (17). This report compares the results of conventional identification methods and culture with PCR techniques for the identification and direct detection of *M. tuberculosis*. A procedure which could be used in routine or specialist laboratories to identify *M. tuberculosis* complex organisms within 4 h or to screen for tuberculosis in clinical samples within 3 days is described.

A total of 211 isolates identified as mycobacterium species by conventional methods between August and December 1990 were tested blind by the PCR method. A colony was picked into 100 μl of purified water and heated at 94°C for 10 min. A 5-μl volume of each suspension was inoculated into 20 μl of prepared reaction mixture containing 65 mM Tris hydrochloride buffer (pH 8.8 at 25°C), 16 mM ammonium sulfate, the four deoxyribonucleoside triphosphates (200 μM each), 200 μg of gelatin per ml, 2 mM magnesium chloride, 0.4% Triton X-100, 30 ng of each of the oligonucleotide primers (TB-1A, 5′-GAACAATCCGGAGTTGACAA-3′; TB-1B, 5′-AGCACGCTGTCAATCATGTA-3′), and 1 U of *Taq* DNA polymerase (Biotech International, Bentley, Western Australia, Australia) covered with 50 μl of paraffin oil. Samples were subjected to 30 cycles of denaturation at 94°C for 30 s and to annealing and extension at 62°C, for 1 min. Five microliters of the reaction product was mixed with equal volumes of gel loading buffer and subjected to electrophoresis on a 2% agarose gel at 100 V for 30 min. Gels were stained with ethidium bromide and photographed on a short- to medium-wavelength UV transilluminator. Samples were considered positive for pathogenic *M. tuberculosis* complex when a single band of DNA 372 bp in size was evident in the ethidium bromide-stained gels.

Clinical specimens were composed of 127 microscopy-positive samples submitted to the Mycobacteria Reference Laboratory for routine culture between September and November 1989 and 50 microscopy-negative samples submitted during March 1990. Samples included sputum (n = 132), urine (n = 15), bronchial washing (n = 14), feces (n = 4), lung tissue (n = 4), and miscellaneous (n = 8) specimens. Multiple specimens were collected from some patients. Bacteriology and microscopy were performed by standard methods (7, 15). Culture deposits from the microscopy-positive and -negative samples were stored at −20°C for 6 to 8 months and at 4°C for a week, respectively, before they were tested by the PCR.

Each sample was washed once in water and heated at 75°C for 45 min to inactivate the mycobacteria and any other potential pathogens in the samples. Samples were lysed with 2 mg of lysozyme per ml and then with 1% sodium dodecyl sulfate–100 μg of proteinase K per ml and were extracted

* Corresponding author.

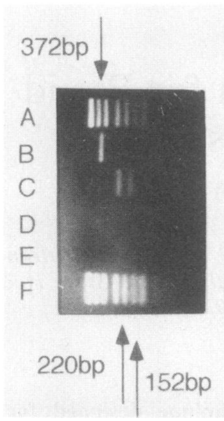

FIG. 1. Results of the PCR for detection of *M. tuberculosis* before and after digestion with *SacI* restriction endonuclease. Lanes A and F, 1 μg of pUC19 restricted with *HpaII* used as a size marker; lane B, amplified product obtained from a specimen from which *M. tuberculosis* was isolated on culture; lane C, the amplified product from lane B after digestion with *SacI*; lane D, amplified product from *Escherichia coli* negative control; lane E, amplified product from lane D after digestion with *SacI*.

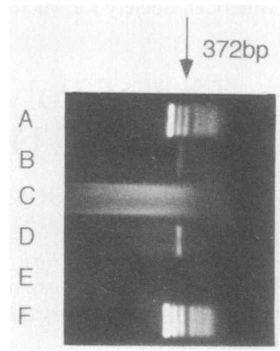

FIG. 2. Results of amplification of DNAs from clinical specimens. Lanes A and F, 1 μg of pUC19 restricted with *HpaII* used as a size marker; lane B, amplified product from an isolate of *M. tuberculosis* used as a positive control; lane C, smear resulting from the amplification of DNA from a specimen of sputum from which *M. tuberculosis* was isolated by culture; lane D, results of amplification of specimen from lane C when the specimen was diluted 1/10 before amplification; lane E, results of PCR for specimen from which *M. intracellulare* was isolated.

with 10% hexadecyltrimethyl ammonium bromide in 0.7% sodium chloride (16) and phenol-chloroform. DNA was precipitated with salt and isopropanol and dissolved in 50 μl of Milli-Q water. A 5-μl volume was inoculated into the reaction mixture detailed above, and amplification was performed by using 40 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 62°C, and extension for 1 min at 72°C. A 5-μl volume of the amplified mixture was examined by electrophoresis as described above. All samples were tested blind at least twice. After amplification and electrophoresis, any sample which had a visible band was subjected to enzyme digest with *SacI* (Toyobo, Osaka, Japan). Products from this digest were examined by electrophoresis on a 3.5% agarose gel.

Isolates. Of the 211 mycobacterium isolates tested, 101 were identified as *M. tuberculosis* complex by the PCR. These 101 isolates were identified as *M. tuberculosis* (n = 80), *M. bovis* (n = 19), and *M. bovis* BCG (n = 2) by conventional methods. The remaining isolates, which gave negative results in the PCR, were identified as *M. intracellulare* (n = 67), *M. avium* (n = 29), *M. gordonae* (n = 4), *M. chelonae* (n = 3), *M. kansasii* (n = 3), *M. terrae* (n = 2), *M. smegmatis* (n = 1), and *M. scrofulaceum* (n = 1), giving a sensitivity and specificity of 100% compared with those of conventional identification methods.

Clinical specimens. Positive samples and positive controls produced a single band of DNA 372 bp in size. On subsequent digestion with *SacI* two bands of DNA of expected sizes 220 and 152 bp were evident (Fig. 1), which strongly suggests that the amplified product contains sequences of *M. tuberculosis*. The negative control run with each set of samples produced no visible product on ethidium bromide-stained agarose gels. In about 15% of the specimens, a smear of DNA was visible, and when this occurred, the sample was diluted 1/10 in purified water and reanalyzed (Fig. 2). In 10 specimens giving such a smear, a 372-bp band of DNA was then visible on the stained gel. A total of 67 of the microscopy-positive samples tested were submitted from 14 patients with tuberculosis confirmed by isolation of *M. tuberculosis* on at least one occasion (Table 1). Culture detected 64 (95.5%) of these samples as positive for *M. tuberculosis*. Of the 67 samples, 65 (97%) were positive for *M. tuberculosis* complex when tested by the PCR. A total of 110 specimens were from patients for whom tuberculosis was not confirmed by culture. The PCR was negative for 83 of these specimens, giving a specificity of 75.5% compared with culture results. Results of the PCR and the culture are summarized in Table 1. A computer search conducted with series 61 of GenBank revealed no published mammalian, primate, or bacterial sequences which matched the synthetic primers or the targeted 372-bp sequence.

The results of this study suggest that the PCR is highly specific for the identification of *M. tuberculosis* complex organisms, confirming the findings obtained in a previous study (3) in which the PCR was used for the identification of *M. bovis*. In some developing countries, even reference laboratories have been known to make a high number of errors in the cultural identification of mycobacteria (4). Because the PCR is extremely sensitive (3), a single colony is sufficient for the test. The technique is simple to perform and could be used by any laboratory with a thermal cycler for the rapid and accurate identification of *M. tuberculosis* complex organisms.

For the direct detection of *M. tuberculosis* in specimens, the PCR was more sensitive than traditional culture methods for those patients known to have tuberculosis. These results compare favorably with those reported by Brisson-Noel et al. (2) and Pao et al. (10), in which the PCR was positive for all culture-positive specimens in studies with 15 and 49 positive specimens, respectively, and primers were directed at fragments of a gene encoding the 65-kDa mycobacterial antigen.

The PCR offers considerable advantages in speed over the traditional methods of identification and culture of *M. tuberculosis*. Specimens could be screened for *M. tuberculosis* in 2 to 3 days and identification of an isolate could be completed within 4 h. This contrasts with the requirement of between 2 and 8 weeks for successful cultivation and 2 to 3 weeks for an identification by conventional methods. When excess DNA appeared to mask the PCR, as was seen in some of the samples, it was a simple matter to dilute the extracted DNA and repeat the test. Since results can be available in 3

TABLE 1. Comparison of detection of *M. tuberculosis* in 177 specimens by PCR and culture

Patient group and organism	No. of patients	No. of specimens	No. of specimens culture positive for *M. tuberculosis* and:		No. of specimens culture negative for *M. tuberculosis* and:	
			PCR positive	PCR negative	PCR positive	PCR negative
Microscopy positive						
M. tuberculosis[a]	14	67	63	1	2	1
M. intracellulare[b]	17	51	0	0	16	35
M. avium[c]	5	8	0	0	2	6
Culture negative	1	1	0	0	1	0
Microscopy negative	30	50	0	0	8	42
Total	67	177	63	1	29	84

[a] Patients with confirmed *M. tuberculosis* infection.
[b] Patients with confirmed *M. intracellulare* infection.
[c] Patients with confirmed *M. avium* infection.

days, the PCR could be used as a rapid screening method for the presence of *M. tuberculosis* organisms in specimens. The use of BACTEC isolation combined with the Gen-Probe test can decrease the time required for diagnosis of tuberculosis (1) and has been reported to detect 83% of *M. tuberculosis* culture-positive specimens within 18 days (12). Because of its sensitivity, the PCR may also have an application in the rapid detection of *M. tuberculosis* complex organisms from BACTEC media, similar to the Gen-Probe method.

Compared with culture results, the specificity of 75.5% for the PCR in this report was an improvement on the 62.6% specificity found by Pao et al. (10). Some specimens from which *M. avium* or *M. intracellulare* were isolated, or which were negative on culture, produced a positive result by the PCR. All of the specimens tested were collected from patients or outpatients suspected of having tuberculosis because of their clinical history, characteristic radiographs, or history of exposure to tuberculosis. If we assume that they are true positives, then the numbers of *M. tuberculosis* complex organisms present may have been too few to detect by conventional means, or they may have been overgrown by a greater number of other mycobacteria in cases in which they were identified by conventional culture methods. In unpublished work (2a), it has been found that decontamination with 2% sodium hydroxide for 20 min can lower the number of viable mycobacteria in solution by at least 100-fold. Hence, it is theoretically possible for the sodium hydroxide treatment to have destroyed the viability of low numbers of *M. tuberculosis* organisms in specimens for which a positive PCR result was obtained.

False positives in the PCR can be caused by contamination in the system; however, negative controls run with each batch of samples failed to identify any contaminating DNA. False positives can also be caused by using too many amplification cycles or an annealing temperature which may be too low to allow specific amplification. Amplification with a decrease in the number of cycles was tried, but it resulted in a decrease in sensitivity in the positive samples. Increasing the annealing temperature above 63°C gave unreliable results. Although a search of GenBank to find similar sequences was unsuccessful, it is possible that there is a source of DNA in the specimens that may be very similar to the primer sequences used in this PCR, hence causing the amplification of a false signal.

Although the use of restriction endonucleases to detect a known restriction site within the amplified region provides strong evidence that the amplified product was that of the known sequence, the development of a probe to specifically detect a small DNA sequence internal to the amplified product would provide further evidence of specific amplification. Preliminary results of hybridizations with a digoxigenin-labelled probe internal to the amplified sequence support the results obtained by restriction analysis. This probe will continue to be evaluated in further studies by the PCR method.

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