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Detection and Identification of *Mycobacterium tuberculosis* by DNA Amplification

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The polymerase chain reaction (PCR) was used to identify mycobacterial DNA sequences in uncultured clinical specimens. Two oligonucleotide primers derived from the sequence of a gene that codes for the 65-kilodalton antigen of *Mycobacterium tuberculosis* amplified DNA from all 11 species of mycobacteria tested. Amplified DNAs of nontuberculosis mycobacteria were found to be approximately 20 to 40 bases shorter than those from *M. tuberculosis* and *Mycobacterium bovis* BCG. DNA equivalent to that present in as few as 40 *M. tuberculosis* cells either alone or in the presence of DNA equivalent to that in 10^6 human cells could be detected. Results from analysis of cultured bacteria and clinical specimens showed PCR was sensitive and specific both in detecting mycobacteria and in differentiating *M. tuberculosis* and BCG from other species of mycobacteria. The PCR method with the primers reported here may become a useful tool in the early and rapid detection of mycobacterial infections in uncultured clinical specimens.

Diagnosis of mycobacterial infections is still a long process that traditionally depends on the isolation of the pathogen *Mycobacterium tuberculosis*, which can take up to 8 weeks because of the slow-growing nature of mycobacteria. Direct microscopy and Ziehl-Neelsen staining of clinical specimens lack sufficient sensitivities and specificities (1). The radiometric BACTEC system (Johnston Laboratories, Inc., Towson, Md.) has greatly reduced the time needed for detection, but it is still growth dependent. A major limitation of noncultural methods, such as the immunodiagnostic approach, is the lack of sensitivity. We and others have recently reported the direct identification of the presence of mycobacterial DNA with DNA probes (3, 6, 7, 10). However, the radioisotopes used in the labeling of DNA probes and the considerable time and expertise needed by DNA hybridization procedures are some of the obstacles of DNA probe technology to be accepted by clinical laboratories. More recently, a technique known as the polymerase chain reaction (PCR) (8), which can amplify specific target DNA sequences many thousands of times in a matter of hours, has been applied in the rapid amplification and identification of many microorganisms, including mycobacteria (2, 4).

Here we report the use of a pair of 24-base synthetic oligonucleotides that bracket a 165-base region of a gene that codes for a 65-kilodalton antigen of *M. tuberculosis* to serve as primers to amplify the mycobacterial DNA with a high degree of sensitivity and specificity. We also demonstrated that these primers can identify and differentiate *M. tuberculosis* and *Mycobacterium bovis* BCG (bacillus Calmette-Guérin) from many other species of mycobacteria by a simple DNA amplification method.

MATERIALS AND METHODS

Mycobacterial cultures and isolates. The following mycobacterial and nonmycobacterial reference bacterial strains were obtained from the American Type Culture Collection (ATCC; Rockville, Md.) for use in PCR amplification and were grown according to the instructions of ATCC: *M. tuberculosis* ATCC 25177, *M. avium* ATCC 19074, *M. bovis* ATCC 19210, *M. chelonae* ATCC 14472, *M. fortuitum* ATCC 6841, *M. gordonae* ATCC 14470, *M. kansasii* ATCC 12478, *M. paratuberculosis* ATCC 19698, *M. phlei* ATCC 11758, *M. smegmatis* ATCC 19420, and *M. xenopi* ATCC 19250. Clinical isolates of *M. tuberculosis* and other nontuberculosis mycobacterial species were obtained from the Microbiology Section of the Clinical Pathology Department, Chang Gung Memorial Hospital. Only one isolate from each patient was included in the study. Procedures for culturing mycobacteria from clinical specimens have been described in a previous report (6). All clinical isolates were identified by conventional testing, which included growth rate; gross and microscopic colony morphology; pigmentation; and tests for niacin, catalase, nitrate reduction, and urease.

DNA amplification by PCR. Procedures for the isolation and preparation of DNA from cultured mycobacterial and nonmycobacterial bacterial cells have been described earlier (6). The procedures for PCR have also been described earlier (5). Briefly, 50 ng of purified total cellular DNA was amplified with a thermostable *Taq* DNA polymerase in a thermal cycler (Perkin-Elmer Cetus; Norwalk, Conn.) to establish *M. tuberculosis* positivity. The 100-μl DNA amplification reaction mixture contained 10 mM Tris hydrochloride (pH 8.3); 50 mM potassium chloride; 1.5 mM magnesium chloride; 0.01% gelatin; 20 pmol of each of the two primers; 2.5 nmol of each of the four deoxyribonucleoside triphosphates; 1 U of *Taq* DNA polymerase (Perkin-Elmer Cetus); and appropriate amounts of specimen DNA, positive control DNA, or negative control DNA. The temperature of the reaction mixture was first raised to 94°C for 20 s, to denature the DNA, and was then cooled down to 63°C for 20 s. The temperature of the reaction mixture was then raised to 72°C for 1 min, to extend DNA chain growth. This process was repeated 32 times, with a 10-min incubation at 72°C at the end. One-tenth of the amplified reaction mixture was fractionated electrophoretically in a 2% agarose gel containing 0.5 μg of ethidium bromide per ml and was visually in-

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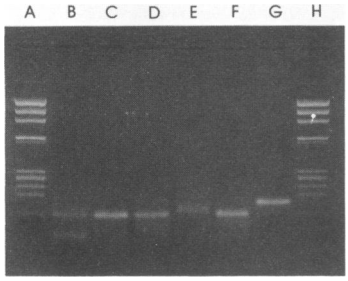

FIG. 1. Determination of mycobacterial DNAs in cultured bacterial cells by PCR amplification. Procedures for DNA preparation and PCR amplification and the sequences of primers are given in the text. DNA was visually inspected through UV light after agarose gel electrophoresis, and a DNA band of 165 base pairs indicated the presence of *M. tuberculosis* DNA. Lanes A and H, Restriction endonuclease *Hae*III-digested phage φX174 DNA used as DNA size standards; the sizes were 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 base pairs; lanes B through G, PCR DNA amplification analysis of mycobacterial DNA prepared from *M. avium*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. phlei*, and *M. tuberculosis*, respectively.

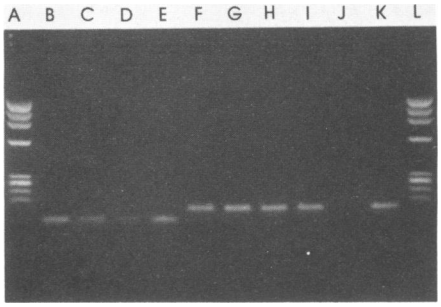

FIG. 2. Amplification of *M. tuberculosis* and nontuberculosis mycobacterial DNAs prepared from cultured clinical specimens. Lanes A and L, Restriction endonuclease *Hae*III-digested phage φX174 DNA used as DNA size standards; the sizes were 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 base pairs; lanes B through E, four independent isolates of nontuberculosis mycobacteria; lanes F through I, four independent isolates of *M. tuberculosis*; lane J, a negative control of 10 ng of human DNA; lane K, DNA prepared from ATCC reference strain *M. tuberculosis* ATCC 25177.

spected under UV light for bands of DNA of appropriate sizes. If the initial result was negative, a portion (usually one-fifth) of the first amplified reaction mixture was amplified for another 32 cycles under the same conditions with freshly supplemented deoxyribonucleoside triphosphates, primers, and *Taq* DNA polymerase. Alternatively, the DNA from the initial round of PCR was subject to Southern blot hybridization analysis with an internal oligonucleotide labeled at the 5′ end, with ^{32}P used as a probe (see below).

Primers. A pair of 24-base synthetic oligonucleotides that bracket a 165-base region of a gene codes for a 65-kilodalton antigen was synthesized (9). The sequences of the oligonucleotide primers were (from the 5′ to the 3′ ends) CTAG GTCGGGACGGTGAGGCCAGG and CATTGCGAAGT GATTCCTCCGGAT. Another oligonucleotide of 40 bases in length located between the two primers was synthesized to be used as an internal probe, and its sequence was (from the 5′ to the 3′ ends) AGCGTAAGTAGCGGGGTTGCCGT CACCCGGTGACCCCCGT.

Southern blot hybridization. A ^{32}P-labeled oligonucleotide probe was used for Southern blot hybridization analysis of PCR products (11).

RESULTS

DNA prepared from *M. tuberculosis* and 10 other nontuberculosis mycobacteria, including *M. avium*, *M. bovis* BCG, *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. paratuberculosis*, *M. phlei*, *M. smegmatis*, and *M. xenopi*, were used for PCR amplification. DNAs from all mycobacteria tested were amplified with the two primers used in this study. The size of DNA produced was 165 base pairs when *M. tuberculosis* and BCG DNAs were amplified. DNAs from the other nine species of mycobacteria tested produced amplified DNA that were approximately 20 to 40 base pairs shorter than that from *M. tuberculosis* (Fig. 1). DNAs prepared from 10 independent *M. tuberculosis* isolates cultured from clinical specimens gave DNAs of 165 base pairs after amplification, and another 10 similarly cultured isolates of nontuberculosis mycobacteria gave DNAs that were shorter in length (Fig. 2). DNA from a total of 18 bacteria of nonmycobacterial origin, including organisms commonly found in the respiratory tract, did not yield any detectable amplification product (data not shown). These nonmycobacterial microorganisms tested were *Aeromonas hydrophila*, *Citrobacter freundii*, *Corynebacterium diphtheriae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterococcus* spp., *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Nocardia asteroides*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella typhi*, *Serratia marcescens*, *Staphylococcus aureus*, beta group *Streptococcus* spp., and *Streptomyces* spp.

The authenticities of the amplified DNAs were established by two independent methods. Southern blot hybridization with an internal oligonucleotide DNA probe showed that the 165-base-pair DNAs from both *M. tuberculosis* and BCG can hybridize with the probe that was derived from the sequence that was located between the two primers (Fig. 3). Comparison of the actual restriction endonuclease pattern of the amplified DNA products with that predicted from the known amplified DNA sequences and the locations of the restriction

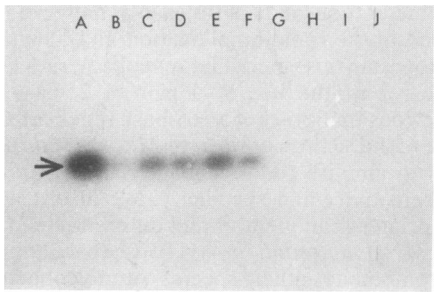

FIG. 3. Southern blot hybridization of amplified products with the internal oligonucleotide probe. The preparation of DNA and procedures of amplification, as well as the sequence of the internal oligonucleotide probe, were described in the text. Lane A, Amplification of DNA prepared from ATCC reference strain *M. tuberculosis* ATCC 25177; lanes B through F, DNA prepared from six clinical isolates identified as *M. tuberculosis*; lane G, amplification without DNA template; lane H, human DNA which served as a negative control; lanes I and J, *Taq* polymerase- and primer-negative controls, respectively.

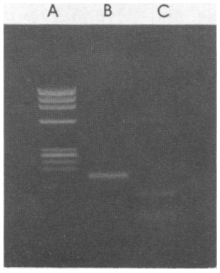

FIG. 4. Agarose gel electrophoresis of amplified *M. tuberculosis* DNA before and after restriction endonuclease digestion. A 20-μl reaction mixture of amplified *M. tuberculosis* DNA was purified by phenol-chloroform extraction and was used for restriction endonuclease *Hpa*II digestion. The conditions for the digestion were those recommended by the enzyme manufacturers. Lane A, Restriction endonuclease *Hae*III-digested phage φX174 DNA used as DNA size standards; the sizes were 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 base pairs; lanes B and C, amplified *M. tuberculosis* DNA before and after restriction endonuclease *Hpa*II digestion, respectively. The 165-base-pair DNA in lane B disappeared after *Hpa*II treatment, and two DNA bands of 85 and 79 base pairs appeared in lane C.

sites was illustrated by the disappearance of the original band of 165 base pairs after restriction endonuclease *Hpa*II digestion and the appearance of two new bands of 85 and 79 base pairs. These results strongly suggest that the amplified DNA contains DNA sequences of *M. tuberculosis* (Fig. 4).

The level of detection of mycobacterial DNA by PCR with the primers used in this study was determined by amplification serial dilutions of a purified *M. tuberculosis* DNA stock solution with a known concentration of about 0.1 pg of *M. tuberculosis* DNA, or approximately 40 cells, even in the presence of DNA equivalent to that in as many as 10^6 human cells (data not shown).

The sensitivity of detection of *M. tuberculosis* by PCR was determined by comparing the culture and DNA PCRs on a total of 284 clinical specimens (236 sputum and 48 pleural effusion specimens), and the results are given in Table 1. *M. tuberculosis* DNA sequences could be found in 118 of 284 (41.5%) uncultured sputum or pleural effusion specimens by PCR, whereas the traditional growth-dependent culture method showed only a 17.3% positive rate for the same specimens ($\chi^2 = 40.28$; $P < 0.001$). Nontuberculosis mycobacterial DNA sequences were detected in another five sputum and one pleural effusion specimen. All culture-negative but PCR DNA-positive specimens were collected from patients who had clinical signs indicative of tuberculosis, including characteristic radiographs, typical clinical manifestations of the disease, positive exposure history or past history of tuberculosis, and/or clinical response to antituberculosis chemotherapy.

TABLE 1. Comparison of the presence of *M. tuberculosis* DNA in sputum and pleural effusion specimens determined by culture and PCR

Source of specimen	No. of specimens:			
	Culture positive		Culture negative	
	PCR positive	PCR negative	PCR positive	PCR negative
Sputum	38	0	61	137
Pleural effusion	11	0	8	29

DISCUSSION

The data reported here suggest that DNA amplification is a superior method with a high degree of sensitivity and specificity for the detection of mycobacterial DNA sequences. Based on available culture and PCR DNA results, PCR has an overall sensitivity of 100% and a specificity of 62.6% when compared with culture results. Some of the clinical specimens that were positive by PCR but that were negative in culture were obtained from patients who were being treated for suspected tuberculosis. This is consistent with the fact that treated patients can still harbor mycobacteria long after culture for mycobacteria has become negative (2). This may suggest that the DNA amplification method could detect mycobacteria that are unable to grow in vitro. The perfect sensitivity and seemingly low specificity of clinical specimen assays further illustrated the low efficiency of the growth-dependent culture method and the unique amplification nature of PCR. The detection limit of PCR was much better than that of any other technique available in detecting mycobacteria, including the DNA probe method (6, 10). The high degree of sensitivity of PCR when combined with a low detection limit should be very useful in the early detection of mycobacterial infections, when bacterial cells may not be abundant enough for efficient or dependable direct staining and culture screening.

We found that the primers used in this study did not amplify DNA from the nonmycobacterial cells that we tested, including those commonly found in the respiratory tract. The fact that *M. tuberculosis* and BCG produced amplified DNAs with the same sizes was expected since the 65-kilodalton antigens of these two bacteria are identical, despite some reported differences in the DNA sequences in the region 3′ to the open reading frame (9, 12, 13). The ability of the primers evaluated in this study to differentiate *M. tuberculosis* and BCG from many other common nontuberculosis species of mycobacteria by a simple PCR is an added advantage of using PCR in terms of treating mycobacterial infections. The results here also suggest that it may be possible to design specific primers when more DNA sequence data become available that will allow the amplification and identification of specific species of nontuberculosis mycobacteria, either alone or in the presence of other microorganisms. The fact that DNA amplification can detect mycobacterial DNA sequences in the presence of vast excess amounts of human DNA makes it especially useful when quick results are required in certain clinical circumstances. Amplification may also be useful when large-scale screening of mycobacteria is indicated, such as in areas where tuberculosis is still a public health problem.

In summary, the results reported here suggest that with properly designed primers, DNA amplification may potentially be a very useful and valuable tool for the rapid detection of mycobacteria in uncultured clinical specimens.

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