Use of a Reamplification Protocol Improves Sensitivity of Detection of *Mycobacterium tuberculosis* in Clinical Samples by Amplification of DNA

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We have compared the sensitivity and specificity of quantitative mycobacterial culture against results obtained by using the polymerase chain reaction for the detection of DNA from organisms of the *Mycobacterium tuberculosis* complex in 82 clinical specimens from patients suspected of having tuberculosis. Two amplification protocols were used, a standard amplification protocol, which amplifies a segment of the gene coding for the 65-kDa antigen, and a protocol in which the initial amplification products are reamplified with a second set of nested oligonucleotide primers. Although the standard amplification protocol gave positive results for 18 of 18 samples which grew >100 CFU/ml and gave positive results in 4 of 35 specimens from patients with tuberculosis which were negative by culture, only 1 of 6 samples which grew <100 CFU/ml was positive. This lack of sensitivity could not be explained by the presence of inhibitors of *Taq* polymerase present in the original samples. In contrast, the reamplification protocol gave positive results for 24 of 24 samples which were positive by culture as well as for 13 of 35 samples from patients with tuberculosis which were negative by culture (overall sensitivity, 63%, $P < 0.02$, compared with the standard amplification protocol and routine culture). Two of 23 samples from patients not diagnosed as having tuberculosis gave positive results when the standard amplification protocol was used, but no additional false-positive results were seen with the reamplification protocol (overall specificity, 91%). We conclude that the use of a reamplification protocol improves the sensitivity of detection of mycobacterial DNA in clinical samples without sacrificing specificity. The sensitivity of this approach appears to be superior to that of standard culture techniques.

Standard techniques for the diagnosis of tuberculosis are suboptimal. The identification of mycobacteria by microscopic examination is insensitive and, even when positive, does not permit the identification of the species of mycobacteria observed. Similarly, culture techniques require 3 to 6 weeks before results are available, and multiple samples are required to maximize the chance of obtaining a positive result (1, 2). As a consequence, patients suspected of having tuberculosis may be subjected to invasive diagnostic procedures to establish or exclude the diagnosis or may receive possibly inappropriate empiric antituberculous therapy while awaiting results of cultures. All diagnostic tests may ultimately be negative for some patients thought to have tuberculosis on clinical grounds, despite extensive evaluation (1, 3).

In an effort to overcome these limitations in the diagnosis of tuberculosis, we (5, 12) and others (4, 8, 9, 14, 17, 18, 20, 23, 24, 27) have recently developed procedures based on the amplification of mycobacterial DNA by use of the polymerase chain reaction. Preliminary studies have indicated that this procedure can be used to rapidly identify the presence of DNA from *Mycobacterium tuberculosis* in clinical samples. The diagnosis of tuberculosis is especially difficult in cases where only a few mycobacteria are present. Thus, a new diagnostic approach which permits the detection of small numbers of mycobacteria would be particularly useful. In

this context, the sensitivity for the detection of mycobacteria in clinical samples containing only small numbers of organisms by standard bacteriological techniques and the sensitivity of detection by techniques based on the amplification of mycobacterial DNA have been compared in relatively few studies.

In this study, we have compared the sensitivity and specificity of two amplification protocols for the detection of *M. tuberculosis* in clinical samples from patients suspected of having tuberculosis. The results demonstrate that a standard amplification protocol, which amplifies a segment of the gene coding for the 65-kDa antigen (5, 12), could detect the presence of mycobacterial DNA from organisms of the *M. tuberculosis* complex in samples containing more than 100 mycobacteria per ml but was frequently negative with samples containing fewer mycobacteria. The inefficient amplification of mycobacterial DNA during this amplification procedure was not due to the presence of inhibitors present in the initial sample and could be overcome by reamplifying an aliquot of the original amplification products by use of a second set of nested oligonucleotide primers recognizing DNA sequences contained within the originally amplified mycobacterial DNA sequence. When this reamplification protocol was used, the sensitivity for detection of *M. tuberculosis* was superior to that of standard culture techniques.

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MATERIALS AND METHODS

Clinical specimens. By using standard procedures, specimens of sputum or gastric lavage were obtained from 32 patients admitted to Hôpital Bichat-Claude Bernard (Paris) or Hôpital Louis Mourier (Colombes) on suspicion of having pulmonary tuberculosis. Four samples were subsequently eliminated from the study because of a technical error during the extraction of DNA. The final study group contained 70 sputum samples and 12 gastric aspirates. Samples were decontaminated by use of the sodium dodecyl sulfate (SDS) method (26), neutralized, and concentrated by centrifugation. An aliquot of the sample was examined for acid-fast bacilli by using an auramine-fluorochrome stain (25), and the remaining sample was divided into two equal portions. One part was inoculated into Löwenstein-Jensen medium by using quantitative culture techniques (6). The second part was stored at -80°C prior to extracting DNA.

Diagnosis of tuberculosis. All patients were initially suspected of having tuberculosis either because of the presence of constitutional symptoms (fever and/or weight loss) and compatible abnormalities on chest radiograph or because of known exposure to a close family member with active tuberculosis and recent positive conversion of their skin test response to intermediate-strength purified protein derivative. Nineteen of the 32 patients were ultimately diagnosed as having tuberculosis. For the purposes of the study, this diagnosis was based on the following two criteria: (i) the final clinical diagnoses established by the primary physician included tuberculosis, and (ii) the patient was treated for tuberculosis by use of a standard therapeutic protocol. The diagnoses were established without knowledge of the results of the DNA amplification procedures evaluated in this study. One or more culture specimens ultimately grew *M. tuberculosis* in 11 of 19 patients diagnosed as having tuberculosis.

Extraction of DNA. In most cases, DNA was extracted from the frozen aliquots of the concentrated clinical samples as previously described (12, 16). Briefly, the specimen was centrifuged and the precipitate was heated at 95°C for 15 min in a solution of 0.1 N NaOH-2 M NaCl-0.5% SDS and centrifuged, after which the DNA was purified from the supernatant by extraction with phenol-chloroform and ethanol precipitation and redissolved in 100 μl of 10 mM Tris hydrochloride (pH 7.4) containing 0.1 mM EDTA.

In some cases, a portion of the sample was treated as described above and the DNA was extracted from the remaining portion by using a modification of the procedure of Higuchi (15). In this procedure, the sample was washed once with 1 ml of phosphate-buffered saline and incubated for 12 h at 55°C in 0.1 ml of amplification buffer (see below) containing 60 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) per ml, 0.45% Tween-20 (Sigma), and 0.45% Triton X-100 (Sigma). The sample was then heated at 95°C for 10 min.

Amplification and detection of mycobacterial DNA. The amplification of mycobacterial DNA was performed as previously described (5, 12, 22). The final reaction mixture (50 μl) contained 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 2.5 mM MgCl_2 , 100 μg of gelatin per ml, 0.3 mM (each) deoxynucleotides, 2.5 U of *Taq* polymerase, 50 pmol each of the oligonucleotide primers TB-1 (5'-GAGATCGAGCTG GAGGATCC) and TB-2 (5'-AGCTGCAGCCCAAAAGGT GTT) (12), and 10 μl of sample. Samples were sequentially incubated at 95°C (1.5 min), 60°C (2 min), and 72°C (2 min extended by 4 s after each cycle) for 50 cycles with an

automated thermal cycler (The Perkin-Elmer Corp., Norwalk, Conn.).

To reamplify mycobacterial DNA, 1 μl of the original amplification products was transferred by use of a piston-type pipette (Microman; Gilson Instruments, Villiers-le-Bel, France) to tubes containing the same reaction mixture described above except that the oligonucleotide primers TB-1 and TB-2 were replaced by oligonucleotides TB-28 (5'-CCAT CGATCCGAGACCCTGCTCAAGGGC) and TB-29C (5'-TG CTCTAGACTCCTCGACGGTGATGACG), which recognize sequences contained within the mycobacterial sequence amplified by TB-1 and TB-2 (12). The samples were subsequently amplified for an additional 50 cycles as described above. Because the sequence of the gene coding for the 65-kDa antigen corresponding to the 3' end of TB-28 differs between *M. tuberculosis* and most other mycobacteria, the reamplification system favors the amplification of DNA from *M. tuberculosis* complex organisms (data not shown).

The presence of amplified mycobacterial DNA sequences was detected as previously described (5, 12). Briefly, aliquots of the amplification products were electrophoresed in 2% agarose gels, the DNA was transferred to nylon membranes, and the membranes were hybridized with 3×10^5 cpm of ^{32}P -5'-labeled (specific activity, 1 to 3 mCi/pmol) oligonucleotide TB-4 (12), washed, and exposed to radiographic film. A sample was considered positive if a distinct signal corresponding to an amplified product of the appropriate size was seen in autoradiograms exposed for 18 h. Control samples containing no DNA were included with each amplification procedure and were always negative.

To test for the presence in extracts of clinical samples of substances capable of inhibiting the amplification of DNA, a fixed amount of purified mycobacterial DNA (containing approximately 1,000 genomes) was added to 1 to 12.5 μl of the solution obtained after extraction of clinical samples. The samples were amplified as described above, and the results were compared with those obtained for the amplification of mycobacterial DNA alone.

The sensitivity and specificity of the different procedures were compared by use of a Σ test.

RESULTS

Detection of mycobacterial DNA by a standard amplification protocol. Fifty-nine of the 82 samples evaluated in this study were obtained from the 19 patients ultimately diagnosed as having tuberculosis. Culture for *M. tuberculosis* was positive for 24 of these 59 samples; no other species of mycobacteria were grown from these samples. The number of mycobacteria grown from positive cultures was quite variable, ranging from 10^6 to 2 CFU/ml (Fig. 1a).

The results for the detection of mycobacterial DNA in these samples after amplification by our standard amplification protocol are shown in Fig. 1a. Mycobacterial DNA could be detected in 18 of 18 samples which grew more than 100 CFU/ml. Acid-fast organisms were also identified in all of these samples by microscopic examination (Fig. 1a). In contrast, the results of the amplification procedure were negative for five of six samples which grew fewer than 100 CFU/ml, and the only sample in this group which was positive by the amplification procedure was also positive by microscopic examination. The amplification procedure gave positive results in 4 of 27 samples obtained from patients not receiving antituberculous therapy but for which both culture and microscopic examination were negative. The overall sensitivity of the amplification procedure (39%) was not

FIG. 1. Comparison of the results of quantitative cultures and those obtained by the standard amplification protocol (a) and the reamplification protocol (b) for the detection of *M. tuberculosis* in 59 clinical samples from patients diagnosed as having tuberculosis. Samples which were positive by direct microscopic examination (triangles) and negative by direct examination (circles) are shown. Solid symbols represent samples which were positive by the given amplification protocol.

significantly different from that of culture techniques (41%) or microscopic examination (41%), but samples containing few mycobacteria were likely to be negative by the amplification procedure.

Twenty-three of the 82 samples studied were obtained from patients diagnosed as not having tuberculosis. None of these samples was positive by culture. Two of the 23 samples did, however, give positive results by the amplification procedure. In both cases, only one of three samples from two different patients was positive by the amplification procedure (overall specificity, 91%).

Detection of mycobacterial DNA by a reamplification protocol. An aliquot of the original amplification products was reamplified by use of a set of nested oligonucleotide primers which would specifically recognize sequences contained within the amplified mycobacterial DNA but not those present in the spurious amplification products. The results obtained by this reamplification protocol are shown in Fig. 1b. All 24 samples from patients with tuberculosis which were positive by culture were positive when the reamplification procedure was used, including the 4 samples which grew fewer than 100 CFU/ml and which were negative both by direct microscopic examination and by the standard amplification protocol. Furthermore, of the 35 samples from patients with tuberculosis which were negative by culture, 13 samples were positive by the reamplification procedure. This group included 2 samples from patients receiving treatment for tuberculosis and 11 samples from patients who had never received antituberculous therapy. The overall sensitivity of the reamplification procedure for the detection of *M. tuberculosis* (63%) was significantly greater than that of culture (41%, $P < 0.02$) and that of direct microscopic examination (41%, $P < 0.02$).

Of the 13 samples from patients with tuberculosis which were positive by the reamplification protocol but which were negative by culture, 5 were obtained from patients for whom other samples evaluated in this series were positive both by culture and the reamplification procedure. The remaining

eight samples were obtained from four different patients who were diagnosed as having tuberculosis despite the fact that no clinical specimen eventually grew *M. tuberculosis*. More than one specimen was positive by the reamplification protocol in three of these four cases (three of three, two of three, two of four, and one of two positive specimens, respectively). All four of these patients were children whose mothers had been recently diagnosed as having tuberculosis and who had had a recent conversion in their skin test reactivity, but none had received antituberculous therapy before the time that the specimens were obtained.

The two samples from patients diagnosed as not having tuberculosis, but which were positive by the standard amplification protocol, were also positive after reamplification. No additional samples from patients not having tuberculosis were positive when the reamplification protocol was used. Thus, the overall specificity of the reamplification procedure was the same as that of the standard amplification protocol (91%).

For all 35 of the samples which were positive when the reamplification protocol was used, a band whose molecular weight corresponded to that expected for the amplification product of mycobacterial DNA was visible when the amplification products were electrophoresed into 2% agarose gels and stained with ethidium bromide (Fig. 2). Furthermore, when the reamplification products were transferred to nylon membranes and hybridized with the probe specific for *M. tuberculosis*, a very strong signal was observed on autoradiograms in all cases, even after an exposure time of only 2 h (Fig. 2). In contrast, when samples were amplified by the standard amplification protocol, a band corresponding to the amplification product of mycobacterial DNA was observed in 19 of 35 cases (Fig. 2), and, in some cases, the signals observed on autoradiograms, even after an 18-h exposure period, were relatively weak.

Evaluation of techniques used for preparation of samples. When aliquots of samples prepared by the alkaline lysis method, and which were negative when tested alone by the standard amplification protocol, were "spiked" with purified mycobacterial DNA, all samples gave positive results. Furthermore, the intensity of the signal observed for tubes containing up to 12.5 μ l of sample plus purified mycobacterial DNA were always at least 50% of that observed for samples containing the purified mycobacterial DNA alone ($n = 8$; data not shown).

In contrast, when samples were prepared by use of a procedure based on proteolytic digestion, and in which the DNA was not subsequently purified by phenol extraction and ethanol precipitation, the addition of 12.5 μ l of sample completely inhibited the amplification of purified mycobacterial DNA in 9 of 13 samples, and, in 5 cases, the addition of as little as 1 μ l of the sample to the purified mycobacterial DNA resulted in no detectable amplification of mycobacterial DNA.

DISCUSSION

We have evaluated the sensitivity and specificity of a procedure that is based on the amplification and subsequent detection of mycobacterial DNA from organisms of the *M. tuberculosis* complex for the detection *M. tuberculosis* in clinical specimens from patients suspected of having tuberculosis. We have found that when a standard amplification protocol is used, the technique can detect DNA from *M. tuberculosis* in samples containing >100 CFU/ml but is frequently negative for samples containing fewer organisms.

FIG. 2. Amplification of mycobacterial DNA. Samples from patients with tuberculosis (lanes 1 to 4) which were positive by culture (300, 10, 2, and 60 CFU/ml, respectively) and samples from patients without tuberculosis (lanes 5 to 7) were amplified by using the standard amplification protocol (a) or the reamplification protocol (b), and a 7- μ l aliquot of the reaction products was electrophoresed into 2% agarose gels containing ethidium bromide and photographed (a and b, top). The DNA was then transferred to nylon membranes, and the membranes were hybridized with 32 P-5'-labeled oligonucleotide TB-4, washed, and used to prepare autoradiograms (a and b, bottom). The autoradiogram for the standard amplification protocol was exposed for 18 h, and that for the reamplification protocol was exposed for 2 h. The arrows indicate the position of migration expected for the amplified fragment produced by the amplification protocol (a, 383 bp) and reamplification protocol (b, 155 bp).

Thus, the test was negative in all samples which were negative by direct examination but positive by culture. This lack of sensitivity, which does not appear to be due to the presence of inhibitors of the *Taq* polymerase in the original samples, can be dramatically improved by using a protocol in which the initial amplification products are reamplified by use of a set of nested oligonucleotide primers recognizing sequences contained within the sequence of mycobacterial DNA amplified during the initial amplification reaction. The sensitivity of detection of *M. tuberculosis* when the reamplification protocol was used was superior to that obtained when standard culture techniques were used, and the use of reamplification protocol did not reduce the specificity of the test.

The lack of sensitivity of our amplification protocol could

be explained neither by a failure to extract mycobacterial DNA from the original clinical material (e.g., most samples from patients with tuberculosis were shown to contain *M. tuberculosis* DNA by using the reamplification protocol) nor by the presence of inhibitors of *Taq* polymerase in the original samples. The most likely explanation is that in reactions containing low numbers of target molecules, non-specific amplification products (e.g., amplified human DNA and primer-dimers) are generated which compete with the mycobacterial DNA (21). This could be overcome by reamplifying the initial amplification products with primers which recognize the inefficiently amplified mycobacterial DNA but do not recognize the nonspecific amplification products. This approach has previously been used to increase the sensitivity of DNA amplification for the detection of other target sequences (7, 10, 13), and a similar approach has recently been described by Plikaytis et al. for the detection of *Mycobacterium leprae* and *M. tuberculosis* (20).

In addition to greatly increasing the sensitivity of detection of mycobacterial DNA, the reamplification protocol also facilitates the subsequent identification of the amplified mycobacterial DNA. When the standard amplification protocol was used, many positive samples gave relatively weak positive signals on autoradiograms exposed for 18 h. In contrast, when the reamplification protocol was used, a band corresponding to the amplified *M. tuberculosis* DNA was visible on ethidium bromide-stained gels in all cases and all samples gave strong positive signals on autoradiograms exposed for as little as 2 h. Thus, the time required to reamplify samples can be recovered by reducing the time necessary for detecting positive samples. Overall, the reamplification procedure described here requires 3 days to obtain final results. The large amount of mycobacterial DNA present in reamplified samples could also facilitate the use of less sensitive but more easily handled nonradioisotopic detection systems.

A striking result in this study was the finding that the reamplification procedure could detect the presence of *M. tuberculosis* in many clinical samples from untreated patients with tuberculosis which were negative by culture. Furthermore, eight such samples were obtained from four patients for whom no clinical specimens grew mycobacteria. It is noteworthy that all of these patients were children whose mothers had active tuberculosis and who had had recent positive conversions of their tuberculin skin tests. That these patients actually had tuberculosis is strongly supported by the finding that, in all but one case, two or more samples from the same individual were positive when the reamplification procedure was used. Since decontamination procedures are known to reduce the viability of mycobacteria (19), it is possible that the processing of these samples killed the small number of viable mycobacteria present. Alternatively, some individuals may shed mycobacteria which are largely nonviable (e.g., because of the host immune response). In this context, mycobacterial DNA has been detected in culture-negative clinical samples which had not been decontaminated (e.g., pleural fluid and cerebrospinal fluid) (8, 17, 23, 27). Further studies comparing the results obtained by culture with those based on the detection of mycobacterial DNA should permit better assessment of the viability of mycobacteria before and after processing for mycobacterial culture.

Recently, several other groups have described systems based on the polymerase chain reaction for the detection of mycobacteria. Most of these systems, like that described here, are specific for DNA from organisms of the *M.*

tuberculosis complex but cannot distinguish individual members of this group (4, 5, 9, 14, 17, 20, 24, 27), although two systems have been described which are apparently specific for *M. tuberculosis* (8, 18). When tested against purified mycobacterial DNA, several of these systems have been shown to detect as little as 1 fg of mycobacterial DNA (equivalent to less than one mycobacterial genome) (4, 9, 18, 20, 27) and can detect the presence of fewer than 20 mycobacteria added to samples prior to extraction of DNA (4, 8, 9, 12, 20, 24). Those systems which amplify either a target sequence present in multiple copies in the *M. tuberculosis* genome (8, 9, 14, 18, 27) or rRNA from *M. tuberculosis* after reverse transcription (5×10^3 copies per mycobacterium) (4) may prove to be more sensitive than systems based on the detection of single copy sequences, such as the gene coding for the 65-kDa antigen used as a target in this study. In this context, Thierry et al. (27) demonstrated that a system based on the detection of the repetitive sequence IS6110 (10 to 15 copies per genome) was approximately 10-fold more sensitive than that of a system based on the detection of the 65-kDa antigen which was similar to the standard amplification protocol used in our study. Further work is required to compare the relative sensitivities of these different systems and to determine the extent to which the use of a reamplification protocol might increase their sensitivity.

For two different patients diagnosed as not having tuberculosis, one of three samples was positive when the standard amplification protocol was used. Because no evidence for contamination of the reagents used for amplification were observed in this series (i.e., amplification of samples containing no DNA always gave negative results), these presumably false-positive results are likely to have resulted from the contamination of the samples during the extraction of DNA. It is noteworthy, however, that the use of the reamplification protocol did not result in additional false-positive results in this study. The contamination of samples might be minimized by developing simpler procedures for extracting DNA. In this study, we used as starting material for the purification of DNA clinical samples which had been decontaminated for culture, thus permitting the direct comparison of results obtained with the amplification procedure and standard culture techniques. A disadvantage of this approach is that such samples have been treated with SDS. The presence of this potent inhibitor of *Taq* polymerase (11) may explain why samples prepared by the shorter proteolytic digestion method often inhibited the amplification of purified mycobacterial DNA and necessitated the use of additional purification steps before DNA amplification. It is possible that simpler extraction procedures may work if samples which have not been decontaminated are used as a starting material and thereby diminish the risk of sample contamination.

This study demonstrates that the sensitivity of the detection of *M. tuberculosis* by use of the polymerase chain reaction is highly dependent on the techniques used to prepare the DNA for amplification and the type of amplification protocol employed. In the reamplification procedure described here, the sensitivity of the test appears to be superior to that of routine culture and this approach does not appear to adversely affect its specificity. These findings support the conclusion that this approach will prove to be useful in the rapid detection of *M. tuberculosis* in clinical samples containing only small numbers of mycobacteria. It should be stressed, however, that the reamplification procedure described here is relatively expensive (for example, the

reagents alone necessary for amplification cost more than \$5 per sample) and labor intensive. Furthermore, this test will not obviate the need for cultures, the only established means of defining antibiotic susceptibility. Thus, further studies are needed to define the role of this test in clinical practice.

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