JOURNAL OF CLINICAL MICROBIOLOGY, May 1996, p. 1209–1215 Vol. 34, No. 5
0095-1137/96/$04.00+0

Sequence Capture-PCR Improves Detection of Mycobacterial DNA in Clinical Specimens

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Received 22 November 1995/Returned for modification 3 January 1996/Accepted 22 February 1996

The rapid identification of mycobacterial DNA in clinical samples by PCR can be useful in the diagnosis of tuberculous infections, but several large studies have found that the sensitivity of this approach is not better than that of culture. In order to improve the sensitivity of detection of mycobacterial DNA in clinical specimens from patients with paucibacillary forms of tuberculosis, we have developed a procedure permitting the specific capture of mycobacterial DNA in crude samples prior to amplification, thereby concentrating the target sequences and removing irrelevant DNA and other potential inhibitors of the amplification reaction (sequence capture-PCR). By using this approach to capture and amplify two different sequences specific for organisms of the *Mycobacterium tuberculosis* complex (IS*6110* and the direct repeat region), it was possible to detect as little as one genome of mycobacterial DNA in samples containing up to 750 μg of total DNA, representing a 10- to 100-fold increase in sensitivity compared with that obtained by purifying total DNA prior to amplification. Detection of the IS*6110* sequence in pleural fluid samples from patients with tuberculous pleurisy by sequence capture-PCR gave positive results in 13 of 17 cases, including 3 of 3 culture-positive samples and 10 of 14 culture-negative samples. In contrast, when total DNA was purified from these samples by adsorption to a silica matrix prior to amplification, only the three culture-positive samples were positive by PCR. The sensitivity of detection of the direct repeat sequence in these samples by sequence capture-PCR was similar to that of IS*6110* and, in addition, permitted immediate typing of the strains from some patients. We conclude that sequence capture-PCR improves the sensitivity of detection of mycobacterial DNA in paucibacillary samples. This approach should be useful in detecting rare target sequences from organisms implicated in other pathologic processes.

Tuberculosis remains a major worldwide health problem and, because of its protean manifestations, must be considered in the differential diagnosis of numerous patients (2, 3, 15). Unfortunately, the standard methods used in the diagnosis of tuberculosis have several important limitations. Microscopic identification of acid-fast mycobacteria is insensitive and, when positive, does not permit identification of the species of mycobacterium identified. Mycobacterial culture may require several weeks to obtain positive results and frequently gives negative results for paucibacillary forms of tuberculosis. These limitations create a variety of problems in the clinical management of patients suspected of having tuberculosis and may lead to delays in initiating appropriate treatment and/or the use of invasive procedures to firmly establish or exclude this diagnosis.

In an effort to overcome these problems, a number of laboratories have evaluated the usefulness of the detection of mycobacterial DNA in clinical samples by techniques based on PCR in the diagnosis of tuberculosis. Several large studies have found that this approach can be used to rapidly diagnose tuberculous infections with a sensitivity that is equivalent to or somewhat less than that of mycobacterial culture (7, 8, 12, 13, 21, 25, 28). Unfortunately, most studies have found that not all samples which are direct examination negative and culture positive are also positive by PCR and that only a minority of culture-negative samples from patients ultimately shown to have tuberculosis are positive by this approach. Thus, in clinical situations in which improvements in diagnostic techniques are most needed (paucibacillary forms of tuberculosis), current PCR techniques have not been of considerable help.

Two obstacles have limited the sensitivity of this approach in the diagnosis of tuberculosis. First, the presence of too much DNA can inhibit PCR, and many clinical specimens (blood, bronchoalveolar lavage fluids, pleural fluids, bone marrow aspirates, tissue biopsies, etc.) contain large numbers of immune and inflammatory cells, a source of large amounts of DNA. Thus, it is necessary to dilute these samples (and consequently the mycobacterial DNA present) prior to amplification. Second, to obtain optimal sensitivity, it is necessary to eliminate inhibitors of the amplification reaction present in clinical samples. Unfortunately, the multistep processes required to obtain highly purified DNA are difficult to apply in routine practice.

To overcome these problems, we have developed an approach that permits the specific capture of mycobacterial DNA in crude samples containing large numbers of human cells, thereby permitting the removal of irrelevant DNA and potential inhibitors present in the original sample prior to amplification. Using this technique, we have demonstrated that this enrichment leads to the anticipated increase in the sensitivity of detection of mycobacterial DNA in standard samples containing known amounts of mycobacterial DNA and in paucibacillary clinical samples from patients with tuberculous pleurisy.

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MATERIALS AND METHODS

Materials. The oligonucleotides used for amplification of a 123-bp fragment of the IS*6110* insertion element (IS1 and IS2) and the direct repeat (DR) region of *M. tuberculosis* (DRa and DRb) have been previously described (11, 16, 23). Oligonucleotides IS3 (13) and DRc (5′-CCCAAAACCCCGAGAGGG) were used for the detection of amplification products by Southern blotting. Capture oligonucleotides for the IS*6110* sequence were Cap-1, 5′-AAAAACGAACG GCTGATGACCAAACTC, and Cap-2, 5′-AAAAAGGAGGTGGCCATCGT GGAAG. These oligonucleotides are complementary to IS*6110* sequences 97 bases upstream of that recognized by IS1 and 39 bases downstream of that recognized by IS2 and therefore do not recognize products amplified by IS1 and IS2. The oligonucleotides were positioned to hybridize with regions devoid of inverted repeat sequences identified by using the STEMLO program. Because the repetitive sequence in the DR region is only 36 bp long, the oligonucleotides used for the capture of DR sequences were identical to the oligonucleotides, DRa and DRb, used to amplify this region, except that 5 adenosine residues were added to the 5′ ends. All oligonucleotides were synthesized by Genset (Paris, France). Capture oligonucleotides were synthesized with a biotinylated 5-carbon spacer arm attached to the 5′-end and were purified by high-pressure liquid chromatography. In preliminary experiments evaluating the efficiency of capture of biotinylated oligonucleotides by avidin-coupled magnetic beads, capture oligonucleotides were labelled at their 3′ ends with [α-^{32}P]dCTP (Amersham, Slough, United Kingdom) by using terminal deoxytransferase (10).

To evaluate the presence of inhibitory substances in amplification reactions, an internal standard in which the sequences recognized by IS1 and IS2 were added to opposite ends of a 403-bp fragment of plasmid pGEM-3 and which generated a 443-bp fragment when amplified by primers IS1 and IS2 was constructed. Serial dilutions were tested, and the last dilution which gave consistently positive results when amplified in the presence of 0.5 μg of highly purified human DNA (5 μl of a 10^{-8} dilution) was used to verify that specimens could support amplification.

DNA from *M. tuberculosis* H37Rv was purified and quantified by densitometry, and serial dilutions were prepared by using a solution containing 100 μg of human DNA (human placental DNA; Sigma, St. Louis, Mo.) per ml to produce standards containing 0.1 to 100 genomes per 5 μl, assuming a molecular mass of 2.5×10^9 Da for 1 mycobacterial genome (e.g., 1 genome = 3 to 4 fg). To evaluate techniques used for the extraction of mycobacterial DNA, *M. tuberculosis* H37Rv was grown in suspension culture in 7H9 medium, organisms were quantified by limiting-dilution culture, and aliquots containing <10 viable organisms were added to tissues prior to DNA extraction.

Pleural fluid samples. Samples of pleural fluid also submitted for mycobacterial culture were obtained from 17 patients with tuberculous pleurisy evaluated at Hôpital Tenon, Paris, France (age, 38.6 ± 14.5 years; 13 men and 4 women). For 11 patients, the diagnosis was established on the basis of positive culture(s) for *M. tuberculosis* of samples of sputum, pleural fluid, and/or pleural biopsies. For six patients, all mycobacterial cultures were negative and the diagnosis was based on the demonstration of caseating granulomas in pleural biopsies. Cultures of pleural biopsies, performed on seven patients, were positive in four cases. None of the patients had a positive serologic test for human immunodeficiency virus, and none had any other disease known to produce immunosuppression. The volume of pleural fluid obtained from these patients was 5 to 1,000 ml (average, 185 ± 319 ml). Acid-fast staining and mycobacterial culture were performed as previously described (22), except that sputum samples were decontaminated by treatment with 4% sodium hydroxide.

To serve as controls, pleural fluid samples from 25 patients (age, 56.6 ± 15.4 years; 21 men and 4 women) without tuberculosis were also evaluated. The causes of pleural effusion in these patients were as follows: metastatic carcinoma (n = 13), mesothelioma (n = 2), parapneumonic pleural effusion (n = 8), and lymphoma (n = 2). The volume of pleural fluid obtained from these patients ranged from 8 to 1,000 ml (average, 132 ± 253 ml). In seven cases, two different aliquots of pleural fluid were used as control samples.

Solubilization of samples. Pleural fluid samples were centrifuged (2,240 × g; 30 min). Cell pellets or fragments of tissue biopsies were suspended in 500 μl of 100 mM Tris-HCl containing 150 mM NaCl and 50 mM EDTA (pH 7.4), and transferred to 2-ml screw-cap tubes (Eppendorf, Freemont, Calif.) containing 0.5 ml of 0.1-mm-diameter glass microspheres (Biospec Products, Bartlesville, Okla.) and 50 μl of 20 mg of proteinase K (Interchim, Montluçon, France) per ml. Samples were agitated (Mini-BeadBeater; Biospec) for 50 s, allowed to digest overnight at 50°C (Thermomixer; Eppendorf), and agitated again for 50 s, and the supernatant (crude extract) was recovered by centrifugation. Preliminary experiments performed with samples containing small numbers of intact mycobacteria demonstrated that this procedure was highly efficient in releasing mycobacterial DNA.

The DNA in crude extracts was measured by spectrofluorometric assay, as previously described (5). An aliquot containing 5 μg of DNA was removed, and DNA was purified by adsorption to a silica matrix (Geneclean II; BIO 101, Inc., La Jolla, Calif.) as previously described (4, 12). Purified DNA was eluted from the silica matrix into 30 μl of distilled water, and 10-μl aliquots were used for amplification.

Sequence capture. Crude extracts from tissues and cells (final volume, 0.55 ml, containing up to 750 μg of total DNA) were transferred to 1.5-ml Eppendorf tubes, heated at 100°C for 10 min, and cooled to 0°C on ice, and 0.2 ml of 3.75 M NaCl–2.5 pmol each of biotinylated capture oligonucleotides Cap-1 and Cap-2 was added (final volume, 0.75 ml in 1 M NaCl). Tubes were incubated with agitation (Thermomixer) at 60°C for 3 h to allow hybridization. Ten microliters of M-280 Streptavidin Dynabeads (Dynal, Oslo, Norway), washed according to the manufacturer's instructions, was added, and the incubation was continued for 2 h at 20°C. Magnetic beads were captured (Dynal magnetic-particle concentrator), washed twice with 10 mM Tris-HCl–0.1 mM EDTA (pH 8), and resuspended in water. Two aliquots, each containing 5 μl of beads in 10 μl of water, were used for amplification. Capture of the DR region was performed by analogous techniques, except that the Cap-DRa and Cap-DRb oligonucleotides were used and hybridization performed at 42°C. The procedure is summarized in Fig. 1.

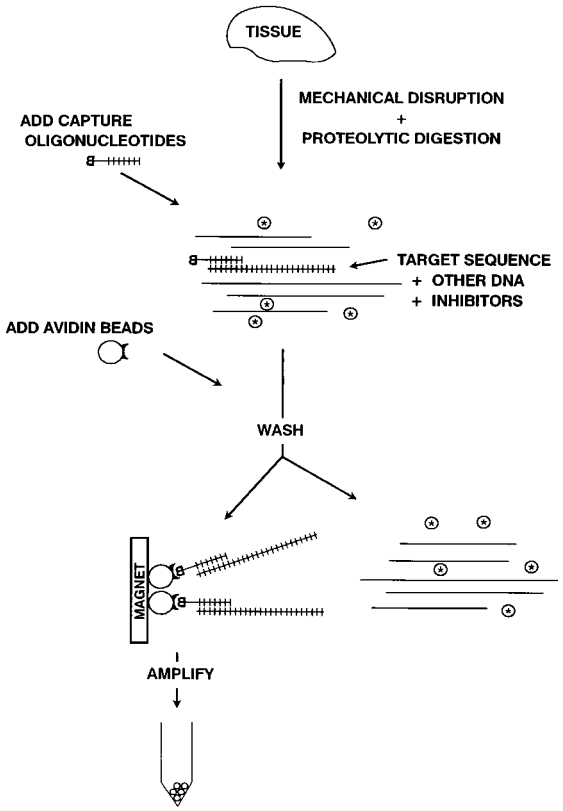

FIG. 1. Schematic representation of sequence capture-PCR. DNA is liberated from tissues or cells, producing a crude extract containing the specific target DNA sequence (hatched bar), human DNA (lines), and potential inhibitors of the amplification reaction (stars). The target sequence is specifically captured by the sequential addition of biotinylated capture oligonucleotides and avidin-coupled magnetic beads. The beads are added directly to the amplification reaction mixture.

Amplification and detection of mycobacterial DNA. Samples for amplification (see above) were suspended in a final volume of 45 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 100 μg of gelatin per ml; 0.2 mM (each) dATP, dGTP, dCTP, and dUTP; 12.5 pmol of each oligonucleotide primer; and 1 U of uracil-*N*-glycolase (Gibco BRL, Gaithersburg, Md.). Samples were incubated at 37°C for 10 min, heated to 95°C for 10 min, and cooled to 80°C in a thermal cycler (Perkin-Elmer, Norwalk, Conn.). Five microliters of a solution containing 1 U of *Taq* DNA polymerase (Appligene, Illkirch, France) was added by using a positive-displacement pipette prior to amplification. For amplification of the IS*6110* insertion element (oligonucleotides IS1 and IS2), the cycling parameters were 95°C for 40 s, 65°C for 40 s, and 72°C for 15 s for 50 cycles. For amplification of the DR region (oligonucleotides DRa and DRb), 2.0 mM $MgCl_2$ was used; the cycling parameters were 95°C for 40 s, 55°C for 40 s, and 72°C for 15 s for 50 cycles. Amplification products were electrophoresed onto agarose gels and transferred to nylon membranes, membranes were hybridized with ^{32}P-labelled oligonucleotides, and positive signals were detected by autoradiography as previously described (23).

Mycobacterial typing. To type mycobacterial DNA amplified in clinical specimens, the spacer oligotyping method described by Kamerbeek et al. (16) was used. Briefly, a 5-μl aliquot of amplification products from positive reactions were reamplified for 25 cycles by using the DR primer set in which the DRa oligonucleotide was biotinylated at the 5′ extremity. Aliquots of the amplified

products were hybridized (60°C, 60 min) in a reverse line blotting assay (17) by using a membrane to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *Mycobacterium bovis* BCG had been covalently linked. Membranes were washed at 60°C to remove unbound amplification products and incubated with horseradish peroxidase-labelled streptavidin (Boehringer, Mannheim, Germany), and positive hybridization was revealed by reaction with ECL detection reagents and by exposure of ECL hyperfilm (Amersham, Hertogenbosch, The Netherlands).

Interpretation of results. In experiments evaluating clinical samples, each sample of pleural fluid from a patient with tuberculosis was processed in parallel with four control samples during all steps of the procedure (solubilization of DNA, purification of DNA by sequence capture and adsorption to silica matrix, and amplification). Two types of control specimens, spleen fragments from Wistar rats and pleural fluid samples from patients without tuberculosis, were used. Two identical aliquots of DNA purified by sequence capture or silica matrix adsorption from the same sample were amplified in all cases. Samples were considered positive if one or both of the reactions gave a positive signal on autoradiography. Statistical comparisons were made by using the χ^2 test.

RESULTS

Optimization of PCR. To minimize false-positive results due to carryover of amplified products from prior reactions, all PCRs were performed with dUTP instead of dTTP, and new reaction mixtures were pretreated with uracil-*N*-glycosylase prior to amplification (19). After optimization of reaction conditions, positive results were obtained for amplification of the IS*6110* fragment in 55 of 60 samples containing one genome of DNA from *M. tuberculosis* in 500 ng of human DNA (final volume, 50 μl), 28 of 60 samples containing as little as 0.1 genome, and 0 of 60 samples without mycobacterial DNA. This sensitivity is similar to that we obtained by amplifying this sequence with dTTP (23) and approaches the maximal theoretical sensitivity of the test. (Assuming that *M. tuberculosis* H37Rv contains 15 copies of the IS*6110* sequence and that DNA was fragmented during purification such that each sequence was on a separate fragment, 78 of 100 samples containing 0.1 genomes would contain an amplifiable target.) As previously reported (18), optimal sensitivity was strictly dependent on the total amount of DNA present. When one genome of mycobacterial DNA was added to <1 μg of human DNA, 10 of 10 amplifications were positive, but 3 of 10 and 0 of 5 reactions were positive when the same amount of mycobacterial DNA was amplified in the presence of 2 and 5 μg of human DNA, respectively.

Development of techniques for sequence capture-PCR. Because the presence of excess human DNA impairs the sensitivity of detection of mycobacterial DNA, we developed an approach to selectively purify mycobacterial DNA prior to amplification. Commonly, biotinylated oligonucleotides are attached to avidin-coated beads and subsequently incubated with denatured DNA containing sequences to be captured (direct capture). Positive results can be obtained by this approach for samples containing large amounts of mycobacterial DNA (≥100 genomes). We found, however, that direct capture rarely gave positive results for samples containing 10 or fewer mycobacterial genomes (data not shown), and this technique was abandoned in favor of the two-step capture procedure depicted in Fig. 1.

To ensure that all captured sequences are present in the amplification reaction mixture, it is desirable to directly add magnetic beads containing the captured sequences to the PCR reaction mixture. The addition of up to 5 μl of magnetic beads had no deleterious effect on the amplification of mycobacterial DNA, although larger amounts of beads had progressively prominent inhibitory effects. Thus, capture was performed with 10 μl of beads; beads were subsequently divided into two equal aliquots (5 μl each) prior to amplification. This amount of magnetic beads could completely bind up to 5 pmol of each capture oligonucleotide, but the binding of larger amounts of oligonucleotides was incomplete (Fig. 2). The efficiencies of capture of small amounts of mycobacterial DNA (≤10 genomes) by using 1 and 2.5 pmol each of the two biotinylated capture oligonucleotides were compared and found to be equivalent (data not shown). These results indicate that the use of 2.5 pmol of each oligonucleotide was sufficient to ensure that the concentration of capture oligonucleotides was not a limiting factor in the efficient capture of mycobacterial DNA.

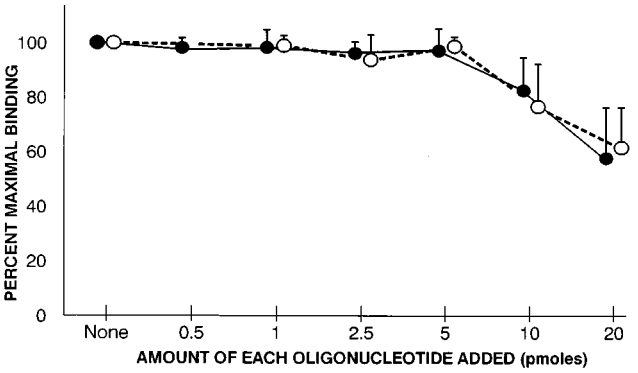

FIG. 2. Binding of capture oligonucleotides by avidin-coupled magnetic beads. Biotinylated capture oligonucleotides were labelled with ^{32}P at their 3′ ends by using terminal transferase, and tracer amounts of radiolabelled oligonucleotide were incubated with 10 μl of avidin-coupled magnetic beads for 2 h at 20°C in the absence (None) or presence of the indicated amounts of each unlabelled capture oligonucleotide, Cap-1 and Cap-2 (solid symbols; $n = 4$) or Cap-DRa and Cap-DRb (open symbols; $n = 3$). Data are the means ± standard deviations of the maximum percentage of oligonucleotide bound, which represented >85% of total radioactivity.

Numerous other factors affecting the efficiency of sequence capture (e.g., solubilization of DNA, composition of the hybridization solution, and times and temperatures during hybridization and binding of oligonucleotides to beads) were also evaluated. To test the overall sensitivity of the conditions defined in these studies, fragments of animal tissues or human immune and inflammatory cells obtained by centrifugation of pleural fluid samples were digested by the established protocol and small amounts of mycobacterial DNA were added to some samples prior to performing capture and subsequent amplification of the IS*6110* sequence. In these studies, 4 of 4 samples containing 100 mycobacterial genomes, 27 of 29 samples containing 10 mycobacterial genomes, and 8 of 13 samples containing 1 mycobacterial genome gave positive results, whereas none of the samples containing no added mycobacterial DNA was positive (Table 1). The positive samples used in these studies contained up to 750 μg of DNA. Thus, it was possible to detect mycobacterial DNA in samples containing as little as 0.001 mycobacterial genome per μg of total DNA, representing a 10- to 100-fold increase in sensitivity over that obtained by amplifying samples without prior enrichment of mycobacterial DNA.

Detection of mycobacterial DNA in pleural fluid samples from patients with tuberculous pleurisy. To determine whether the improved sensitivity of the sequence capture technique would improve the detection of mycobacterial DNA in clinical samples, it was important to use specimens containing only small numbers of mycobacteria. Pleural fluid samples from patients with tuberculous pleurisy were chosen for this purpose. Compatible with the results for prior series (6, 9), mycobacteria were not observed in pleural fluid samples from patients with tuberculous pleurisy by acid-fast staining and only 3 of 17 of these samples were positive by culture (Table 2). For

TABLE 1. Comparison of the sensitivities of sequence capture-PCR in detecting two different mycobacterial sequences, IS*6110* and the DR region[a]

Type of sample	No. of samples positive/no. tested	
	IS*6110*	DR region
Animal tissues or human cells with purified mycobacterial DNA added[b]		
100 genomes	4/4	ND[c]
5–10 genomes	27/29	25/26
1–2 genomes	8/13	2/6
Pleural fluids from patients with tuberculosis[d]	11/15	10/15
Control tissues	0/34	0/25

[a] $P > 0.3$ for all comparisons between IS*6110* and the DR region by Fisher's exact test.
[b] The total DNA was ≤750 μg.
[c] ND, not done.
[d] Only samples for which sequence capture-PCR using both systems was performed.

the detection of mycobacterial DNA by PCR, fluid samples obtained by thoracentesis were centrifuged and DNA was extracted from the cell pellet by mechanical disruption and proteolytic digestion. DNA was purified from an aliquot of the sample by adsorption to a silica matrix, and the remainder of the DNA, up to a limit of 750 μg (total), was used for sequence capture (375 ± 278 μg per sample; $n = 17$).

When total DNA purified by adsorption to a silica matrix was used for amplification of the IS*6110* sequence by the IS1 and IS2 primer pair, only 3 of the 16 samples evaluated were positive; the positive samples corresponded to those that were also positive by culture. To ensure that negative samples could support amplification, an internal standard that generates a 443-bp product when amplified by the IS1 and IS2 oligonucleotides was added to an identical aliquot of each sample prior to amplification. The presence of an amplification product of the expected size was observed in 16 of 16 samples, indicating that the presence of inhibitory substances could not explain the negative results obtained with these samples.

In contrast, when DNA was enriched for mycobacterial DNA by the sequence capture technique prior to amplification of the IS*6110* sequence, positive results were obtained for 13 of 17 samples from patients with tuberculous pleurisy, including the 3 samples that were positive by culture and 10 of the 14 samples that were culture negative ($P < 0.01$; comparing results for DNA purified by sequence capture and adsorption to silica). It is noteworthy that for six of these patients, mycobacteria were never isolated by culture from any specimen submitted. For three of these culture-negative patients, pleural fluid samples gave positive results by sequence capture-PCR; these findings represented the only direct evidence for the presence of *M. tuberculosis* in specimens from these patients.

For each sample from a patient with tuberculosis, three or four control samples were processed in parallel during all steps of the procedure (solubilization of samples, purification of mycobacterial DNA by sequence capture, and amplification). Two fragments of a rat spleen were evaluated to ensure that reagents were not contaminated with mycobacterial DNA and that no transfer of mycobacterial DNA occurred during processing. In addition, one ($n = 2$) or two ($n = 15$) samples of pleural fluid from patients without tuberculosis were tested to evaluate the possibility that mycobacterial DNA could be recovered from individuals without active tuberculosis. None of these control samples gave positive results (0 of 34 animal tissue and 0 of 32 nontuberculous pleural fluid samples).

Amplification of the DR sequence from the *M. tuberculosis* complex by sequence capture-PCR. Sequences present in multiple copies in the *M. tuberculosis* genome are particularly attractive targets for sequence capture. Although most strains of *M. tuberculosis* contain multiple copies of IS*6110*, some strains have few copies; in certain geographical areas, strains not containing IS*6110* are prevalent (27). Therefore, we also developed a sequence capture technique that targets an alternative mycobacterial sequence, the DR sequence. This sequence, which is also specific for the *M. tuberculosis* complex, is present as multiple highly conserved tandem repeats of 36 bp, each separated by a 35- to 41-bp spacer sequence (14). Unlike the DRs, each of these spacers has a unique sequence. Oligonucleotides DRa and DRb, which amplify fragments of variable lengths between two different DR sequences (including the intervening spacer and DR sequences), were used to amplify this region (16).

When samples containing known amounts of purified mycobacterial DNA in 500 ng of human DNA were amplified, positive results were obtained for 11 of 11 samples containing 2 to 10 mycobacterial genomes, 17 of 28 samples containing 1 genome, and 0 of 9 samples containing 0.1 genome. The lower-level sensitivity of the DR system compared with that of the IS*6110* system for the detection of purified mycobacterial DNA is expected. Unlike the IS*6110* sequence, which is dispersed in multiple copies throughout the mycobacterial genome of the mycobacterial strain used as a standard in these studies, the repeated DR sequences are present at a single locus and therefore are likely to be present on a single DNA fragment. Thus, at limiting dilutions (≤1 genome per sample), individual aliquots are less likely to contain fragments with the DR sequence than fragments containing the IS*6110* sequence.

When the sequence capture-PCR protocol was used, however, marked differences in sensitivity between the DR and

TABLE 2. Comparison of the detection of mycobacteria in clinical samples by standard bacteriological techniques and amplification of mycobacterial DNA

Patients[a]	No. of positive sputum samples/no. tested		Result with pleural fluid[b]			
			Bacteriology		Amplification of IS*6110*	
	Acid-fast stain	Culture	Acid-fast stain	Culture	Silica adsorption	Sequence capture
1	0/3	3/3	−	+	+	+
2	0/3	0/3	−	+	+	+
3	0/2	0/2	−	+	+	+
4	0/3	1/3	−	−	−	+
5	0/3	2/3	−	−	−	+
6‡	0/3	0/3	−	−	−	+
7	3/3	3/3	−	−	−	+
8‡	0/3	0/3	−	−	−	+
9	0/3	1/3	−	−	−	+
10	3/3	3/3	−	−	−	+
11‡	0/3	0/3	−	−	−	+
12	0/3	1/3	−	−	−	+
13	0/3	3/3	−	−	ND	+
14‡	0/3	0/3	−	−	−	−
15†	0/3	0/3	−	−	−	−
16‡	0/3	0/3	−	−	−	−
17‡	0/3	0/3	−	−	−	−

[a] †, patient for whom culture of pleural biopsy was positive; ‡, patient for whom all cultures submitted were negative for mycobacteria.
[b] +, positive result; −, negative result; ND, not done.

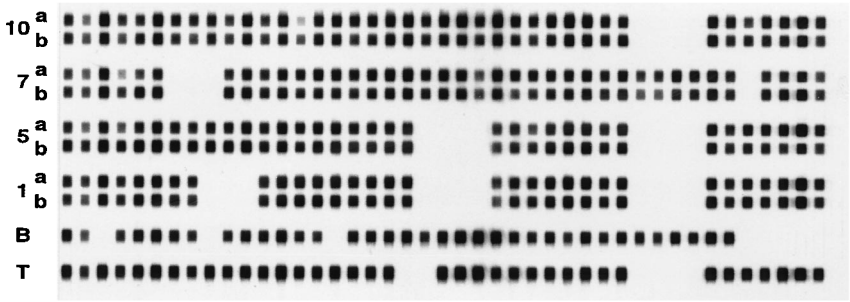

FIG. 3. Typing of mycobacterial strains by spoligotyping after sequence capture-PCR. Sequence capture-PCR targeting the DR region was performed as outlined in Fig. 1 on pleural fluid samples from patients with tuberculous pleurisy. For samples from individuals for which two independent reactions gave positive results, aliquots of the amplification products were reamplified by the DRa-DRb primer pair in which the DRa oligonucleotide was biotinylated. The amplification products were then hybridized to membranes to which synthetic oligonucleotides complementary to each of the 43 different spacers present in the DR cluster of *M. tuberculosis* H37Rv and/or *M. bovis* BCG had been covalently linked, and positive hybridization reactions were identified by detecting the presence of biotinylated amplification products using the ECL detection system (Boehringer Mannheim). Spacer oligonucleotides are displayed in numerical order from left to right on the membrane. Shown are the results for four of the five patients (patients 1, 5, 7, 8, and 10) for which the spoligotyping profiles from two independent reactions (a and b) were identical. Note that the profiles are unique for each patient and distinct from those obtained with DNAs from *M. tuberculosis* H37Rv (T) and *M. bovis* BCG (B).

IS*6110* systems were not observed. First, sequence capture-PCR targeting the DR sequence was performed on samples containing small amounts of mycobacterial DNA added to crude extracts of animal tissues or human immune and inflammatory cells containing up to 750 μg of human DNA. Positive results were obtained for 25 of 26 samples containing 10 mycobacterial genomes and 2 of 6 samples containing 1 mycobacterial genome, results not significantly different from those obtained by using the IS*6110* system (Table 1). Similarly, crude extracts of DNA recovered from pleural fluid samples of patients with tuberculous pleurisy, available from 15 patients evaluated by using the IS*6110* system, were also tested by the DR sequence capture technique. Positive results were obtained for 10 of 15 specimens, including all three samples that were culture positive. For 12 samples, the results were concordant between the two systems, although 2 samples positive by using the IS*6110* system were negative by using the DR system and 1 sample positive by using the DR system was negative by using the IS*6110* system.

Typing of mycobacterial strains after sequence capture-PCR. Although all strains of *M. tuberculosis* contain the DR sequence, the spacer sequences present are different for different strains. Kamerbeek et al. (16) have used this observation to develop a technique to type mycobacterial strains on the basis of the hybridization of amplification products of the DR region to a panel of synthetic oligonucleotides specifically recognizing different spacer sequences (spoligotyping). To determine whether the amplification products obtained from the pleural fluid samples of patients with tuberculous pleurisy were adequate to permit rapid typing, this approach was applied to these samples.

It has previously been shown that when extremely small amounts of mycobacterial DNA are used, amplification of only a portion of the DR region may occur, producing incomplete spoligotyping profiles. Although this is not a problem when DNA is extracted from cultured mycobacteria, it is a potential problem when spoligotyping is applied to mycobacterial DNA obtained from paucibacillary clinical samples such as those studied here. To guard against this possibility, typing was restricted to samples for which positive results were obtained for both of the independent amplification reactions and for which the spoligotyping profiles were identical for two independent reactions. These criteria were met for 5 of the 10 pleural fluid samples that were positive for mycobacterial DNA after amplification of the DR region, and the spoligotyping profiles are shown in Fig. 3. In each case, the profiles were distinct and different from that of *M. tuberculosis* H37Rv, the strain used as a positive control in these experiments. Thus, none of the patients was infected with the same mycobacterial strain, and in no case could positive results be explained by the inadvertent contamination of the sample with DNA from another patient or the control strain.

DISCUSSION

In this study, we have developed a new PCR-based strategy, sequence capture-PCR, that permits the rapid enrichment of mycobacterial DNA present in crude extracts of clinical samples prior to amplification and thereby results in a substantial increase in sensitivity of detection of mycobacterial DNA in these specimens. By using samples containing known amounts of DNA, this approach was shown to be 10 to 100 times more sensitive than are procedures in which total DNA is extracted prior to amplification. Furthermore, this improved sensitivity was shown to result in a much higher proportion of positive results when clinical samples from patients with tuberculous pleurisy were tested; only sequence capture-PCR permitted the detection and typing of mycobacteria in a majority of culture-negative specimens from patients with tuberculosis.

The specific capture of nucleic acids by immobilized oligonucleotides has numerous applications in molecular biology but has not found wide application in diagnostic tests. Muir et al. (20) used oligonucleotides coupled to magnetic beads to capture enteroviral RNA prior to reverse transcription-PCR. They found that although this method was simpler to perform, the sensitivity was similar to that obtained by traditional extraction techniques. We found, however, that when oligonucleotides recognizing mycobacterial DNA were directly coupled to beads (direct capture), the efficiency of capture of mycobacterial DNA was much less than that when the biotinylated oligonucleotides were hybridized to mycobacterial DNA in solution and subsequently bound to avidin-coated beads (two-step capture). The reasons that direct capture was less efficient were not studied, but it may result from poor diffusion of the immobilized oligonucleotides and/or steric interference by the large beads. In practice, two-step capture was no more difficult to perform; the only disadvantage is the risk that endogenous biotin could impair efficient binding of biotinylated oligonucleotides. Endogenous biotin was not found in clinical specimens of lungs, lymph nodes, pleural fluids, or

peripheral blood leukocytes. When present (e.g., biopsies of livers and kidneys), it could be removed by pretreating samples with avidin-Sepharose prior to capture (unpublished data).

Our study confirms prior reports (18) that the sensitivity of detection of rare target sequences by PCR is highly dependent on the amount of total DNA in the sample; the sensitivity of detection of mycobacterial DNA was clearly lower in samples containing more than 1 to 2 μg of total DNA in a 100-μl reaction mixture. Because many clinical samples, such as the pleural fluid samples studied here, contain several milligrams of DNA, only a small fraction of the sample can be used when total DNA is studied. In contrast, sequence capture-PCR eliminates essentially all cellular DNA, thereby permitting the analysis of all or the majority of the sample in a single reaction. We have demonstrated that mycobacterial DNA can be detected in a variety of clinical samples, including samples containing large amounts of DNA (e.g., sputum, tissue biopsies, and peripheral blood cells). In addition, sequence capture eliminates potential inhibitory substances present in crude samples. For example, we found that mycobacterial DNA present in tissues containing large amounts of hemoglobin or those extracted with 1% sodium dodecyl sulfate, both strong inhibitors of *Taq* polymerase, could be successfully amplified after sequence capture.

An important finding in the present study was the observation that sequence capture-PCR permitted the detection of mycobacterial DNA in the majority of culture-negative pleural fluid samples from patients with tuberculosis. Prior studies have reported detecting mycobacterial DNA in culture-negative specimens from patients with tuberculosis (7, 11–13, 19, 22, 24), indicating that nonviable organisms can be present in these samples because of the mycobactericidal action of inflammatory cells or loss of viability attendant with sample processing. Nevertheless, in previous studies by us and other groups in which total DNA was amplified, only occasional culture-negative samples gave reproducibly positive results. In contrast, sequence capture-PCR gave positive results for 10 of 14 culture-negative samples. For three of the patients studied here, the detection of mycobacterial DNA by sequence capture-PCR was the only direct evidence for the presence of *M. tuberculosis* in these patients, as multiple sputum, pleural fluid, and pleural biopsy cultures were negative.

Systems permitting the amplification of two different mycobacterial sequences, IS*6110* and the DR region, were developed in these studies. Both were shown to be highly efficient in detecting DNA from as few as 10 mycobacteria in 750 μg of total DNA, and the sensitivities of these two systems for the detection of mycobacterial DNA in tuberculous effusions were not different. These results suggest that sequence capture-PCR can be applied to a variety of different target sequences. Further studies will be needed to rigorously compare the sensitivities of the two systems described here in clinical practice, but two potential advantages of the DR system merit mention. First, the DR sequence is always present in organisms of the *M. tuberculosis* complex in multiple copies; strains not containing this sequence have not been identified. In contrast, the IS*6110* sequence is present in only one or two copies in many *M. tuberculosis* strains and strains lacking IS*6110* have been reported (1, 26, 27). Second, as confirmed in this study, amplification products generated by amplifying the DR region can be used to type the mycobacterial strain detected, thereby permitting rapid identification of community outbreaks or nosocomial infection. Current work in our laboratory is directed at automating the sequence capture-PCR procedure, thereby permitting routine clinical use of this highly sensitive approach.

ACKNOWLEDGMENTS

These studies were supported in part by grants from the Caisse Nationale de l'Assurance Maladie des Travailleurs Salariés (CNAMTS) and the Fondation pour la Recherche Médicale.

We thank Annelies Bunschoten for excellent technical assistance and Veronique Vincent-Lévy-Frébault for providing the standard mycobacterial DNA.

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