JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1993, p. 646–652
0095-1137/93/030646-07$02.00/0

Vol. 31, No. 3

Elimination of Contaminating DNA within Polymerase Chain Reaction Reagents: Implications for a General Approach to Detection of Uncultured Pathogens

ALBRECHT MEIER,[1] DAVID H. PERSING,[2] MARION FINKEN,[1] AND ERIK C. BÖTTGER[1]*

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 3000 Hannover 61, Germany,[1] and Section of Clinical Microbiology, Department of Laboratory Medicine and Pathology, Mayo Foundation, Rochester, Minnesota 55905[2]

Received 12 October 1992/Accepted 22 December 1992

Analysis based on comparisons of 16S rRNA sequences provides a rapid and reliable approach to identifying human pathogens. By directing oligonucleotide primers at sequences conserved throughout the eubacterial kingdom, bacterial 16S ribosomal DNA sequences of virtually any member of the eubacterial kingdom can be amplified by polymerase chain reaction and subsequently analyzed by sequence determination. Indeed, automated systems for broad-range amplification, sequencing, and data analysis are now feasible and may form the basis of the next generation of automated microbial identification systems. However, identification of pathogens by this strategy is hampered by the frequent contamination of reagents used for the amplification reaction, in particular *Taq* polymerase, with exogenous bacterial DNA. Here, we describe detailed investigations on the use of 8-methoxypsoralen and long-wave UV light to eliminate contaminating DNA in polymerase chain reaction reagents. The clinical utility of the developed procedure was demonstrated in a case of paucibacillary osteomyelitis, for which no specific bacterial agent had been cultured.

rRNA sequences are an appealing target for detection, identification, and classification of bacteria (45). Comparative analysis of the 16S rRNA sequences from different microorganisms revealed stretches of highly conserved primary structure and others with a considerable amount of variability. Several investigators have shown that organism-specific oligonucleotides can be directed successfully at the variable regions within the 16S rRNA (for examples, see references 1, 3, 14, 17, 34, 35, and 40). However, even when labelled with ^{32}P, these probes can detect no fewer than 10^3 to 10^4 cells. Oligonucleotide probes directed at rRNA are therefore not sensitive enough to detect small populations of bacteria. To improve the sensitivity of rRNA probes, several investigators have taken the approach of using the polymerase chain reaction (PCR) primed with genus- or species-specific oligonucleotides to amplify a ribosomal DNA (rDNA) gene fragment (2, 3, 5, 6, 16, 19, 28, 39, 43).

In 1989, we described the use of primers complementary to conserved sequences within the 16S rRNA to amplify and subsequently analyze by sequence determination rDNA of microorganisms (9, 15); other studies using this approach have since been reported (4, 12, 41, 44). In theory, broad-range eubacterial primers permit amplification of any bacterial 16S rDNA and offer advantages over more narrowly specific probes for detecting organisms whose identities are unknown. Such an approach would be particularly valuable for detecting unculturable eubacteria of unknown identity in disease in which there is circumstantial evidence for a bacterial etiology. However, this approach is hampered by problems due to contamination. Tiny amounts of PCR product carried over from one reaction to the next are a well-known cause of a false-positive results (23). In the case of bacterial 16S rDNA, additional contamination problems exist, as conventional contamination of the reagents by any sort of eubacterial DNA represents a serious problem. In particular, *Taq* polymerase has been found to be frequently contaminated with bacterial DNA (10, 29, 37). Obviously, methods directed at eliminating product carryover (13, 24, 27) are largely ineffective in this instance.

We describe here detailed investigations on the use of 8-methoxypsoralen to inactivate contaminating DNA in reagents used for broad-range amplification by PCR. The elimination of contaminating DNA facilitates definitive analysis of organisms by broad-range amplification. It is especially suited for detection and identification of small numbers of pathogens that are difficult or impossible to culture.

MATERIALS AND METHODS

Nucleic acid preparation. Genomic DNA from *Mycobacterium tuberculosis* H37 cultures was prepared by a lysozyme–proteinase K–sodium dodecyl sulfate procedure as described recently (6). The concentration of nucleic acids was determined spectrophotometrically at 260 nm and verified by judging the relative intensities of bands after electrophoresis on 0.8% (wt/vol) agarose gels. For estimation of the number of bacterial genomes, it was assumed that a single bacterium contains 5 fg of nucleic acid (6).

Nucleic acids were extracted from a patient's sample after homogenization. Briefly, the cell pellet was dissolved in guanidine isothiocyanate lysis solution (8) and transferred to a 1.5-ml screw-top plastic microcentrifuge tube containing acid-washed glass beads with a diameter of 100 μm (Sigma, Deisenhofen, Germany). An equal volume of buffered phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) was added, and the samples were placed for 5 min in a tissue disintegrator (H. Mickle, Gomshall, Surrey, United Kingdom) at maximal speed to disrupt the cells. After centrifugation in a microcentrifuge for phase separation, the aqueous phase was transferred to a fresh tube and the organic phase was reextracted with guanidine isothiocyanate lysis solution.

* Corresponding author.

TABLE 1. Oligonucleotides used

Oligonucleotide	Sequence (5′ to 3′)	16S rRNA[a] position	Strand orientation	Melting temp (°C)	Reference
246	AGA GTT TGA TCC TGG CTC AG	8–28	+	60	6
240	CCG TCA ATT CCT TTG AGT TT	928–908	−	56	15
264	TGC ACA CAG GCC ACA AGG GA	1046–1027	−	64	6
244	CCC ACT GCT GCC TCC CGT AG	361–342	−	68	15

[a] Corresponding *Escherichia coli* position.

The combined aqueous phases were reextracted with buffered phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and nucleic acids were precipitated with 0.05 volume of 3 M sodium acetate (pH 5.2) and 1 volume of cold isopropanol. The pellet was washed twice with cold 70% EtOH (vol/vol), dried, and resuspended in 50 μl of H_2O. A 1-μl aliquot was used for PCR. HeLa cells and a lymph node from a patient with lymphoma were used as sources of human control DNA.

PCR amplification. The PCR was performed with a total volume of 50 μl with 1.25 U of native *Taq* polymerase (Perkin-Elmer Cetus, Überlingen, Germany) in 50 mM KCl–10 mM Tris-HCl (pH 8.3)–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–200 μM each deoxynucleoside triphosphates (dATP, dCTP, dGTP, and dTTP)–50 pmol of each of the two primers. The 45-μl mixture was covered with 50 μl of light mineral oil (Perkin-Elmer Cetus). DNA was always added last in a 5-μl volume after or before the complete PCR mixture was irradiated with UV light.

The thermal profile involved 39 cycles of denaturation at 93°C for 1 min, primer annealing at 56 or 60°C for 2 min, and extension at 72°C for 2 min. The oligonucleotides used for amplification are shown in Table 1. Primers 246 and 240 represent broadly reactive eubacterial 16S rDNA primers; primer 264 is specific for mycobacteria. A combination of primers 246 and 240 will result in amplification of any eubacterial 16S rDNA (15), and a combination of primers 246 and 264 will specifically amplify mycobacterial nucleic acids (6).

8-Methoxypsoralen (Sigma, Munich, Germany) was dissolved in dimethyl sulfoxide (DMSO) to result in a stock of 2.5 mg/ml. Appropriate dilutions of 8-methoxypsoralen in DMSO were prepared to result in a final concentration of 1% DMSO in the PCR assay, as more than 5% DMSO was found to inhibit the PCR. The 0.5-ml transparent plastic microcentrifuge tubes (Sarstedt; Nümbrecht, Germany) were irradiated from the side at a distance of 1 cm with long-wave (366 nm) UV at room temperature by using a conventional UV hand monitor (Hanau Fluotest; Heraeus, Osterode, Germany). Pyrogen-free water for injection (Pfrimmer, Erlangen, Germany) was autoclaved twice and used in all procedures. The buffers used for PCRs were autoclaved twice, and plugged pipette tips were used throughout all the procedures.

Hybridization and analysis of amplified samples. Aliquots of amplified samples (5 μl) were electrophoresed through 0.8% agarose gels, and the DNA was visualized by UV fluorescence after ethidium bromide staining.

For slot blot analysis, 10-μl aliquots of amplified samples were denatured by the addition of 100 μl of 0.5 M NaOH–25 mM EDTA, incubated for 30 min at room temperature, and neutralized with 110 μl of 2 M ammonium acetate. The samples were loaded into wells of a manifold (Minifold II; Schleicher & Schuell, Dassel, Germany) fitted with a nitrocellulose membrane (BA 85; Schleicher & Schuell) previously wetted in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Each well was rinsed with 0.4 ml of 10× SSC, and the membranes were heated for 2 h at 80°C. Hybridization was performed by standard techniques. In brief, the membranes were rinsed in 6× SSC and prehybridized in 6× SSC–5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin)–0.1% sodium dodecyl sulfate–100 μg of tRNA (Boehringer, Mannheim, Germany) ml^{-1} for 1 to 2 h at 50°C. Hybridization was performed overnight with the same solution containing 2×10^6 to 1×10^7 cpm of 5′-end-labeled oligonucleotide ml^{-1}. The blots were washed three times for 20 min each at room temperature in 6× SSC and three times for 20 min each in 6× SSC–0.1% sodium dodecyl sulfate at the melting temperature, (in degrees Celsius) of the oligonucleotide. The blots were exposed to XAR-5 films (Eastman Kodak Co., Rochester, N.Y.) in cassettes containing an amplifying screen for various lengths of time at −70°C.

The oligonucleotide probe was labeled in 15 μl of 50 mM Tris (pH 7.6)–10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM spermidine–0.1 mM EDTA with 8 pmol of synthetic oligonucleotide, 100 μCi of [γ-^{32}P]ATP (specific activity, >5,000 Ci/mmol; Amersham, Braunschweig, Germany), and 10 U of T4 polynucleotide kinase (New England BioLabs, Schwalbach, Germany) by incubation for 1 h at 37°C. The kinase was inactivated by incubation for 10 min at 75°C, the nucleotides were extracted by phenol, and the labeled oligonucleotide was purified and separated from residual [γ-^{32}P]ATP with push columns (Stratagene, Heidelberg, Germany). The resulting specific activity of the labeled oligonucleotide was 2×10^8 cpm/μg.

Direct sequence determination of amplified gene fragments was performed by using biotinylated primer 246 in PCR and magnetic beads coated with streptavidin (Dynabead; Dynal, Hamburg, Germany) as described previously (20).

RESULTS AND DISCUSSION

Contamination of PCR reagents with exogenous bacterial DNA represents a serious problem for amplification of eubacterial 16S rDNA sequences with broad-range eubacterial 16S rDNA primers. It was through the use of such broad-range primers that it was found that *Taq* polymerase is frequently contaminated with bacterial DNA (10, 29, 37). We and others (37) have been unable to eliminate or reduce the apparent contamination by using previously described procedures (21, 36). Indeed, it was found that the recommended treatments frequently damage *Taq* polymerase (26).

Psoralens are known to intercalate into double-stranded nucleic acids and form a covalent interstrand cross-link after photoactivation with light at 320 to 400 nm. Jinno and

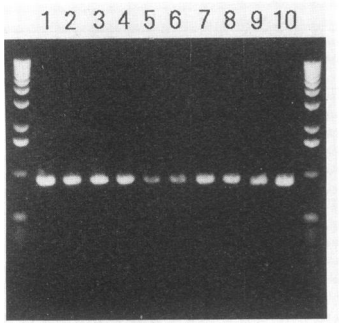

FIG. 1. Effect of 8-methoxypsoralen on the amplification reaction. Bacterial nucleic acids (2.5 pg) were added to reaction mixtures 1 to 10. Primer 246 and reverse primer 240 were used for amplification and to direct the synthesis of a 920-bp fragment. In addition, 1% DMSO was added to reaction mixtures 3 and 4; 50 μg of 8-methoxypsoralen per ml was added to reaction mixtures 5 and 6; 25 μg of 8-methoxypsoralen per ml was added to reaction mixtures 7 and 8; and 12.5 μg of 8-methoxypsoralen per ml was added to reaction mixtures 9 and 10. Amplification products were resolved by electrophoresis in 0.8% agarose gels. The molecular mass marker is the 1-kb ladder (GIBCO-BRL, Eggenstein, Germany).

coworkers originally suggested the use of 8-methoxypsoralen to extinguish the template activity of contaminating DNAs (21). The recommended concentrations of 8-methoxypsoralen (100 μg/ml) and irradiation with long-wave (365 nm) UV light for 1 h, however, were detrimental to the *Taq* polymerase and/or the target DNA to be amplified (Fig. 1 and 2). Here, we report our detailed investigations of the use of 8-methoxypsoralen to eliminate DNA contamination of PCR reagents and characterize several effects of a combined 8-methoxypsoralen-UV treatment on DNA template and *Taq* polymerase activity.

To determine whether 8-methoxypsoralen itself without UV treatment affects the amplification reaction, 8-methoxypsoralen at different concentrations was added to the PCR mixture prior to the addition of 2.5 pg of target DNA (corresponding to 500 bacterial genomes). As can be seen in Fig. 1, lanes 5 and 6, 8-methoxypsoralen at a concentration of 50 μg/ml severely inhibits amplification of the target DNA, indicating that this concentration of 8-methoxypsoralen is detrimental to the amplification reaction. As 8-methoxypsoralen at 25 μg/ml (Fig. 1, lanes 7 and 8) did not interfere with the amplification of target DNA, this concentration was chosen for further experiments.

Next, we optimized the time of UV treatment. As can be seen in Fig. 2A and B (lanes 1 and 2), 8-methoxypsoralen at a concentration of 25 μg/ml without prior UV treatment does not affect amplification of contaminating DNA with broad-range 16S rRNA primers. Note that PCRs in Fig. 2A and B were performed without adding target DNA. Irradiating the 8-methoxypsoralen-containing reaction mixtures for 3, 4, 5, and 10 min (Fig. 2A and B, lanes 7 to 14) eliminated amplification of the contaminating DNAs. The effect of combined 8-methoxypsoralen and long-wave UV treatment on the activity of *Taq* polymerase was measured by adding 500 target DNA molecules to the reaction mixture after UV treatment. Figure 2C and D illustrate that UV treatment in excess of 5 min is detrimental to the enzyme. From these experiments, we conclude that 8-methoxypsoralen at a concentration of 25 μg/ml in combination with long-wave UV treatment for 3 to 5 min clearly eliminates contaminating

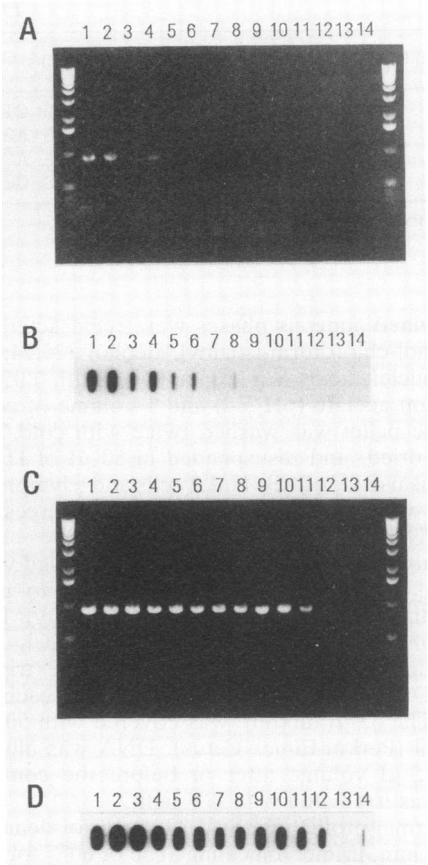

FIG. 2. Effect of UV treatment on the amplification reaction. Primer 246 and reverse primer 240 were used for amplification, and 8-methoxypsoralen was added at a final concentration of 25 μg/ml. (A) Reaction without added template DNA. Lanes: 1 and 2, no UV treatment; 3 and 4, UV treatment for 1 min; 5 and 6, UV treatment for 2 min; 7 and 8, UV treatment for 3 min; 9 and 10, UV treatment for 4 min; 11 and 12, UV treatment for 5 min; 13 and 14, UV treatment for 10 min. (B) Slot blot analysis of panel A with the internal broad-range oligonucleotide 244 (15) as a probe. (C) Bacterial nucleic acids (2.5 pg) were added to the amplification reaction mixture after UV treatment. Lanes same as for panel A. (D) Slot blot analysis of panel C with the above-described oligonucleotide as a probe.

DNAs in PCR reagents without significantly affecting the activity of *Taq* polymerase. 8-Methoxypsoralen, DMSO (Fig. 1, lanes 3 and 4), or 3 to 5 min of UV treatment alone had no effect on the template activity of the contaminating DNA, nor did DMSO plus 8-methoxypsoralen without UV treatment (Fig. 2, lanes 1 and 2) or DMSO plus 3 to 5 min of UV treatment (data not shown), indicating that elimination of contaminating DNA is a specific effect of combined 8-methoxypsoralen-UV treatment.

The effect of combined 8-methoxypsoralen-UV treatment on the activity of *Taq* polymerase was investigated in more detail. For this purpose, different amounts of mycobacterial nucleic acids (equivalent to 200, 20, 7, and 2 bacterial genomes) were added as target for amplification after irradiating the PCR mixtures. A primer combination (246-264) which would specifically amplify the input mycobacterial 16S rDNA was chosen (6). Performing PCR without adding mycobacterial DNA does not result in an amplified gene

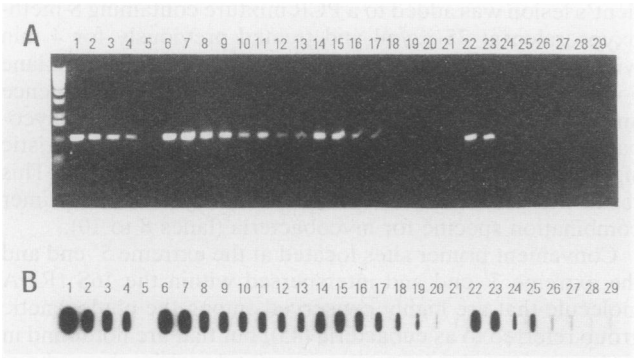

FIG. 3. Effect of 8-methoxypsoralen-UV treatment on the sensitivity of the amplification reaction. Primer 246 and reverse primer 264 were used for specific amplification of mycobacterial 16S rDNA and to direct the synthesis of a 1,020-bp fragment. Mycobacterial DNA corresponding to the indicated amounts of bacterial genomes was added after UV treatment. (A) Lanes: 1 to 5, no 8-methoxypsoralen, no UV treatment; 6 to 13, 25 μg of 8-methoxypsoralen per ml and 3 min of UV treatment; 14 to 21, 25 μg of 8-methoxypsoralen per ml and 4 min of UV treatment; 22 to 29, 25 μg of 8-methoxypsoralen per ml and 5 min of UV treatment. Lanes: 1, 6, 7, 14, 15, 22, and 23, 200 genomes; 2, 8, 9, 16, 17, 24, and 25, 20 genomes; 3, 10, 11, 18, 19, 26, and 27, 7 genomes; 4, 12, 13, 20, 21, 28, and 29, 2 genomes. Lane 5 is the negative control reaction with no mycobacterial DNA added. (B) Slot blot analysis of panel A using the same oligonucleotide as in Fig. 2.

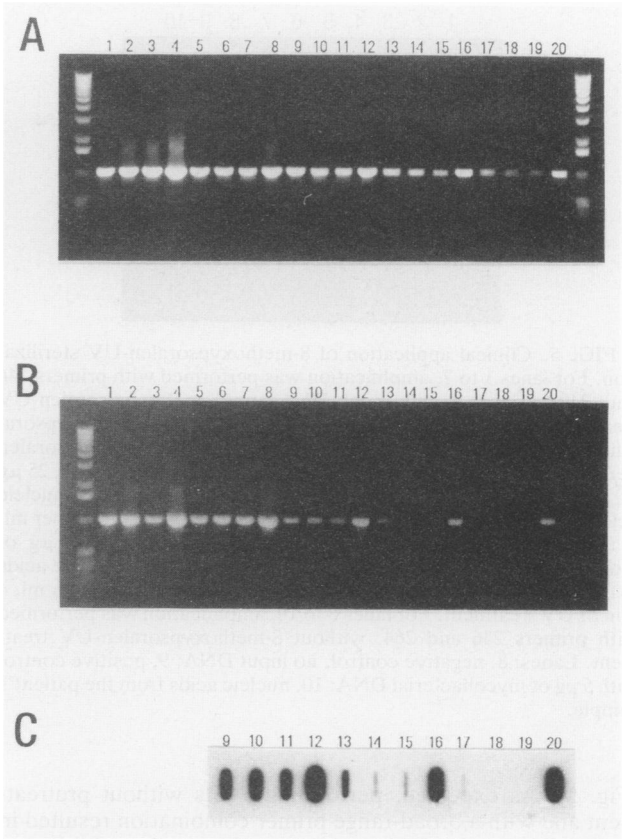

FIG. 4. Determination of the amounts of target DNA that can be eliminated by combined 8-methoxypsoralen-UV treatment. Amplification was performed with primer 246 and reverse primer 264. 8-Methoxypsoralen was present at a final concentration of 25 μg/ml. (A) The indicated amounts of mycobacterial genomes were added after UV treatment. (B) The indicated amounts of mycobacterial DNA were added before UV treatment. Lanes: 1 to 4, 1×10^7 genomes; 5 to 8, 1×10^6 genomes; 9 to 12, 1×10^5 genomes; 13 to 16, 1×10^4 genomes; 17 to 20, 1×10^3 genomes. Lanes: 1, 5, 9, 13, and 17, 3 min of UV treatment; 2, 6, 10, 14, and 18, 4 min of UV treatment; 3, 7, 11, 15, and 19, 5 min of UV treatment; 4, 8, 12, 16, and 20, no UV treatment. (C). Slot blot analysis of lanes 9 to 20 from panel B with the oligonucleotide described in the legend to Fig. 2.

fragment with this primer combination (Fig. 3A and B, lane 5). By comparing the results of control amplification reactions without added 8-methoxypsoralen and UV treatment (Fig. 3A and B, lanes 1 to 4) with the results of reactions with 8-methoxypsoralen at 25 μg/ml and UV light for 3 min (Fig. 3A and B, lanes 6 to 13), 4 min (Fig. 3A and Fig. 3B, lanes 14 to 21), and 5 min (Fig. 3A and Fig. 3B, lanes 22 to 29), the effects of the decontamination procedure on *Taq* polymerase activity can be determined. With untreated PCR mixtures, the assay is able to detect two mycobacterial genomes (Fig. 3, lanes 4). As can be seen in Fig. 3, *Taq* polymerase exhibits sensitivity to UV proportionally to the time used for irradiation. While a 5-min UV treatment resulted in a 10-fold reduction in the amount of target molecules detected, a 3- to 4-min exposure only marginally affected the sensitivity, by a factor of 2 to 3.

The quantitative reduction of contaminating DNA was assessed in a model system. For this purpose, different amounts of mycobacterial nucleic acids (equivalent to 10^7, 10^6, 10^5, 10^4, and 10^3 bacterial genomes) were added to a PCR mixture prior to UV treatment with the mycobacterium-specific primer combination 246-264. To control for amplification efficiency, the same amount of target DNA was added to another set of tubes after UV treatment (compare Fig. 4A and B) as well as to PCR mixtures without UV treatment (Fig. 4A and B, lanes 4, 8, 12, 16, and 20). The results in Fig. 4B and C demonstrate that a 5-min combined 8-methoxypsoralen-UV treatment eliminates template activity of 1×10^4 target DNA molecules and that a 3- to 4-min treatment eliminates 1×10^3 target DNA molecules. As the activity of *Taq* polymerase and the capability to destroy a given number of target DNA molecules appear to show an inverse correlation in this procedure, we have chosen 4 min as the optimal time for UV treatment for elimination of contaminating DNA (Fig. 2A, lanes 9 and 10) because this treatment simultaneously affects *Taq* polymerase activity only minimally (Fig. 3A and B).

To demonstrate that contamination of *Taq* polymerase with bacterial DNA is not limited to a specific producer, different preparations of *Taq* polymerase, including recombinant *Taq* polymerase and "low-DNA" *Taq* polymerase, which was generously provided by Roche Molecular Systems, Alameda, Calif., were investigated. All enzymes were found to be contaminated with bacterial DNA, with low-DNA *Taq* polymerase being quantitatively the least contaminated enzyme (data not shown).

The method of elimination of contaminating DNA within PCR reagents was applied to a case of osteomyelitis, the clinical details of which will be reported elsewhere (18). Smears from the patient's lesions sporadically showed isolated structures resembling acid-fast microorganisms, but repeated attempts to culture the pathogen failed. Nucleic acids were extracted from a lesion, and amplification was performed with a broad-range primer combination (246-240)

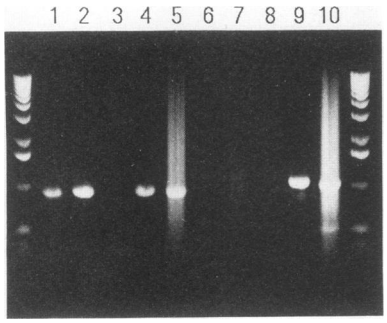

FIG. 5. Clinical application of 8-methoxypsoralen-UV sterilization. For lanes 1 to 7, amplification was performed with primers 246 and 240. Lanes: 1, no input DNA, no 8-methoxypsoralen-UV treatment; 2, 5 pg of mycobacterial DNA, no 8-methoxypsoralen-UV treatment; 3, no input DNA, 25 μg of 8-methoxypsoralen per ml, 4 min of UV treatment; 4, 5 pg of mycobacterial DNA, 25 μg of 8-methoxypsoralen per ml, 4 min of UV treatment; 5, nucleic acids from the patient's sample, 25 μg of 8-methoxypsoralen per ml, 4 min of UV treatment; 6, 400 ng of human DNA, 25 μg of 8-methoxypsoralen per ml, 4 min of UV treatment; 7, nucleic acids extracted from a lymph node, 25 μg of 8-methoxypsoralen per ml, 4 min of UV treatment. For lanes 8 to 10, amplification was performed with primers 246 and 264, without 8-methoxypsoralen-UV treatment. Lanes: 8, negative control, no input DNA; 9, positive control with 5 pg of mycobacterial DNA; 10, nucleic acids from the patient's sample.

(Fig. 5). As expected, performing PCRs without pretreatment and with a broad-range primer combination resulted in an amplified gene fragment even without the addition of extraneous DNA (lane 1), reflecting the contamination of *Taq* polymerase with bacterial DNA. Treatment with 8-methoxypsoralen and UV completely eliminated contaminating DNA (lane 3). Using human DNA as a template does not result in specific amplification products with this primer combination (lane 6 and 7). DNA extracted from the patient's lesion was added to a PCR mixture containing 8-methoxypsoralen at 25 μg/ml and treated previously for 4 min with UV (lane 5). The amplified fragment of 920 bp from lane 5 was gel purified and sequenced directly (20, 32). Sequence analysis revealed the presence of regions specific for mycobacteria at the genus level (6) as well as the characteristic signature sequence of *Mycobacterium ulcerans* (Fig. 6). This result was corroborated by sequence analysis with a primer combination specific for mycobacteria (lanes 8 to 10).

Convenient primer sites located at the extreme 5′ end and the extreme 3′ end and interspersed within the 16S rRNA molecule that are highly conserved among the phylogenetic group referred to as eubacteria (45), but that are not found in eucaryotes, archaebacteria, or mitochondria, have been described (4, 9, 12, 15, 44). These primers allow the selective amplification of rDNA gene fragments of unknown, eubacterial microorganisms even in the presence of nucleic acids from other types of organisms (Fig. 5). Diagnostic amplification with such highly conserved regions as primer target sites permitting amplification of any bacterial 16S rDNA for detection of bacterial pathogens necessitates reagents that are not contaminated with bacterial DNA. In addition, the tissues chosen for investigation should not be contaminated with indigenous bacterial flora, e.g., from skin or intestinal lumen. The presence of contaminating DNA within PCR reagents, especially *Taq* polymerase, has so far limited the broad-range eubacterial primer approach to samples which show a large number of the microorganism of interest (30, 31, 42). Samples which are clearly positive by microscopy indicate the presence of at least 10^4 to 10^5 target organisms per ml. As commercially available lots of *Taq* polymerase have been estimated to be contaminated with approximately 100 genome equivalents of bacterial DNA per unit of enzyme (29), the excess of target DNA over contaminating DNA will ensure that the target DNA will preferably be amplified. The availability of such samples has allowed the identification of the agent of bacillary angiomatosis (30) and Whipple's disease (31, 42). In infections with small numbers of bacteria,

129	175	
TGA TCT GCC CTG CAC TTC	CGG ATA CCA CCA CGG GAT GCA TG TCT-TGT GGT GGA AAG C	M.tb. complex
CA. ... A..	... ...T .AA ..C ... .-.C.	M.avium
CA.	... ...T TTA .GC ... .-.TA	M.intracellulare
CA.	... ...T TTA ..C ... .-.T.	M.intracellulare serovar 18
CA.	..A ... .T TTA ..C ... .-.T.	M.intracellulare serovar 7
CA.	... ... .TT .GC ... .. C..-...	M.scrofulaceum
CA. ... ... AC.	... ... .TT .GC ... .. C..-...	M.gastri, M.kansasii
.A.	... ... .TT .GC ... .. C..-...	M.simiae
.A.	..A ... .C ..A .GC ... .. C..-..G	M.malmoense
.A.	... ...C ..A .GC ... .. C..-..G	M.szulgai
.A.	... ...T .AA .GC ... .. C..T...	M.haemophilum
.A.	... ... .TT .AA .GC ... .-...	M.leprae
... ...	... ...C-...	M.asiaticum
.A. ... ... A..	..A ... ... .A. ..C A.. .. ..C-...	M.gordonae
.A. ... ... A..	..A ... ... .A. A.C A.. .. ..C-...	M.gordonae
.A. ... ... A..	... ... .A. A.C A.. .. ..C-...	M.gordonae
C..	... ... T.. .. ..C-...	M.ulcerans
C..	... ... T.. .. ..C-...	amplified sequence

FIG. 6. Sequence analysis of the amplified bacterial 16S rDNA gene with nucleic acids extracted from the patient's lesion. Shown is a comparison of the amplified sequence with selected mycobacterial 16S rDNA sequences (7, 22, 33, 38). The region depicted represents the hypervariable region within the 16S rRNA molecule which exhibits species specificity for mycobacteria (32). *M. tuberculosis* (M. tb. complex) is used as the reference sequence, nucleotides different from those of *M. tuberculosis* are indicated, and bars indicate deletions. The numbers indicate the respective *E. coli* 16S rRNA positions. For reasons of space limitation, only selected stretches of 16S rDNA sequences are shown.

however, we have found that contamination of PCR reagents with eubacterial DNA prohibits this approach. To identify a previously unknown and uncultured pathogen causing disseminated infection in patients with AIDS, it was necessary to use more specific primers to avoid problems with contamination, as these primers will restrict amplification to the genera of interest (11, 25).

In the present study, we have successfully eliminated contaminating DNA within PCR reagents. As a demonstration of this procedure, we have amplified a 16S rRNA sequence identical to that of *M. ulcerans* from a case of paucibacillary osteomyelitis, for which no specific pathogen had been cultured. The method of decontamination described herein facilitates the broad-range eubacterial primer approach for identification of bacterial pathogens that infect humans, a strategy that is particularly suited for identification of pathogens that cannot be cultured and otherwise remain elusive. It may allow the use of this technique in infections in which only small numbers of target organisms are present.

ACKNOWLEDGMENTS

We are indebted to Michael Hofer and Bernard Hirschel, Hopital Cantonal Universitaire, Geneva, Switzerland, for providing tissue samples and information on the patient. We thank Philip Kirschner for helpful discussions and critical comments on the manuscript, Dieter Bitter-Suermann for continuous encouragement, Monika Kiekenbeck and Annette Wrede for expert technical assistance, and Susanne Maibom for typing the manuscript. E.C.B. is particularly thankful to I.B. for her gracious unselfishness and for tolerating late-night shifts.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (01 K1 89117), the Commission of the European Communities, and by the Niedersächsischer Verein zur Bekämpfung der Tuberkulose e.V.

REFERENCES

1. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
2. **Anderson, B. E., J. W. Sumner, J. E. Dawson, T. Tzianabos, C. R. Greene, J. G. Olson, D. B. Fishbein, M. Olsen-Rasmussen, B. P. Holloway, E. H. George, and A. F. Azad.** 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J. Clin. Microbiol. **30:**775–780.
3. **Arnoldi, J., C. Schlüter, M. Duchrow, L. Hübner, M. Ernst, A. Teske, H.-D. Flad, J. Gerdes, and E. C. Böttger.** 1992. Species-specific assessment of *Mycobacterium leprae* in skin biopsies by in situ hybridization and polymerase chain reaction. Lab. Invest. **66:**618–623.
4. **Barry, T., R. Powell, and F. Gannon.** 1990. A general method to generate DNA probes for microorganisms. Bio/Technology **8:**233–236.
5. **Blanchard, A., M. Gautier, and V. Mayan.** 1991. Detection and identification of mycoplasmas by amplification of rDNA. FEMS Microbiol. Lett. **81:**37–42.
6. **Böddinghaus, B., T. Rogall, T. Flohr, H. Blöcker, and E. C. Böttger.** 1990. Detection and identification of mycobacteria by amplification of rRNA. J. Clin. Microbiol. **28:**1751–1759.
7. **Böddinghaus, B., J. Wolters, W. Heikens, and E. C. Böttger.** 1990. Phylogenetic analysis and identification of different serovars of *Mycobacterium intracellulare* at the molecular level. FEMS Microbiol. Lett. **70:**197–204.
8. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Ditten, and J. van der Noordaa.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28:**495–503.
9. **Böttger, E. C.** 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **65:**171–176.
10. **Böttger, E. C.** 1990. Frequent contamination of Taq polymerase with DNA. Clin. Chem. **36:**1258.
11. **Böttger, E. C., A. Teske, P. Kirschner, S. Bost, H. R. Chang, V. Beer, and B. Hirschel.** 1992. Disseminated "*Mycobacterium genavense*" infection in patients with AIDS. Lancet **340:**76–80.
12. **Chen, K., H. Neimark, P. Rumore, and C. R. Steinmann.** 1989. Broad range DNA probes for detecting and amplifying eubacterial nucleic acids. FEMS Microbiol. Lett. **57:**19–24.
13. **Cimino, G. D., K. C. Metchette, J. W. Tenman, J. E. Hearst, and S. T. Isaacs.** 1991. Post-PCR sterilization: a method to control carryover contamination for the polymerase chain reaction. Nucleic Acids Res. **19:**99–107.
14. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for identification of single cells. Science **243:**1360–1363.
15. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger.** 1989. Isolation and complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17:**7843–7853.
16. **Gaydos, C. A., T. C. Quinn, and J. J. Eiden.** 1992. Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. J. Clin. Microbiol. **30:**796–800.
17. **Göbel, U., R. Maas, G. Haun, C. Vinga-Martins, and F. J. Stanbridge.** 1987. Synthetic oligonucleotide probes complementary to rRNA for group- and species-specific detection of mycoplasmas. Isr. J. Med. Sci. **23:**742–746.
18. **Hofer, M., B. Hirschel, P. Kirschner, M. Baghetti, A. Haelin, C.-A. Siegrist, S. Suter, A. Teske, and E. C. Böttger.** Disseminated *Mycobacterum ulcerans* osteomyelitis: diagnosis by culture free microbiology. Submitted for publication.
19. **Hoshina, S., S. M. Kahn, W. Jiang, P. H. R. Green, H. C. Neu, N. Chin, M. Morotomi, P. LoGerfo, and I. B. Weinstein.** 1990. Direct detection and amplification of *Helicobacter pylori* ribosomal 16S gene segments form gastric endoscopic biopsies. Diagn. Microbiol. Infect. Dis. **13:**473–479.
20. **Hultman, T., S. Stahl, E. Hornes, and M. Uhlén.** 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as support. Nucleic Acids Res. **17:**4937–4946.
21. **Jinno, Y., K. Yoshiura, and N. Niikawa.** 1990. Use of psoralen as extinguisher of contaminated DNA in PCR. Nucleic Acids Res. **18:**6739.
22. **Kirschner, P., and E. C. Böttger.** 1992. Microheterogeneity within rRNA of *Mycobacterium gordonae*. J. Clin. Microbiol. **30:**1049–1050.
23. **Kwok, S., and R. Higuchi.** 1984. Avoiding false positives with PCR. Nature (London) **339:**237–238.
24. **Longo, M. C., M. S. Berninger, and J. L. Hartley.** 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reaction. Gene **93:**125–128.
25. **Nadal, D., R. Caduff, R. Kraft, M. Salfinger, T. Bodmer, P. Kirschner, E. C. Böttger, and U. B. Schaad.** Invasive infection with *Mycobacterium genavense* in children with the acquired immunodeficiency syndrome. Eur. J. Clin. Microbiol. Infect. Dis., in press.
26. **Ou, C.-Y., J. L. Moore, and G. Schochetman.** 1991. Use of UV-irradiation to reduce false positivity in polymerase chain reaction. BioTechniques **10:**442–444.
27. **Persing, D. H.** 1991. Polymerase chain reaction: trenches to benches. J. Clin. Microbiol. **29:**1281–1285.
28. **Persing, D. H., S. R. Telford, P. N. Rys, D. E. Dodge, T. J. White, S. E. Malawista, and A. Spielman.** 1990. Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes damnini* ticks. Science **249:**1420–1423.
29. **Rand, V. H., and H. Houck.** 1990. Taq polymerase contains bacterial DNA of unknown origin. Mol. Cell. Probes **4:**445–450.
30. **Relman, D. A., J. S. Lovtit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis. An approach to the identification of uncultured pathogens. N. Engl. J. Med. **323:**1573–1580.
31. **Relman, D. A., T. M. Schmidt, R. P. MacDermatt, and S. Falkow.** 1992. Identification of the uncultured bacillus of Whipple's disease. N. Engl. J. Med. **327:**293–301.

32. **Rogall, T., T. Flohr, and E. C. Böttger.** 1990. Differentiation of mycobacterium species by direct sequencing of amplified DNA. J. Gen. Microbiol. **136:**1915–1920.
33. **Rogall, T., J. Wolters, T. Flohr, and E. C. Böttger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. Int. J. Syst. Bacteriol. **40:**323–330.
34. **Romaniuk, P. J., and T. J. Trust.** 1989. Rapid identification of *Campylobacter* species using oligonucleotide probes to 16S ribosomal RNA. Mol. Cell. Probes **3:**133–142.
35. **Rossau, R., E. Van Mechelen, J. de Ley, and H. van Heyverswijn.** 1989. *Neisseria gonorrhoeae* DNA probes derived from ribosomal RNA. J. Gen. Microbiol. **135:**1735–1745.
36. **Sarkar, G. A., and S. S. Sommer.** 1990. Shedding light on PCR contamination. Nature (London) **343:**27.
37. **Schmidt, T. M., B. Pace, and N. R. Pace.** 1991. Detection of DNA contamination in Taq polymerase. BioTechniques **11:**176–177.
38. **Teske, A., J. Wolters, and E. C. Böttger.** 1991. The 16S rRNA nucleotide sequence of *Mycobacterium leprae*: phylogenetic position and development of DNA probes. FEMS Microbiol. Lett. **80:**231–238.
39. **Wakefield, A. E., L. Guiver, R. F. Miller, and J. M. Hopkin.** 1991. DNA amplification on induced sputum samples for diagnosis of *Pneumocystis carinii* pneumonia. Lancet **337:**1378–1379.
40. **Waters, A. P., and T. F. McCutchan.** 1989. Rapid, sensitive diagnosis of malaria based on ribosomal RNA. Lancet **i:**1343–1346.
41. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
42. **Wilson, K. H., R. Blitchington, R. Frothingham, and J. A. P. Wilson.** 1991. Phylogeny of the Whipple's-disease-associated bacterium. Lancet **338:**474–475.
43. **Wilson, K. H., R. Blitchington, P. Shah, G. McDonald, R. D. Gilmore, and L. P. Mallavia.** 1989. Probe directed at a segment of *Rickettsia rickettsii* rRNA amplified with polymerase chain reaction. J. Clin. Microbiol. **27:**2692–2696.
44. **Wilson, K. H., R. B. Blitchington, and R. C. Greene.** 1990. Amplification of bacterial 16S ribosomal DNA sequences with polymerase chain reaction. J. Clin. Microbiol. **28:**1942–1946.
45. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.