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Detection of *Mycobacterium tuberculosis* by PCR Amplification with Pan-*Mycobacterium* Primers and Hybridization to an *M. tuberculosis*-Specific Probe

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Nucleic acid amplification techniques such as the PCR are very useful in the rapid diagnosis of infections by *Mycobacterium tuberculosis*. However, recent studies have shown that the accuracy of results can vary widely when tests are performed with nonstandardized reagents. We have developed a PCR assay for the detection of *M. tuberculosis* that is both rapid and accurate. The assay reagents are standardized and quality controlled. False-positive results due to carryover contamination are prevented by the incorporation of dUTP coupled with uracil-*N*-glycosylase restriction. This assay also employs pan-*Mycobacterium* amplification primers, allowing for flexibility in the mycobacterial species that can be identified from a single amplification reaction. The amplification is very sensitive; amplification products generated from as few as three bacteria can be detected by agarose gel electrophoresis. DNAs isolated from 33 of 34 mycobacterial species tested were amplified efficiently. Only DNA from *Mycobacterium simiae* did not amplify. The amplification is also very specific. Amplification products were generated only from the DNAs of bacteria in closely related genera such as *Corynebacterium*. The nonmycobacterial amplicons do not pose a problem, as they do not hybridize to mycobacterium-specific probes. Hybridization of amplicons to an *M. tuberculosis*-specific probe allows for the unambiguous identification of *M. tuberculosis* complex organisms. The clinical performance of this PCR assay was evaluated against that of culture in 662 respiratory specimens. Sensitivities of 100 and 73.1% were obtained from smear-positive and -negative respiratory specimens, respectively. The corresponding specificities were 100 and 99.8%. The high sensitivity and specificity, coupled with the potential for detecting a wide range of mycobacteria, make this assay a useful tool in the clinical management of mycobacterial infections.

The recent resurgence in tuberculosis (TB) cases poses a serious public health problem. Effective TB management requires the rapid detection and identification of the etiologic agent. The currently accepted "gold standard" laboratory method for detecting and identifying *Mycobacterium tuberculosis* is a combination of acid-fast bacillus (AFB) smear for initial screening and culture for bacterial isolation and identification. Microscopic examination of AFB smears can yield a result within 24 h. Unfortunately, smear is neither specific for *M. tuberculosis* nor very sensitive, requiring $>10^3$ to 10^4 organisms per ml of sputum (10). Mycobacteria can also be detected and identified directly from sputum specimens by analyzing the mycobacterial mycolic acid by high-performance liquid chromatography. However, the sensitivity of this method is low, and direct detection from sputum is possible only in smear-positive specimens (14). Bacterial culture is superior to AFB smear and mycolic acid analysis, both in terms of sensitivity and specificity. However, mycobacteria are fastidious organisms with very strict growth requirements. As a result, culture-based diagnostic methods are slow. The need to perform biochemical testing to identify the mycobacterial species adds to the time required to obtain a diagnosis. Even when nucleic acid probes are used for "rapid" species identification from cultures, results are often not available for 2 to 8 weeks. Clearly, a more rapid test for the detection of pulmonary TB and other nontuberculous mycobacterial infections would greatly benefit patient management.

The PCR has proven to be a very useful tool in the rapid diagnosis of infectious diseases and specifically mycobacterial infections (28). Many of the mycobacterial PCR assays employ species-specific primers that allow for the detection of a single or a limited number of mycobacterial species (3–6, 8, 12, 23–25). Species-specific amplification assays are useful in the detection and identification of mycobacterial species. However, only a limited number of mycobacterial species can be detected. This limits the usefulness of these assays, since additional amplifications are needed to detect other species. There are other limitations associated with these assays. The most commonly used of these assays is one targeting the insertion element IS*6110* found only in species within the *M. tuberculosis* complex (6). However, there are isolates of *M. tuberculosis* that do not harbor IS*6110* (30), although they are uncommon. False-positive results have also been reported in specimens negative for *M. tuberculosis* (16).

Several multispecies PCR assays have been described elsewhere (1, 11, 20). However, the breadth of the species coverage of most of these assays cannot be determined because of the limited sequence information available. A pan-*Mycobacterium* assay based on the amplification of rRNA was described by Böddinghaus et al. (1). However, the detection limit of the assay was only 30 organisms when the gene encoding the rRNA (present in one or two copies per cell) was used as the template. Single-organism sensitivity was achieved only when the rRNA (present in 10^3 to 10^4 copies per cell) was used as the template. The use of RNA as an amplification target is less desirable because of the need to perform a reverse transcription step prior to amplification.

For any assay to be useful in a clinical diagnostic setting, careful optimization of amplification conditions and standard-

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TABLE 1. Mycobacterial species tested[a]

Mycobacterium species (isolate[b])	Amplification result[c]	Hybridization signal[d]
M. africanum (ATCC 25420)	+	4.000
M. asiaticum (ATCC 25276)	+	0.107
M. aurum (ATCC 23366)	+	0.119
M. avium (ATCC 25291)	+	0.118
M. bovis (ATCC 19210)	+	4.000
M. brunense (ATCC 23434)	+	ND
M. chelonae (ATCC 35752)	+	ND
M. chitae (ATCC 19627)	+	0.082
M. cookii (ATCC 49103)	+	0.061
M. fallax (ATCC 35219)	+	0.093
M. flavescens (ATCC 14474)	+	0.085
M. fortuitum (ATCC 6841)	+	0.087
M. gastri (ATCC 15754)	+	0.116
M. gordonae (ATCC 14470)	+	0.099
M. intracellulare (ATCC 13950)	+	0.122
M. kansasii (ATCC 12478)	+	0.114
M. komossense (ATCC 33013)	+	0.062
M. malmoense (ATCC 29571)	+	0.105
M. marinum (ATCC 11565)	+	0.120
M. microti (ATCC 11152)	+	4.000
M. neoaurum (ATCC 25795)	+	0.088
M. nonchromogenicum (ATCC 19530)	+	0.078
M. phlei (ATCC 11758)	+	0.093
M. scrofulaceum (ATCC 19981)	+	0.108
M. senegalense (ATCC 35796)	+	0.131
M. simiae (ATCC 25275)	−	0.054
M. smegmatis (ATCC 19420)	+	0.087
M. sphagni (ATCC 33027)	+	0.088
M. szulgai (ATCC 35799)	+	0.124
M. terrae (ATCC 15755)	+	0.089
M. thermoresistibile (ATCC 19527)	+	0.082
M. triviale (ATCC 23292)	+	0.112
M. tuberculosis (ATCC 27294)	+	4.000
M. xenopi (ATCC 19250)	+	0.137

[a] Mycobacterial species used to determine the coverage of primers KY18 and KY75 and the specificity of *M. tuberculosis*-specific probe KY172-T3.

[b] ATCC denotes cultures obtained from the American Type Culture Collection.

[c] Amplification results were determined by agarose gel electrophoresis. Symbols: +, presence of amplification products; −, absence of amplification products.

[d] The hybridization data are the means of two replicate amplifications. Test cutoff, 0.350. ND, not done.

ization of protocols and reagents are needed to ensure proper performance. Results from a recent study comparing the performance of seven clinical laboratories highlight the laboratory-to-laboratory variability that can occur when PCR is performed by individual laboratories using different PCR tests and nonstandardized reagents and protocols (19).

We have developed a rapid, simple, and reliable PCR assay for the detection and identification of *M. tuberculosis* complex organisms from respiratory specimens. In this assay (AMPLICOR MTB), reagents are standardized and quality controlled to assure consistent performance. dUTP and uracil-*N*-glycosylase have also been incorporated into the assay to prevent false-positive results from carryover contamination (18). The current assay targets the gene encoding the small-subunit rRNA (16S rRNA). The availability of 16S rRNA gene sequence information of a large number of bacterial species (both mycobacterial and nonmycobacterial) enabled us to design a single primer pair that can amplify DNA from almost all *Mycobacterium* species, giving our assay a wide breadth of species coverage. Identification of *M. tuberculosis* complex organisms is made by hybridizing amplification products (amplicons) to an *M. tuberculosis*-specific probe.

The incidence of infections by nontuberculous mycobacteria has increased with the rise in AIDS cases (7, 9, 22, 27, 29). A pan-*Mycobacterium* amplification assay allows for greater flexibility in the species that can be detected. Mycobacterial species other than *M. tuberculosis* can be detected and identified simply by hybridizing products from the same amplification reaction to different species-specific probes.

MATERIALS AND METHODS

Cultures and DNA. The mycobacterial species listed in Table 1 were used to determine the coverage of the pan-*Mycobacterium* amplification and the specificity of the *M. tuberculosis*-specific probe. Other bacteria, fungi, and viruses used to determine the specificity of the assay are listed in Table 2. Lysates were prepared from the cultured organisms by the clinical specimen preparation method outlined below. Additional clinical isolates of *Mycobacterium avium*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, and *M. tuberculosis* were kindly provided by E. Fiss (University of California, San Francisco, Calif.). Mycobacterial DNAs were isolated and purified from these clinical isolates by the method described by Patel et al. (21) and used to determine the analytical sensitivity of the amplification. The concentrations of these DNA preparations were determined by A_{260} and by ethidium bromide staining and comparison against DNA standards of known concentrations. Additional purified mycobacterial and nonmycobacterial DNAs were kindly provided by J. W. Zolg (Roche Molecular Systems, Basel, Switzerland).

DNA amplification. Primers and probes were synthesized on the ABD394 DNA synthesizer (Applied Biosystems). Primers KY18 (5′-CACATGCAAGTCGAACGGAAAGG-3′) and KY75 (5′-GCCCGTATCGCCCGCACGCTCACA-3′) were derived from regions of the 16S rRNA gene that are conserved among mycobacterial species. Both primers contain a biotin residue at the 5′ terminus to facilitate amplicon detection in the hybridization assay. The *M. tuberculosis*-specific probe KY172-T3 (5′-GGTGGAAAGCGCTTTAGCGGT-3′) was chosen from a hypervariable region within the 16S rRNA gene. PCR amplifications were carried out in 100-μl reaction mixtures by adding 50 μl of template DNA or lysate to 50 μl of a premade amplification master mixture. Target DNAs were amplified in a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer) as follows. A 2-min incubation at 50°C was followed by two cycles, each cycle consisting of 20 s at 98°C, 20 s at 62°C, and 45 s at 72°C. This was followed by 35 cycles, each cycle consisting of 20 s at 94°C, 20 s at 62°C, and 45 s at 72°C, for a total of 37 cycles. A final incubation at 72°C for ≥5 min was included to allow for completion of strand synthesis.

Amplification products were detected by agarose gel electrophoresis or hybridization to probe KY172-T3 in microwell plates as previously described (17). In the microwell plate assay, amplicons were denatured with 100 μl of denaturation solution. Denatured amplicons (25 μl) were added to wells of a microwell plate coated with probe KY172-T3. Hybridization was carried out at 37°C for 90 min in the presence of 100 μl of hybridization buffer. Detection of hybridized duplex was completed by using an avidin-horseradish peroxidase conjugate-tetramethylbenzidine substrate system. The A_{450} was determined in a microwell plate reader. Absorbance signals greater than or equal to 0.350 were considered to be positive.

Clinical specimens and specimen preparation. Respiratory specimens (662) were collected prospectively from 273 patients and analyzed as they were received. All specimens were decontaminated by the *N*-acetyl-cysteine–NaOH procedure as recommended by the Centers for Disease Control and Prevention

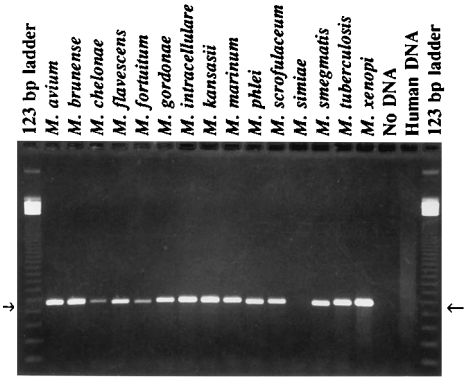

FIG. 1. Amplification of mycobacterial DNAs with primers KY18 and KY75. Approximately 1 pg of each mycobacterial DNA was used as a template in the amplification reactions. Human placental DNA and a reagent blank with no input DNA were included as negative controls. The arrows denote the position of the expected amplification product.

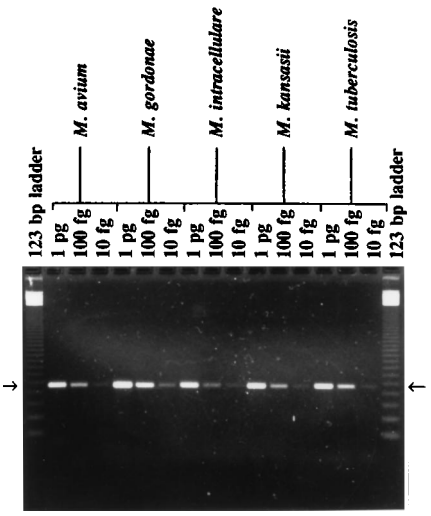

FIG. 2. Analytical sensitivity of amplification with primers KY18 and KY75. DNAs from *M. avium*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, and *M. tuberculosis* were amplified at inputs of 1 pg, 100 fg, and 10 fg. Human placental DNA and a reagent blank with no input DNA were included as negative controls. The arrows mark the location of the expected amplification product.

(15). Preparation of clinical specimens entailed the addition of 100 μl of decontaminated sputum sediment to 0.5 ml of sputum wash buffer and then centrifugation at 12,500 × *g* for 10 min to pellet the organisms. The pellets were resuspended in 100 μl of sputum lysis reagent and incubated for 45 min at 60°C to complete lysis of the mycobacteria. This was followed by the addition of 100 μl of sputum neutralization reagent, making the sample amplification ready. Fifty microliters of the prepared specimen was added to a MicroAmp tube containing the amplification master mixture and amplified as described above.

PCR-positive, culture-negative results were confirmed by amplification of a second locus, the superoxide dismutase gene (31). Discrepant results were further resolved with additional bacteriological data and clinical data. A specimen result was considered truly positive if one of the following criteria were met. (i) Positive cultures on additional specimens collected from the same patient during the current disease episode. (ii) In the absence of bacteriological confirmation, clinical evidence supportive of a diagnosis of tuberculosis including but not limited to abnormal chest X-ray and final discharge diagnosis of tuberculosis. In cases in which the culture result was positive but the PCR result was negative, the culture results were assumed to be correct.

The clinical specimens were analyzed as part of a preclinical evaluation of the AMPLICOR MTB assay. The initial data have been reported separately elsewhere (2, 26). A compilation of the combined data is presented here.

RESULTS

Amplification. To determine the breadth of coverage of the primers, DNAs isolated from the mycobacterial species listed in Table 1 were amplified and analyzed by agarose gel electrophoresis. DNAs from 33 of the 34 species tested amplified efficiently. Only the DNA from *Mycobacterium simiae* failed to generate amplification products (Table 1). An alignment of the *M. simiae* 16S rRNA gene sequence with the downstream primer, KY75, showed 4 mismatches relative to the 3′ terminus of the primer. A representative example of the mycobacterial DNA amplification efficiencies is shown in Fig. 1.

The sensitivity of amplification was determined by amplifying DNAs from five mycobacterial species at different input DNA amounts (10 fg to 1 pg) and analyzing the amplicons by agarose gel electrophoresis. As shown in Fig. 2, amplicons from as little as 10 fg of mycobacterial DNA (the genomic equivalent of three bacteria) were readily detectable by gel electrophoresis for all five mycobacterial species tested. Greater sensitivity can be achieved by hybridization to probes in microwell plates (data not shown).

To assess the specificity of amplification, 10 ng (the genomic equivalent of ~10^6 bacteria and ~10^5 fungi) of DNAs from a subset of the organisms listed in Table 2[5] were used as templates for amplification and the amplification products were analyzed by agarose gel electrophoresis. The organisms tested included organisms that are part of the normal flora of the respiratory tract as well as other respiratory pathogens. Only the DNAs from *Corynebacterium diphtheriae*, *Corynebacterium pseudodiphtheriticum*, *Corynebacterium xerosis*, *Nocardia brevicatena*, and *Rhodococcus equi* generated significant levels of amplification products (Table 2). All of these bacteria belong to genera that are closely related to *Mycobacterium*. The amplification products from the nonmycobacterial DNAs did not hybridize to either a *Mycobacterium* genus-specific probe (data not shown) or the *M. tuberculosis*-specific probe.

Probe hybridization specificity. The mycobacterial isolates listed in Table 1 (with the exception of *M. brunense* and *M. chelonae*) were tested by amplifying the equivalent of approximately 10^6 copies of extracted DNA. The resultant amplification products were hybridized to probe KY172-T3. The isolates tested represented 32 different species of *Mycobacterium*. Only amplicons from *M. tuberculosis* complex organisms (*M. africanum*, *M. bovis*, *M. microti*, and *M. tuberculosis*) gave positive signals (Table 1).

The probe specificity was further tested by amplifying extracted DNAs from the organisms listed in Table 2 and hybridizing the amplification products to probe KY172-T3. This list includes 87 species from 53 genera of bacteria, six fungal species, and 8 virus isolates commonly found in the respiratory tract. Nucleic acids from the equivalent of 10^6 bacteria, 10^5 fungi, and the indicated viral PFU were used. No positive hybridization signals were produced from any of the nonmycobacterial organisms tested (Table 2).

Evaluation of clinical specimens. The compiled results from two preliminary clinical evaluations of the PCR assay are summarized in Tables 3 and 4. The data from testing 662 specimens demonstrated that the AMPLICOR MTB assay has excellent sensitivity and specificity. Of 580 culture-negative specimens tested, 5 were PCR positive (Table 3). The PCR results were confirmed by the successful amplification of a region of the *M. tuberculosis* superoxide dismutase gene (31). The results from these specimens were further resolved by using the criteria outlined in Materials and Methods.

Four of the five PCR-positive, culture-negative results were shown to be true-positive results after discrepant resolution. One specimen was from a patient who had other positive cultures during the current disease episode, and three specimens were from patients who had a clinical diagnosis of tuberculosis. The result from only one specimen was classified as a false positive, resulting in a resolved overall clinical specificity of 99.8% (Table 4). There were seven specimens (all smear negative) which were PCR negative and culture positive, giving an overall clinical sensitivity of 91.9%. The overall specificity and sensitivity for culture were 100 and 95.3%, respectively.

The clinical data were also analyzed according to the smear results. Among smear-positive specimens, the resolved specificity and sensitivity of PCR were both 100%. Culture had a specificity and sensitivity of 100 and 98.3%, respectively, for these specimens. The sensitivities of both PCR and culture were somewhat lower in smear-negative specimens, 73.1 and 88.5%, respectively. In these specimens, the specificities of both methods remained high, 99.8% for PCR and 100% for culture.

The statistical significance of the results from PCR versus culture was determined by the McNemar χ^2 test. The *P* values for all specimens, smear-positive specimens, and smear-negative specimens were >0.75, >0.9, and >0.5, respectively (Table 3).

TABLE 2. PCR assay specificity panel[a]

Bacterium, fungus, or virus (isolate[b])	Amplification result or input PFU/PCR[c]	Hybridization signal[d]
Bacteria		
Acinetobacter calcoaceticus (ATCC 23055)[e]	−	0.091
Actinomadura madurae (ATCC 19425)	ND	0.073
Actinomyces pyogenes (ATCC 19411)	ND	0.113
Actinoplanes italicus (ATCC 27366)	ND	0.084
Aeromonas hydrophila (ATCC 7966)[e]	−	0.059
Arcanobacterium haemolyticum (ATCC 9345)	ND	0.071
Arthrobacter oxydans (ATCC 14358)	ND	0.095
Bacillus subtilis (ATCC 6051)	ND	0.144
Bacillus subtilis (ATCC 6633)[e]	−	ND
Bacteroides fragilis (ATCC 25285)	ND	0.073
Bordetella parapertussis (ATCC 15311)	ND	0.077
Bordetella parapertusis (DSM 4922)[e]	−	ND
Bordetella pertussis (ATCC 9797)[e]	−	0.080
Branhamella catarrhalis (ATCC 25238)[e]	−	0.103
Brevibacterium linens (ATCC 8377)	ND	0.077
Campylobacter jejuni (ATCC 33560)[e]	−	0.084
Chlamydia trachomatis (ATCC VR348B)	ND	0.091
Chromobacterium violaceum (ATCC 12472)	ND	0.070
Citrobacter freundii (ATCC 8090)[e]	−	0.071
Clostridium perfringens (ATCC 13124)	ND	0.050
Corynebacterium aquaticum (ATCC 14665)	ND	0.092
Corynebacterium diphtheriae (ATCC 11913)[e]	+	0.063
Corynebacterium flavescens (ATCC 10340)[e]	−	0.075
Corynebacterium glutamicum (ATCC 13032)	ND	0.066
Corynebacterium jeikeium (ATCC 43734)	ND	0.127
Corynebacterium minutissimum (ATCC 23347)	ND	0.080
Corynebacterium minutissimum (ATCC 23348)[e]	−	ND
Corynebacterium pseudodiphtheriticum (ATCC 10700)[e]	+	0.101
Corynebacterium pseudotuberculosis (ATCC 19410)	ND	0.073
Corynebacterium renale (ATCC 19412)[e]	−	0.135
Corynebacterium striatum (ATCC 6940)[e]	−	0.061
Corynebacterium xerosis (ATCC 373)[e]	+	0.088
Deinococcus radiodurans (ATCC 13939)	ND	0.070
Dermatophilus congolensis (ATCC 14637)	ND	0.087
Derxia gummosa (ATCC 15994)	ND	0.096
Eikenella corrodens (ATCC 23834)	ND	0.079
Enterobacter aerogenes (ATCC 13048)[e]	−	0.132
Enterobacter cloacae (ATCC 13047)[e]	−	0.064
Enterococcus faecalis (ATCC 19433)[e]	−	0.058
Enterococcus faecium (ATCC 19434)[e]	−	0.064
Escherichia coli (ATCC 11775)	ND	0.086
Escherichia coli (ATCC 25922)[e]	−	ND
Fusobacterium nucleatum (ATCC 25586)	ND	0.093
Gordona sputi (ATCC 33610)	ND	0.073
Haemophilus influenzae (ATCC 33391)[e]	−	0.058
Haemophilus parainfluenzae (ATCC 33392)	ND	0.076
Klebsiella pneumoniae (ATCC 13883)[e]	−	0.108
Lactobacillus casei (ATCC 11578)	ND	0.069
Legionella micdadei (ATCC 33204)	ND	0.126
Legionella pneumophila (ATCC 33152)[e]	−	0.070
Microbacterium lactamica (ATCC 8180)	ND	0.116
Mycoplasma hominis (ATCC 23114)[e]	−	0.083
Mycoplasma pneumoniae (ATCC 15531)[e]	−	0.065
Neisseria gonorrhoeae (ATCC 19424)[e]	−	0.057
Neisseria lactamica (ATCC 23970)[e]	−	0.063
Neisseria meningitidis (ATCC 13102)[e]	−	0.130
Nocardia asteroides (ATCC 19247)[e]	−	0.116
Nocardia brasiliensis (ATCC 19296)	ND	0.074
Nocardia brevicatena (ATCC 15333)[e]	+	ND
Nocardia farcinica (W 5218)	ND	0.068
Nocardia nova (W 5194)	ND	0.067
Nocardia otitidiscaviarum (ATCC 14629)	ND	0.104
Nocardia transvalensi (W 4692)	ND	0.084
Oerskovia turbata (ATCC 25835)	ND	0.091
Peptococcus niger (ATCC 27731)	ND	0.089
Peptostreptococcus anaerobius (ATCC 27337)	ND	0.073
Peptostreptococcus magnus (ATCC 15794)	ND	0.126
Porphyromonas asaccharolytica (ATCC 25260)	ND	0.106
Porphyromonas gingivalis (ATCC 33277)	ND	0.095
Prevotella melaninogenica (ATCC 25845)	ND	0.098
Propionibacterium acnes (ATCC 6919)[e]	−	0.076
Proteus mirabilis (ATCC 29906)	ND	0.123
Proteus mirabilis (ATCC 12453)[e]	−	ND
Pseudomonas aeruginosa (ATCC 10145)[e]	−	0.086
Rhodococcus aichiensis (ATCC 33611)	ND	0.106
Rhodococcus bronchialis (ATCC 25592)	ND	0.090
Rhodococcus chubuensis (ATCC 33609)[e]	−	0.096
Rhodococcus equi (ATCC 6939)[e]	+	0.064
Salmonella choleraesuis (ATCC 19430)	ND	0.094
Serratia marcescens (NA)	ND	0.084
Serratia marcescens (ATCC 8100)[e]	−	ND
Staphylococcus aureus (ATCC 12598)	ND	0.077
Staphylococcus aureus (ATCC 25923)[e]	−	ND
Staphylococcus epidermidis (ATCC 14990)[e]	−	0.064
Streptococcus agalactiae (ATCC 13813)[e]	−	0.089
Streptococcus gordonii (ATCC 33399)	ND	0.067
Streptococcus pneumoniae (ATCC 33400)	ND	0.073
Streptococcus pneumoniae (ATCC 6301)[e]	−	ND
Streptococcus pyogenes (ATCC 12344)	ND	0.119
Streptococcus pyogenes (ATCC 19615)[e]	−	ND
Streptomyces griseinus (ATCC 23915)	ND	0.095
Veillonella atypica (ATCC 17744)	ND	0.068
Veillonella parvula (ATCC 10790)	ND	0.066
Vibrio parahaemolyticus (ATCC 17802)	ND	0.110
Xanthomonas maltophilia (ATCC 13637)	ND	0.074
Yersinia enterocolitica (ATCC 9610)[e]	−	0.104
Fungi		
Blastomyces dermatitidis (ATCC 60915)	ND	0.103
Candida albicans (ATCC 18804)	ND	0.058
Candida albicans (ATCC 10231)[e]	−	ND
Coccidioides immitis (NA)	ND	0.073
Cryptococcus neoformans (ATCC 32045)	ND	0.084
Histoplasma capsulatum (NA)	ND	0.084
Pneumocystis carinii (SFG 28914)[e]	−	0.080
Viruses		
Adenovirus (ATCC VR-7)	3.2×10^5	0.066
Cytomegalovirus (ATCC VR-977)	9.8×10^1	0.067
Enterovirus (ATCC VR-1044)	5.6×10^5	0.056
Herpes simplex virus type 1 (ATCC VR-539)	5.6×10^5	0.113
Influenza virus B (ATCC VR-523)	NA	0.063
Parainfluenza virus type 2 (ATCC VR-92)	2.8×10^5	0.071
Respiratory syncytial virus (ATCC VR-955)	2.8×10^1	0.070
Rhinovirus (ATCC VR-284)	2.8×10^4	0.106

[a] Nonmycobacterial organisms used to determine the specificity of amplification and probe hybridization.

[b] ATCC, American Type Culture Collection; W, isolates from J. M. Brown of the Centers for Disease Control and Prevention; DSM, Deutsche Sammlung von Mikroorganismen; SFG clinical specimen from San Francisco General Hospital; NA, not available.

[c] For bacteria and fungi, amplification results determined by agarose gel electrophoresis are shown. Symbols: +, presence of amplification products; −, absence of amplification products. ND, not done. For viruses, input PFU per PCR is shown. NA, not applicable.

[d] The hybridization data are the means of two replicate amplifications. ND, not done.

[e] DNAs from these organisms were used as templates to determine amplification specificity.

TABLE 3. Comparison of PCR results with culture results[a]

Specimen type and PCR result	No. of specimens with the following culture result:		*P* value[b]
	Positive	Negative	
All			>0.75
Positive	75	5	
Negative	7	575	
Smear-positive			>0.9
Positive	59	1	
Negative	0	17	
Smear-negative			>0.5
Positive	16	4	
Negative	7	558	

[a] Summary of the combined clinical data collected at two separate sites.
[b] The *P* values comparing the PCR and culture results were determined by the McNemar χ^2 test.

DISCUSSION

We have developed a PCR assay for the detection of *M. tuberculosis* that is specific and sensitive. The assay uses standardized and quality-controlled amplification and detection reagents to ensure optimal performance and incorporates dUTP and uracil-*N*-glycosylase to effectively control false-positive results from carryover contamination. DNAs from most mycobacterial species are amplified efficiently through the use of pan-*Mycobacterium* primers. The apparently lower amplicon yield from *M. chelonae* and *M. fortuitum* is likely to be indicative of differences in the template DNA concentration estimation rather than real differences in amplification efficiency. The sequences at the primer binding regions from these two species are identical to the corresponding sequences from other species (e.g., *M. smegmatis* and *M. flavescens*) that amplified well. *M. simiae* DNA was not amplified by the primers. *M. simiae* is only rarely associated with disease. Therefore, the clinical impact of not detecting this species is minimal. If amplification of *M. simiae* DNA is desired, it can be achieved by the addition of a second downstream primer (data not shown).

Evaluation of a panel of nonmycobacterial DNAs showed that amplicons were generated only from species that belong to closely related genera. These bacteria are only occasionally found in respiratory tract specimens (13). Even if present, DNAs from these bacteria do not affect amplification of mycobacterial DNA unless they are present in $>10^4$-fold excess over the amount of target DNA (data not shown). These organisms do not affect the specificity of the assay. Unequivocal identification of *M. tuberculosis* complex organisms is achieved by hybridization to an *M. tuberculosis*-specific probe. Only amplicons from *M. tuberculosis* complex organisms generated positive hybridization signals. No cross-hybridization signals were seen when amplicons generated from as many as 10^6 nontuberculous mycobacteria were tested. Amplicons from nonmycobacteria likewise did not hybridize to the probe. The analytical sensitivity of the assay was three or fewer organisms as determined by amplification of purified mycobacterial DNA at various input amounts. Higher sensitivity could be achieved by further hybridization of amplicons to specific probes.

The data in Table 1 show only a single isolate for each species of *Mycobacterium* tested. The amplification and specificity data were confirmed with additional clinical isolates of some of the organisms listed (data not shown).

The clinical performance of the AMPLICOR MTB assay was compared with that of culture. The results from two separate sites involving 662 respiratory specimens indicate that the overall performance of PCR was comparable to that of culture. In cases where the PCR result was positive and the culture result was negative, the final clinical outcome was used to resolve the discrepant results. Using this criterion, four of five PCR-positive, culture-negative results were determined to be truly positive by PCR. The remaining discrepant specimen was positive when tested by PCR for a locus distinct from the 16S rRNA gene. Therefore, the "false-positive" result was not caused by carryover contamination and may indicate the presence of dead or nonculturable bacteria or a subclinical infection. However, the possibility of specimen-to-specimen contamination cannot be ruled out. The 662 clinical specimens included 70 which contained nontuberculous mycobacteria (58 *M. avium-M. intracellulare* complex, 4 *M. kansasii*, 1 *M. gordonae*, 1 *M. fortuitum*, 1 *M. genavense*, 1 unidentified rapid grower, and 4 specimens positive for unidentified AFB). All of these specimens were negative by the AMPLICOR MTB assay. This further confirms the specificity of the assay.

Seven PCR-negative, culture-positive results were noted. All of these were from smear-negative specimens where the organism load is expected to be low. In these cases, the culture results were assumed to be correct. The overall clinical sensitivity and specificity of PCR after resolving discrepant results were 91.9 and 99.8%, respectively. Culture gave an overall sensitivity of 95.3% and specificity of 100%. The difference between culture and PCR was not statistically significant ($P > 0.75$).

When the clinical results were evaluated according to smear status, PCR and culture were found to give equivalent results

TABLE 4. Comparison of PCR and culture results with clinical outcome[a]

Test, specimen type, and test result	No. of specimens with the following clinical outcomes[b]:		Sensitivity (%)[c]	Specificity (%)[c]
	Positive	Negative		
PCR				
All specimens			91.9	99.8
Positive	79	1		
Negative	7	575		
Smear-positive specimens			100	100
Positive	60	0		
Negative	0	17		
Smear-negative specimens			73.1	99.8
Positive	19	1		
Negative	7	558		
Culture				
All specimens			95.3	100
Positive	82	0		
Negative	4	576		
Smear-positive specimens			98.3	100
Positive	59	0		
Negative	1	17		
Smear-negative specimens			88.5	100
Positive	23	0		
Negative	3	559		

[a] Summary of the combined clinical data collected at two separate sites.
[b] The clinical outcome was determined by a consensus of the PCR and culture results or by the criteria outlined in Materials and Methods.
[c] Sensitivity and specificity are relative to clinical outcome.

in smear-positive specimens ($P > 0.9$). In smear-negative specimens, the sensitivity of PCR was somewhat lower than that of culture, 73.1 versus 88.5%. Again, the difference between culture and PCR was not statistically significant ($P > 0.5$). The apparent reduced sensitivity of PCR relative to that of culture in this specimen subset was likely due to low organism load coupled with small specimen input into the PCR. The equivalent of 25 μl of sputum sediment was used per PCR, whereas 100 to 500 μl of sputum sediment was used in culture. Results from experiments where mycobacterial DNA was added to PCR-negative, culture-positive specimen indicate that inhibition was not a major contributing factor.

Standardization of reagents and protocols is important for the routine use of nucleic acid amplification techniques in the diagnosis of mycobacterial infections. The PCR assay that we have developed has proven to have high analytical sensitivity and specificity. Our assay offers several advantages over assays described in the literature. The availability of standardized reagents makes this assay much more reproducible. The pan-*Mycobacterium* coverage endows this assay with great flexibility for future development. Our assay also performed well when compared with bacterial culture. This assay can identify individuals infected with *M. tuberculosis* within 48 h and will be a valuable tool in the management of pulmonary TB.

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