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Genotypic Identification of Mycobacteria by Nucleic Acid Sequence Determination: Report of a 2-Year Experience in a Clinical Laboratory

PHILIP KIRSCHNER, BURKHARD SPRINGER, ULRICH VOGEL, ALBRECHT MEIER, ANNETTE WREDE, MONIKA KIEKENBECK, FRANZ-CHRISTOPH BANGE, AND ERIK C. BÖTTGER*

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, 30623 Hannover, Germany

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Clinical isolates of *Mycobacterium* spp. were identified by direct sequence determination of 16S rRNA gene fragments amplified by polymerase chain reaction. Identification was based on a hypervariable region within the 16S rRNA gene in which mycobacterial species are characterized by species-specific nucleotide sequences. A manually aligned data base including the signature sequences of 52 species of mycobacteria easily allowed rapid and correct identification. The results of this study demonstrate that polymerase chain reaction-mediated direct sequence determination can be used as a rapid and reliable method for the identification of mycobacteria in the clinical laboratory. In addition, the prompt recognition of previously undescribed species is now feasible.

Identification of mycobacteria in the clinical laboratory still remains a fastidious, difficult, and time-consuming procedure. The morphological, cultural, and biochemical tests used for identification require specialized knowledge and well-trained laboratory technicians (17, 33). Additional methods based on lipid analysis, such as high-performance liquid chromatography (HPLC), thin-layer chromatography, and gas-liquid chromatography (GLC), are cumbersome, expensive, and limited in part by the need for standardized growth conditions and are only used in a very few clinical laboratories (8, 23, 25, 27). Identification by use of nucleic acid probes (Gen-Probe, Inc., San Diego, Calif.) is a rapid and widely used procedure but requires a well-grown culture and testing with several probes and covers only a narrow range of mycobacterial species (29).

We previously characterized hypervariable regions within the 16S rRNA molecule that exhibit species-specific characteristics in mycobacteria and proposed that direct sequence determination (3, 9) of these regions may provide a novel and rapid genotypic method for identification of mycobacterial species (34). The pattern of conserved and variable domains within the rRNA molecule offers the unique advantage of performing a single amplification reaction for identification of virtually all *Mycobacterium* spp. The purpose of this investigation was to develop and evaluate a scheme for species identification by nucleic acid sequencing that would be useful in a clinical laboratory. We report here on a 2-year experience with the identification of mycobacteria by 16S rRNA gene sequence determination.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Culture collection strains served as sources for the 16S rRNA data base. These strains were obtained from the American Type Culture Collection (Rockville, Md.) and are in part described previously (2, 20, 35, 40). 16S rRNA gene sequences within hypervariable regions A and B were aligned manually and compiled to result in Fig. 1, 2, and 3. These figures formed the data base for identification based on sequences determined from clinical isolates.

Löwenstein-Jensen slants and BACTEC 12B broth (Becton Dickinson, Sparks, Md.) were inoculated with clinical samples by standard procedures (33). BACTEC bottles were read twice a week by use of the BACTEC 460 (Becton Dickinson). When a growth index of ≥50 was detected, an aliquot was withdrawn from the bottle for Ziehl-Neelsen staining and for inoculation of a blood agar plate, which was subsequently incubated for 24 h at 37°C. Depending on the result of the Ziehl-Neelsen staining (positive or negative for acid-fast bacilli) and on the outcome of the 24-h blood agar culturing (growth or no growth), a scheme for identification by nucleic acid sequence determination was established (Fig. 4). In case of a positive Löwenstein-Jensen slant and a negative BACTEC broth, a small colony was dispersed in 0.5 ml of TE (10 mM Tris [pH 7.4], 1 mM EDTA).

Sample preparation. Nucleic acids were extracted by simple mechanical lysis of bacterial cells. A 0.5-ml aliquot of the broth culture was transferred to a 1.5-ml microcentrifuge tube and incubated for 10 min at 80°C to inactivate the mycobacteria. The sample was centrifuged in a microcentrifuge for 10 min at maximum speed, the supernatant was discarded, and 100 μl of TE and a loopful of acid-washed glass beads with a diameter of 100 μm (Sigma, Munich, Germany) were added. The sample was placed for 2 min at maximum speed in a tissue disintegrator (Mickle Laboratory, Gomshall, United Kingdom) to disrupt the cells. The sample was subsequently centrifuged at maximum speed in a microcentrifuge for 2 min, and a 5-μl aliquot of the supernatant was used in a polymerase chain reaction (PCR).

PCR and sequence analysis. PCR was performed with a 50-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 200 μM each deoxynucleoside triphosphate, 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus, Überlingen, Germany), 30 pmol of primer 264 (5′ TGC ACA CAG GCC ACA AGG GA 3′; corresponding to *Escherichia coli* 16S rRNA from positions 1046 to 1027), and 10 pmol of biotinylated primer 285

* Corresponding author.

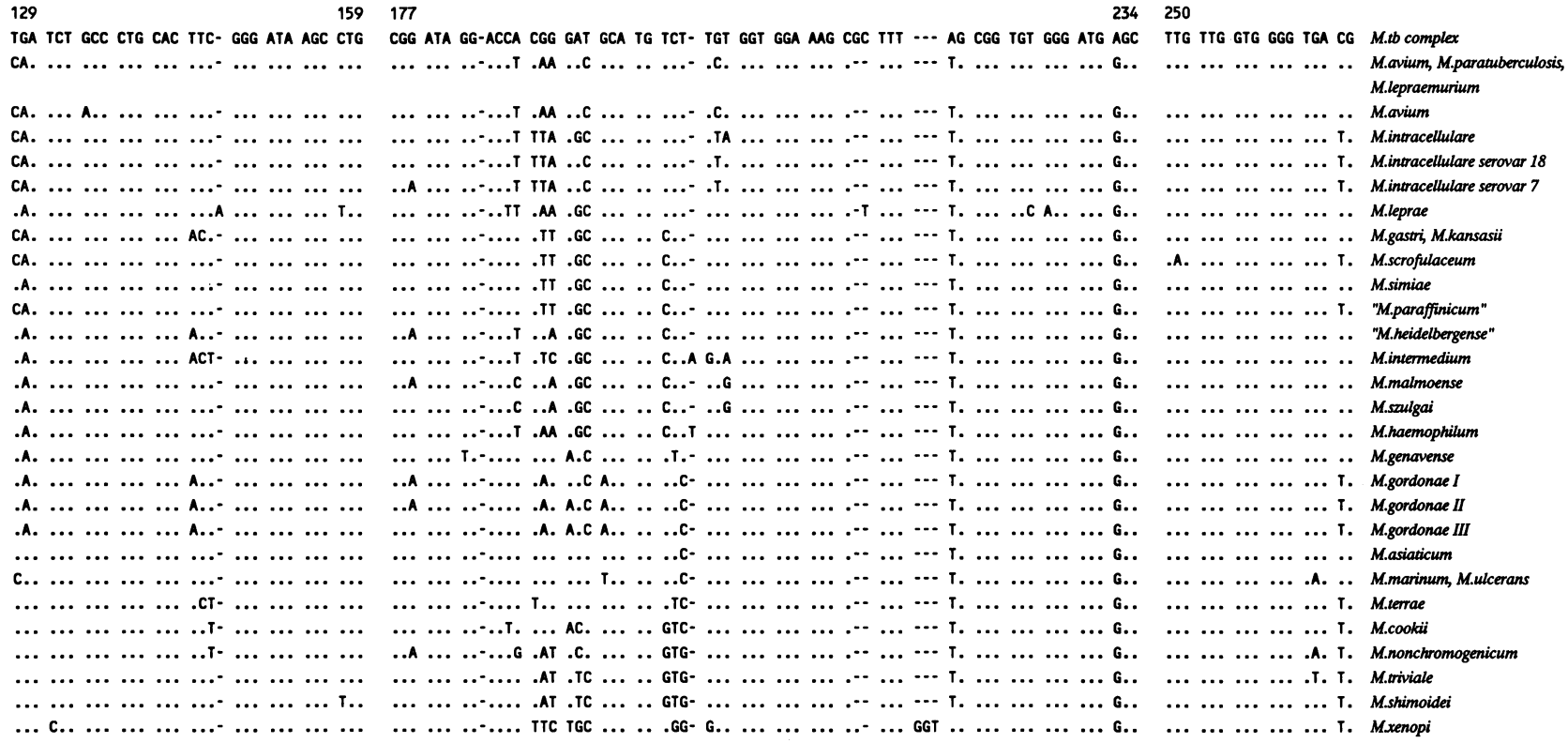

FIG. 1. Alignment of slowly growing mycobacterial 16S rRNA gene sequences within hypervariable region A. *M. tuberculosis* (*M.tb*) was used as a reference sequence. Nucleotides different from those of *M. tuberculosis* are indicated; dashes indicate deletions (2, 6, 10–12, 16, 19, 26, 35, 37, 40; unpublished data); dots indicate identity (35). The numbers indicate the respective *E. coli* 16S rRNA positions. For reasons of space limitation, nt 160 to 176 and nt 235 to 249 are not shown.

129											177																							234	250					
TGA	TCT	GCC	CTG	CAC	TTC-	GGG	ATA	AGC	CTG		CGG	ATA	GG-ACCA	CGG	GAT	GCA	TG	TCT-	TGT	GGT	GGA	AAG	CGC	TTT	---	AG	CGG	TGT	GGG	ATG	AGC		TTG	TTG	GTG	GGG	TGA	CG	*M.tb complex*
...	...	...	...	...	..T-	...	...	...	...		..A	...	TTCC.TA	TT.	.TC	...	..	G.CT	G..	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.flavescens*
...	...	...	...	...	..T-	...	...	...	...		..A	...	CACC.TG	.T.	.TC	...	..	G.CT	G..	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.smegmatis*
...	...	...	...	...	..T-	...	...	...	...		..A	...	CACC.TG	.TN	.TC	...	..	G.CT	N..	..G	...	...	.--	...	-GT	..	...	...	...	...	A..		...	...	...	...	...	T.	*M.thermoresistibile*
...	...	...	...	...	..T-	...	...	...	...		.NN	...	CACC.TG	.TA	.T.	...	.N	G.CT	G..	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.madagaskariense*
		...	...	...	..T-	...	...	...	...		..A	...	CACC.TT	.T.	ATC	...	..	GTCT	G.G	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.confluentis*
...	...	...	...	...	..T-	...	...	...	...		..A	...	CACC.TT	.T.	.C.	...	.N	GTCT	G.G	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	A..	.T.	..	*M.vaccae*
...	...	...	...	...	.CT-	...	...	...	...		...	...	CACC.TT	.T.	.T.	...	..	G..-	G.G	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.phlei*
...	...	...	...	...	..T-	...	...	...	...		..A	...	CACC.TT	TT.	.TC	...	..	G.C-	GT.	A.G	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.agri*
...	...	...	...	...	...-	...	...	...	T..		...	...	T.-....	T..	AC.	T..	N.	GT.-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.komossense*
...	...	...	...	...	..T-	...	...	...	...		..A	...	..-..T.	..C	...	...	..	..G-	...	...	...	...	.--	...	---	T.	...	...	...	...	N..		...	...	...	A..	.T.	..	*M.aurum*
...	...	...	...	...	..T-	...	...	...	...		...	...	..-..T.	..C	...	...	..	..G-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.gadium*
...	...	...	...	...	..T-	...	...	...	...		..A	...	T.-.T..	TC.	.C.	T..	..	GTC-	G..	...	...	...	.--	...	---	T.	...	...	...	...	N..		...	...	...	...	.A.	T.	*M.neoaurum*
...	...	...	...	...	.CT-	...	...	...	...		...	...	..-....	.AC	AC.	T..	..	GTG-	A..	...	.C.	...	.--	...	---	T.	...	...	...	...	A..		...	...	...	...	.A.	T.	*M.chelonae subsp. chelonae*
...	...	...	...	...	.CT-	...	...	...	...		...	...	..-....	.AC	AC.	T..	..	GTG-	A..	...	.C.	...	.--	...	---	T.	...	...	...	...	A..		...	...	...	...	.A.	T.	*M.chelonae subsp. abcessus*
...	...	...	...	...	..T-	...	...	...	...		...	...	..-....	..C	.C.	T..	..	GTG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.senegalense, M.farcinogenes, M.fortuitum third biovariant*
...	...	...	...	...	..T-	...	...	...	...		..A	...	T.-....	..C	.C.	T..	..	GTG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.fortuitum biovar fortuitum*
...	...	...	...	...	..T-	...	...	...	...		..A	...	T.-...G	..C	AC.	T.C	..	GTG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.fortuitum biovar peregrinum*
...	...	...	...	...	.CT-	...	...	...	...		...	...	T.-.T..	T.C	.CC	T..	..	GTG-	...	...	...	...	.--	...	---	T.	...	...	...	...	N..		...	...	...	...	.A.	T.	*M.fallax*
...	...	...	...	...	..T-	...	...	...	...		..A	...	..-...G	.AT	.C.	T..	.N	GTG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	A..	.A.	T.	*M.gilvum*
...	...	...	...	...	..T-	...	...	...	...		..A	...	..-...G	..C	TC.	T..	..	GGG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.fortuitum third biovariant*
.T.	...	...	...	...	..T-	...	...	...	...		..A	...	T.-...G	..C	TC.	T..	..	GGG-	...	...	...	...	.--	...	---	T.	...	...	...	...	NN.		...	...	...	...	.A.	T.	*M.diernhoferi*
...	...	...	...	...	..T-	...	...	...	...		..A	...	T.-.T..	T.C	TC.	T..	..	GGG-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.sphagni*
.A.	...	...	...	...	..T-	...	...	...	...		..A	...	..-.T..	T..	CC.	T..	..	GG.-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.aichiense*
...	...	...	...	...	..T-	...	...	...	...		..A	...	T.-.T..	T..	CC.	T..	..	GG.-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	...	T.	*M.chitae*
...	...	...	...	...	..T-	...	...	...	...		...	...	..-....	...	C..	...	..	.GC-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	A..	.T.	..	*M.chubuense*
...	...	...	...	...	..T-	...	...	...	...		...	...	..-....	...	C..	T..	..	.GC-	...	...	...	...	.--	...	---	T.	...	...	...	...	G..		...	...	...	...	.A.	T.	*M.obuense*

FIG. 2. Alignment of rapidly growing mycobacterial 16S rRNA gene sequences within hypervariable region A comprising helix 10. Dashes indicate deletions (20, 21, 30, 35, 37). N, undetermined nucleotide. Other information is given in the legend to Fig. 1.

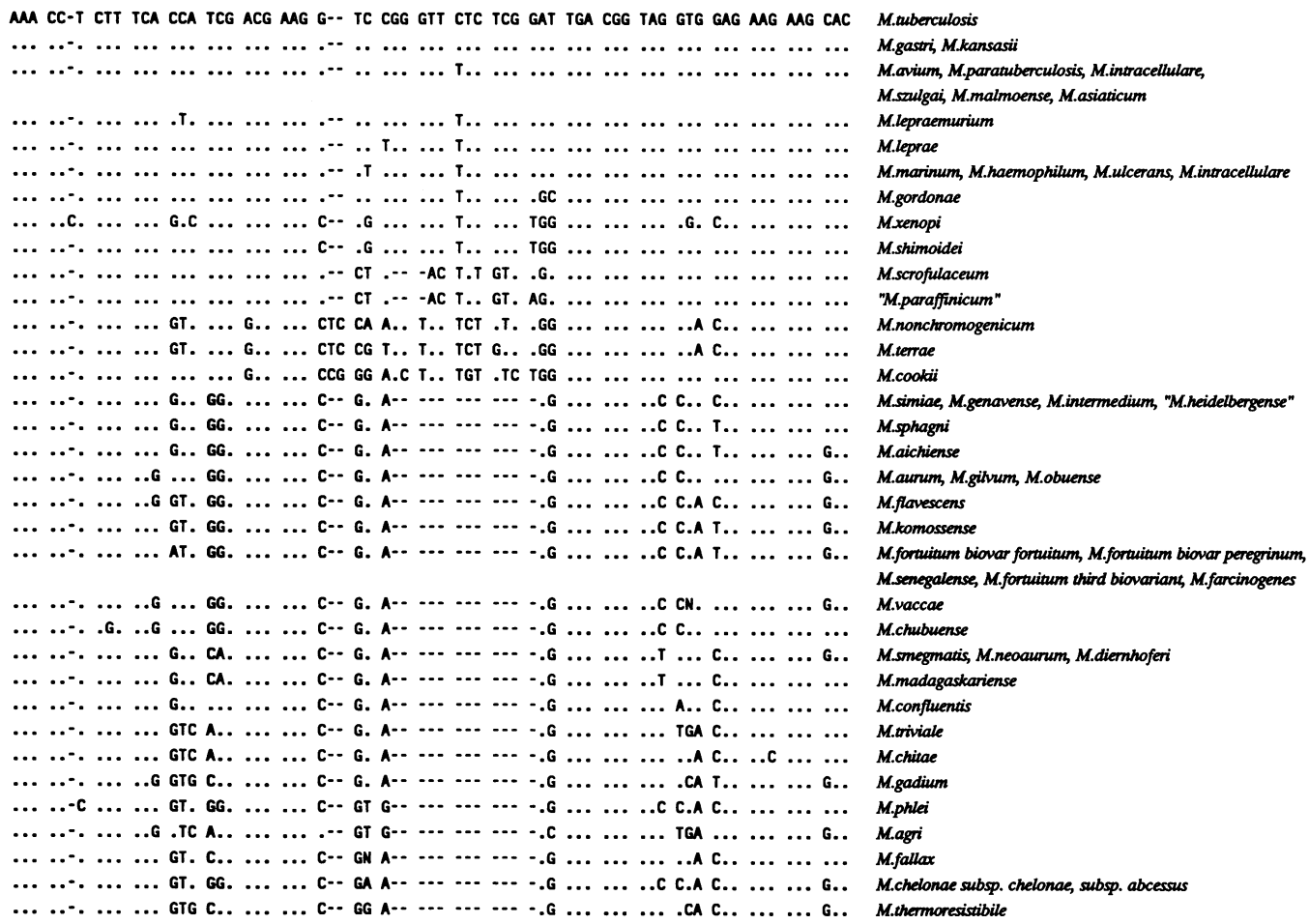

FIG. 3. Alignment of 16S rRNA gene sequences within hypervariable region B comprising helix 18. Because of an insertion of several nucleotides at the primary sequence level, slowly growing mycobacteria typically show a secondary structure with a long helix 18 in this region (35, 37). N, undetermined nucleotide. Other information is given in the legend to Fig. 1.

(5′ GAG AGT TTG ATC CTG GCT CAG 3′; corresponding to *E. coli* 16S rRNA from positions 9 to 30). A 5-μl sample was added to the PCR mixture while the reaction was maintained at 70°C (1). The thermal profile involved 39 cycles with a 1-min denaturation step at 94°C and a 3-min annealing and extension step at 68°C. Successful amplification was controlled by agarose gel electrophoresis as described previously (1).

The biotinylated single-stranded DNA template was prepared by use of Dynabeads M-280–streptavidin (Dynal, Hamburg, Germany) and a Dynal MPC-E magnetic separator essentially as described by the manufacturer. A 20-μl Dynabeads solution (10 μg/μl) was used in each PCR. The beads were resuspended in 20 μl of H_2O. Sequencing was performed with 2 to 5 μl of the Dynabeads single-stranded DNA solution, 2 pmol of sequencing primer 244 (5′ CCC ACT GCT GCC TCC CGT AG 3′; corresponding to *E. coli* 16S rRNA from positions 361 to 341) per reaction, 0.5 to 1.0 μCi of [α-^{32}P]dCTP at 3,000 Ci/mmol (Amersham Buchler, Braunschweig, Germany), and Sequenase version 2.0 (USB, Bad Homburg, Germany) by standard procedures (34, 36; USB and Dynal manuals). Sequencing primer 259 (5′ TTT CAC GAA CAA CGC GAC AA 3′; corresponding to *E. coli* 16S rRNA from positions 609 to 590) was used for determination of the nucleic acid sequence of hypervariable region B. After electrophoresis, gels were fixed in 10% (vol/vol) acetic acid–12% (vol/vol) methanol, dried, and exposed to X-ray film for 6 to 12 h.

RESULTS

Most established mycobacterial species show a unique signature sequence in hypervariable region A (Fig. 1 and 2). Visual inspection of hypervariable region A easily allows one to describe highly discriminative nucleotide positions; e.g., the *Mycobacterium tuberculosis* complex shows a GCGC following position 215 and *M. xenopi* shows an insertion of a GGT at position 221. Members of the *M. tuberculosis* complex, i.e., *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*, exhibit an identical 16S rRNA sequence, reflecting the fact that these taxa have to be separated at a subspecific- or infrasubspecific level (41). The closely related species *M. kansasii* and *M. gastri* show an identical 16S rRNA sequence (34), yet the simple addition of a pigmentation criterion resulted in a specific test for these two taxa. Likewise, *M. ulcerans* and *M. marinum* show a nearly homologous 16S rRNA gene sequence. Hypervariable regions A and B (Fig. 3) do not allow one to distinguish

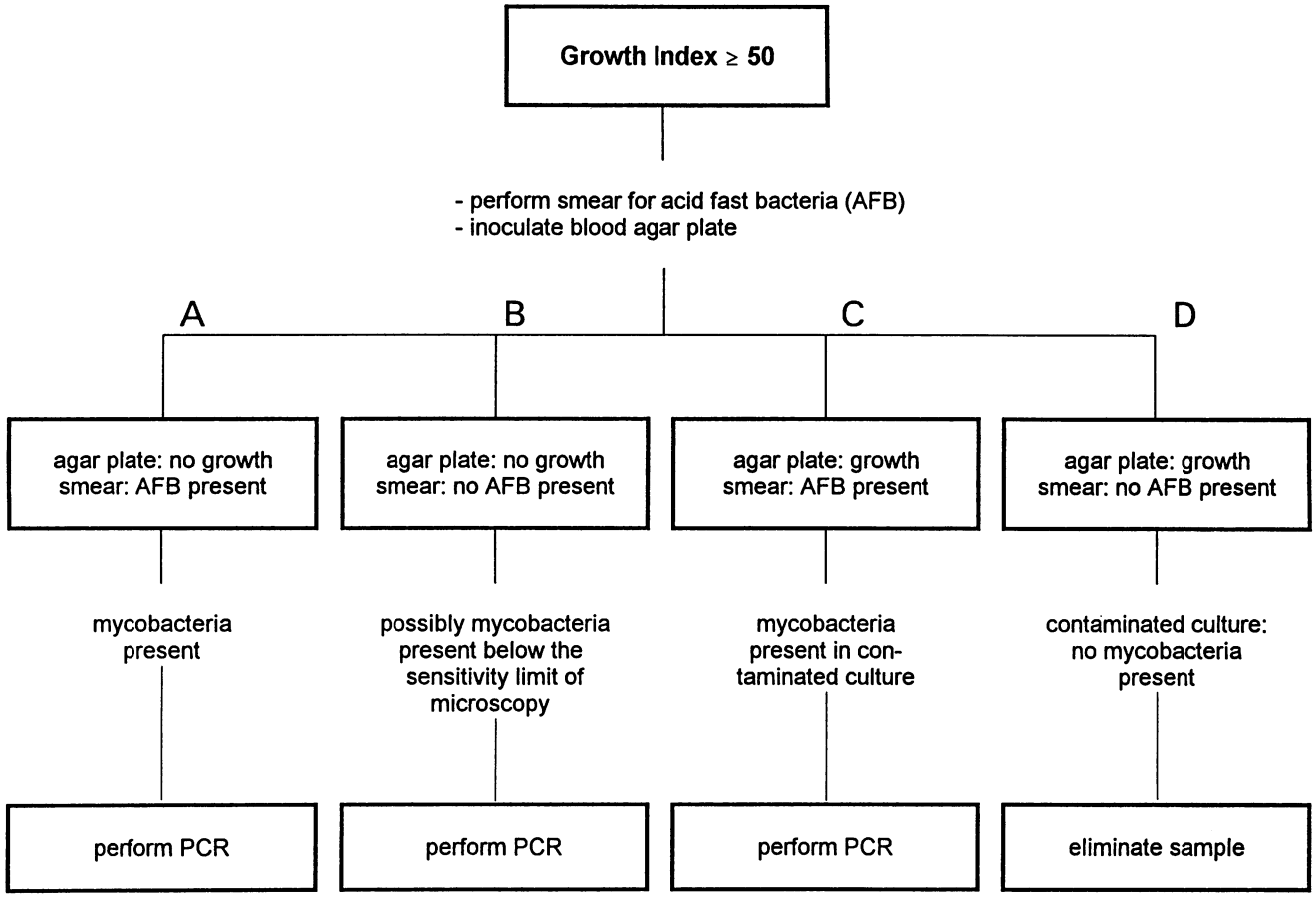

FIG. 4. Flow chart scheme for identification of mycobacteria by nucleic acid sequence determination.

between the two taxa, but sequence determination of nucleic acid positions outside these two regions allows proper identification (12). Among the rapidly growing mycobacteria, *M. chelonae* subsp. *chelonae* and *M. chelonae* subsp. *abscessus* cannot be differentiated within hypervariable regions A and B, although they differ at other 16S rRNA gene positions (unpublished data). *M. gordonae* was the only species encountered that showed intraspecies rRNA gene variability (19) (Fig. 1). This finding of genomic heterogeneity within *M. gordonae* is consistent with results from a restriction enzyme analysis of amplified *hsp-65* gene fragments (31, 39).

A scheme based on a growth index of ≥50 in the BACTEC system was established (Fig. 4). A total of 473 positive BACTEC cultures examined from March 1991 through March 1993 were identified to the species level by nucleic acid sequencing (Fig. 5 shows an overview of the procedure). The growth index ranged from 50 to ≥999 at the time of testing (Table 1). Table 2 shows the identification results of this study; even samples with a growth index of ≤100 (n = 45) as well as contaminated samples (n = 21) were successfully identified. In total, 138 isolates of the *M. tuberculosis* complex and 335 isolates of nontuberculous mycobacteria were found. Correct identification was confirmed by standard biochemical testing (33). We participated regularly in external quality control testing for species identification initiated by the Arbeitskreis Mykobakterien (Working Group on Mycobacteria). The cultures submitted were identified by 16S rRNA sequence determination. All

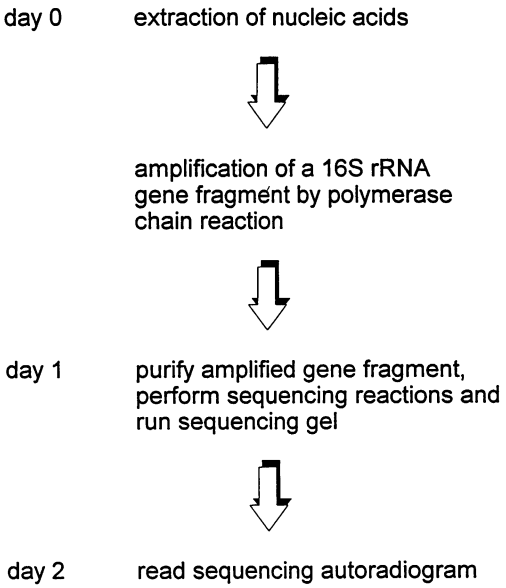

FIG. 5. Schematic overview of the procedure.

TABLE 1. Characteristics of the isolates investigated

Growth index	Smear for acid-fast bacilli	Blood agar plate	No. (%) of isolates
50–100	+	−	38 (8.0)
50–100	−	−	7 (1.5)
100–300	+	−	136 (28.8)
100–300	−	−	24 (5.0)
300–500	+	−	55 (11.7)
300–500	−	−	3 (0.6)
500–999	+	−	189 (40.0)
50–999	+	+	21 (4.4)

isolates (*M. tuberculosis* complex [$n = 7$], *M. simiae* [$n = 2$], *M. kansasii* [$n = 3$], *M. gastri* [$n = 1$], *M. xenopi* [$n = 1$], *M. gordonae* [$n = 2$], *M. chelonae* [$n = 1$], *M. fortuitum* [$n = 2$], *M. phlei* [$n = 1$], *M. avium* [$n = 2$], *M. intracellulare* [$n = 1$], and *M. malmoense* [$n = 1$]) were correctly identified. These isolates were not included in Table 2.

During the course of this study, we encountered isolates that could not be identified by standard biochemical tests. Direct sequencing revealed that a significant proportion of these isolates represented novel species, e.g., *M. confluentis* (21), *M. intermedium* (26), and "*M. heidelbergense*" (10). Secondary structure analysis as well as sequence determination of hypervariable region B (Fig. 3) is especially suited for making higher-order assignments and is particularly useful for the preliminary assignment of isolates that cannot be identified by sequence determination of hypervariable region A and thus may represent hitherto-undescribed species. (i) A distinct branch of rapidly growing mycobacteria that includes, among others, the thermotolerant rapid growers *M. smegmatis*, *M. flavescens*, *M. confluentis*, *M. phlei*, and *M. thermoresistibile* is characterized by an insertion of a nucleotide in helix 10, resulting in a helix containing 11 bp, while all other mycobacteria have a helix that contains 10 bp (21, 30, 37) (Fig. 2). (ii) Rapidly growing mycobacteria have a short helix 18, while slowly growing mycobacteria have a longer helix 18 (5, 35, 37) (Fig. 3). (iii) A steadily expanding group of recently recognized mycobacteria is defined by a unique phylogenetic position. Phenotypically, this group is characterized by slow growth, yet it shows the molecular signature of rapidly growing species, i.e., a short helix 18 (6, 11, 26, 35); these species exhibit in hypervariable region B a nucleic acid sequence that is identical to that of *M. simiae*. (iv) Species belonging to a branch comprising the species *M. terrae*, *M. nonchromogenicum*, and *M. cookii* are characterized by a long helix 18, which is extended by 2 nucleotides (5, 16) (Fig. 3).

TABLE 2. Identification of 473 isolates tested from March 1991 to March 1993

Species	No. (%)
M. tuberculosis complex	138 (29.2)
M. avium	96 (20.3)
M. gordonae	86 (18.2)
M. xenopi	63 (13.3)
M. intracellulare	26 (5.5)
M. genavense	18 (3.8)
M. kansasii	14 (2.8)
M. malmoense	13 (3.0)
M. chelonae	3 (0.6)
M. nonchromogenicum	2 (0.4)
M. szulgai	2 (0.4)
M. fortuitum bv. fortuitum	2 (0.4)
M. fortuitum bv. peregrinum	2 (0.4)
M. terrae	1 (0.2)
M. shimoidei	1 (0.2)
Novel species (11, 26)	2 (0.4)
Anonymous isolates[a]	4 (0.9)

[a] For further details, see the text.

A few isolates that remained anonymous after sequence determination and matching of the obtained sequence with the data base (Table 2) could be assigned to definite phylogenetic branches by use of the higher-order analysis described above. Two isolates were characterized as belonging to the *M. terrae-M. nonchromogenicum-M. cookii* branch (the biochemical reactions of these isolates were compatible with those of either *M. terrae* or *M. nonchromogenicum*), one isolate was assigned to the distinct group of slowly growing mycobacteria that have a short helix 18 (this isolate was unidentifiable by biochemical reactions), and one isolate was found to belong to the phylogenetic branch comprising the thermotolerant rapid growers (this isolate was misidentified as *M. flavescens* by standard biochemical reactions). A complete 16S rRNA gene sequence determination confirmed these preliminary assignments in every case.

DISCUSSION

With the increase in mycobacterial infections over the past decade, there is a great need for rapid methods for identifying *Mycobacterium* spp. In addition, previously unknown or rare forms of mycobacterial disease have been encountered with increasing frequency (42). Using the procedure described here, we correctly identified all 473 positive BACTEC cultures during a 2-year period.

Preparation of the samples was accomplished by simple mechanical disintegration of the bacteria. In contrast to lysis of cells by boiling (10 min at 100°C), which often yielded variable and unreproducible amplification results (data not shown), mechanical disruption with glass beads and a high-speed tissue disintegrator proved to be 100% effective. The isolates were obtained from solid or broth medium without the need for the standardization of growth conditions. A 1-kb fragment of 16S rRNA was amplified with primers 264 and 285. Because of the specificity of primer 264, mycobacterial 16S rRNA gene fragments are preferentially amplified (1), permitting even the correct identification of mycobacteria in contaminated cultures (21 of the 473 cultures investigated). Biotinylation of PCR primer 285 allowed us to use a single-stranded solid-phase sequencing technique (15). We have found this procedure to be much more reliable and convenient than other methods for direct sequencing of amplified gene fragments (e.g., gel purification [34, 43]). Precise identification was possible by comparing manually the determined sequences with the known signature sequences (Fig. 1 and 2). The complete procedure required only 36 h from receipt of a mycobacterial culture to final identification. More advanced instrumentation, which would allow the amplification reaction to be completed in 90 min, and automated on-line nonradioactive sequence determination will permit processing of a sample and final identification within 1 working day. Recent developments in sequencing technologies, such as cycle sequencing, eliminate even the need for purification of the amplified gene fragment, thus allowing sequence determination without prior purification of the PCR product.

The two main problems with the traditional identification of mycobacteria to the species level are the diversity of

techniques and tests that are necessary and the time required for a full identification. The method described here contributes to the solution of these two problems: depending on the instrumentation used, it can be completed within 1 or 2 days, and it represents a universal system of identifying mycobacteria to the species level that does not require specialized knowledge and that even allows nonspecialized microbiologists to make proper identifications.

Direct sequence determination of amplified 16S rRNA gene fragments offers several advantages over conventional biochemical (17, 33), genetic probe (29), restriction enzyme digestion (31, 39), DNA-DNA hybridization (22), and HPLC-GLC (8, 25) identification methods. Commercially available genetic probes only cover a very limited range of mycobacteria. Besides problems with specificity, i.e., false-positive and false-negative hybridization results (7, 14, 18, 19, 24, 38), this approach frequently necessitates the use of a variety of species-specific probes. In practical terms, an isolate that is not identified by the commercially available probes targeting the *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, and *M. gordonae* still must be subjected to traditional and lengthy biochemical testing methods. Another limitation of probe technology is the recognition of mixed cultures. Identification by restriction enzyme digestion of PCR-amplified *hsp-65* gene fragments is a recently introduced technique (31, 32, 39) that suffers mainly from four disadvantages: it requires high-resolution gels and internal standards, since some fragments differ in size only by a few nucleotides; computer-controlled pattern recognition procedures are necessary for interpretation; for many species, the pattern of restriction fragment length polymorphisms has not been determined; and digestion with restriction enzymes must be carefully monitored to avoid misinterpretations as, in the case of no digestion, a failure of the reaction because of, e.g., inappropriate reaction conditions, must be excluded. Identification methods based upon HPLC and GLC require standardized growth conditions, expensive instruments, and sophisticated data analysis and may result in misidentification, even by experienced microbiologists (8).

The procedure described here results in simple sequencing ladders that are easily compared with a data base of signature regions for unambiguous and rapid identification. Mixed mycobacterial infections, which were present in 2 of the 473 samples investigated, are rapidly recognized by the presence of sequence ambiguities, i.e., the simultaneous presence of bands in different lanes of the sequencing gel (data not shown). In contrast to other identification methods, direct sequencing in principle can hardly result in misidentification, as either the sequencing reactions are performed correctly, thus resulting in an appropriate sequence, or the sequencing reactions are done improperly, in which case the autoradiogram yields no readable sequence. A key question concerns the practicability of the method described here. Accordingly, it should be stressed that sequencing presently represents a highly reliable technique that can be easily integrated into almost any major clinical laboratory and does not require costly or sophisticated instrumentation.

16S rRNA gene sequence determination was used successfully to identify extremely fastidious growing mycobacteria which, because of their limited growth, could not be characterized biochemically (4, 6, 13, 28). Standard biochemical identification schemes often yield ambiguous and misleading results, as (i) the tests used may not be highly reproducible and (ii) the phenotype of a species is not an absolute property but may exhibit quite remarkable variability. Because of phenotypic and interassay variability, it can often not be decided whether isolates that cannot be assigned to an established species on the basis of biochemical reactions merely reflect phenotypic or interassay variability or represent a previously unknown species. Likewise, the standard biochemical reactions used for the identification of mycobacteria underestimate the complexity of the genus *Mycobacterium*, as genetically distinct species may exhibit a similar or identical pattern in these reactions, thus resulting in misidentification (10, 11, 36a). In contrast, the 16S rRNA sequence of a species is a stable property specific for microorganisms at the species level (44), resulting in a truly universal system for identification: it allows proper identification of isolates, i.e., assignment to already established taxa, as well as rapid recognition of previously unrecognized species (e.g., 6, 11, 26, 36a). The phenotypic homogeneity and concomitant lack of differential characteristics among members of the genus *Mycobacterium* stand in contrast to the phylogenetic diversity now recognized. We conclude that direct sequence determination of amplified 16S rRNA gene fragments represents a highly accurate and versatile method for the identification of mycobacteria to the species level.

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