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Molecular Technique for Rapid Identification of Mycobacteria

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Identification of mycobacteria through conventional microbiological methods is cumbersome and time-consuming. Recently we have developed a novel bacterial identification method to accurately and rapidly identify different mycobacteria directly from water and clinical isolates. The method utilizes the PCR to amplify a portion of the small subunit rRNA from mycobacteria. The 5′ PCR primer has a fluorescent label to allow detection of the amplified product. The PCR product is digested with restriction endonucleases, and an automated DNA sequencer is employed to determine the size of the labeled restriction fragments. Since the PCR product is labeled only at the 5′ end, the analysis identifies only the restriction fragment proximal to the 5′ end. Each mycobacterial species has a unique 5′ restriction fragment length for each specific endonuclease. However, frequently the 5′ restriction fragments from different species have similar or identical lengths for a given endonuclease. A set of judiciously chosen restriction enzymes produces a unique set of fragments for each species, providing us with an identification signature. Using this method, we produced a library of 5′ restriction fragment sizes corresponding to different clinically important mycobacteria. We have characterized mycobacterial isolates which had been previously identified by biochemical tests and/or nucleic acid probes. An analysis of these data demonstrates that this protocol is effective in identifying 13 different mycobacterial species accurately. This protocol has the potential of rapidly (less than 36 h) identifying mycobacterial species directly from clinical specimens. In addition, this protocol is accurate, sensitive, and capable of identifying multiple organisms in a single sample.

Mycobacteria are slowly growing, acid-fast bacilli. Some members of this group are pathogenic to both humans and animals. *Mycobacterium tuberculosis*, *M. leprae*, and *M. bovis* have long been recognized as human pathogens (12). With the increase in the number of immunocompromised patients, other mycobacterial species, including *M. avium*, *M. intracellulare*, and *M. kansasii*, have been identified as opportunistic pathogens and have become of great interest to clinicians. Also, mycobacterial infections in apparently immunocompetent patients have been reported (6). The diagnosis of mycobacterial infections currently relies on the ability to grow these organisms in the laboratory and their identification either by biochemical tests or by specific nucleic acid probes (4, 12). Because of the long generation time of mycobacteria, an accurate laboratory diagnosis usually takes between 3 and 8 weeks. Recently, rapid PCR-based methods for mycobacterial diagnosis have been described, but they are either costly (7, 8, 10), limited to only a few specific species (5), or capable of resolving only one mycobacterial species per sample (4, 11). We have recently described a method for rapid identification of bacteria by analyzing small subunit (SSU) rRNA restriction fragment length polymorphisms by capillary electrophoresis (1). We have been able to improve this technique by utilizing an automated DNA sequencer, which provides much higher resolution than does capillary electrophoresis analysis, and our improved technique has excellent reproducibility through the use of internal size standards. Our protocol presents a means for rapid and accurate identification of mycobacteria.

We labeled the SSU rRNA from 13 different, clinically important mycobacterial species by PCR amplification and produced a library of 5′ restriction fragment lengths with five different restriction endonucleases. This library was used to characterize 20 unknown mycobacterial species which had been previously identified by conventional methods. By this approach, more than one organism from a single sample can be identified. An inability to perform multiple analyses is a serious limitation with other available methods. When four different previously identified species of mycobacteria were mixed in a single sample, all four were accurately identified by our protocol.

MATERIALS AND METHODS

Mycobacterium cultures and growth conditions. *M. simiae*, *M. kansasii*, *M. avium*, *M. gordonae*, *M. intracellulare*, *M. marinum*, *M. terrae*, *M. tuberculosis*, *M. xenopi*, *M. scrofulaceum*, *M. fortuitum*, *M. bovis*, and *M. chelonae* were used in this study. Stock cultures of mycobacteria (two to five different cultures of each species) were inoculated on slants of Middlebrook 7H10 (Difco, Detroit, Mich.) agar supplemented with cycloheximide (500 μg/ml) or Lowenstein-Jensen medium (Clinical Standards, Carson, Calif.) and were grown at 37°C with 10% CO_2 for 1 month.

DNA extraction. Wooden applicators were used to transfer a small amount of the grown cultures into microcentrifuge tubes containing 500 μl of TE (Tris-EDTA, pH 8.0), 500 μl of buffer-equilibrated phenol (Gibco BRL Life Technologies Inc., Gaithersburg, Md.) and 30 μl of acid-washed glass beads (Sigma, St. Louis, Mo.). The mixture was then subjected to vigorous shaking in a tissue disintegrator for 2 min. After centrifuging for 5 min at maximum speed in a microcentrifuge to separate the phases, the aqueous phase was transferred to a clean microcentrifuge tube. The aqueous phase was reextracted with phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated by adding 2 volumes of 95% ethyl alcohol and 1/2 volume of 7.5 M potassium acetate. The

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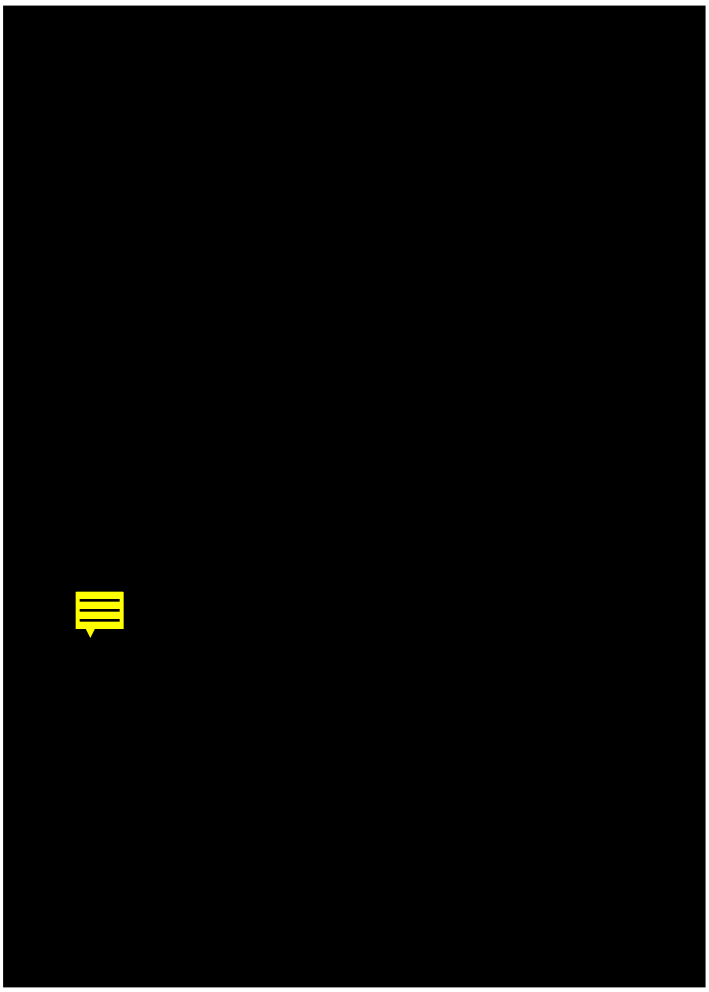

FIG. 1. Gel image produced by ABI 373A automated DNA sequencer. The SSU rRNA PCR products from two *M. avium* samples (lanes 1 to 5 and 6 to 10) and one *M. tuberculosis* sample (lanes 11 to 15) were digested with five different restriction enzymes (*Dpn*II, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I) and were subjected to electrophoresis by an automated DNA sequencer. The yellow bands correspond to the 5′ restriction fragment of the PCR products. The red bands correspond to the Tamara 500 DNA length standards. The lengths of the DNA standards (in base pairs) are shown on the left of the display. The intensities of the small length standards are faint in some lanes of this display but are sufficient for GENESCAN analysis.

precipitated DNA was washed with 70% ethyl alcohol, air dried, and resuspended in 40 μl of TE.

PCR amplification of SSU rRNA and restriction digestion of PCR product. Two primers, with sequences GCCTAACACATGCAAGTCGA (located at the 5′ end of the SSU rRNA sequence) and CGTATTACCGCGGCTGCTGG (located in the middle of the SSU rRNA sequence), were synthesized (Genosis Biotechnologies Inc., The Woodlands, Tex.). These primers hybridize to the conserved 5′ and central regions of SSU rRNA genes of virtually all eubacteria. The 5′ primer has a yellow fluorescent dye label (JOE) at its 5′ end; thus, the PCR-amplified DNA molecules have a fluorescent label at the 5′ end.

Amplification by the PCR was performed with a Perkin-Elmer GeneAmp PCR reagent kit (Roche Molecular Systems, Inc., Branchburg, N.J.) as prescribed by the manufacturer. A 50-ng aliquot of target genomic DNA was suspended in 100 μl of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.01% [wt/vol] gelatin), which contained 200 μM dATP, dGTP, dCTP, and dTTP and 0.5 μM (each) the primers. After 5 min of denaturation at 94°C, 2.5 U of Perkin-Elmer Amplitaq DNA polymerase (Roche Molecular Systems) was added to each reaction mixture and they were overlaid with 50 μl of mineral oil (Sigma). The samples were then subjected to 30 cycles of amplification (denaturation, 94°C for 1 min; annealing, 55°C for 1 min; extension, 72°C for 3 min) and a final 10 min of extension at 72°C. The yield of each amplification reaction was estimated by agarose gel electrophoresis of a small aliquot of the PCR product on a 1% agarose gel run in 0.5× Tris-borate-EDTA buffer for 200 V · h.

The products of the PCR amplifications were purified by WIZARD PCR purification columns (Promega, Madison, Wis.) and eluted in a total volume of 50 μl. One-microliter aliquots of the purified PCR products were separately digested with 5 U of *Dpn*II, *Hae*III, *Hha*I, *Msp*I, and *Rsa*I restriction endonucleases (Gibco BRL Life Technologies and New England Biolabs), in a total volume of 25 μl, for 1 h.

The digested PCR products (1 μl of each) were prepared for electrophoresis by the addition of 2 μl of deionized formamide, 0.5 μl of loading buffer (Applied Biosystems Instruments [ABI], Foster City, Calif.), and 0.5 μl of Tamara 500 DNA length standard (ABI). This mixture was denatured at 94°C for 2.5 min and cooled immediately on ice for 5 min prior to electrophoresis by an automated DNA sequencer.

Restriction fragment analysis by automated DNA sequencer. An ABI 373A automated DNA sequencer and GENESCAN software were used to analyze the fluorescently labeled restriction fragments. A 6% acrylamide gel was used as separation medium on a 24-cm-diameter gel plate, and the sequencer was set on filter set B for recording the fluorescence. The electrophoresis was conducted for 6 h, with limits at 800 V and 30 mA. After electrophoresis, the lengths of the fragments were determined by the GENESCAN analysis software, with Tamara 500 (ABI) as an internal size standard.

Calculation of restriction fragment sizes from mycobacterial sequences found in the Ribosomal Database Project sequence bank. A computer program was developed to simulate our protocol for analyzing restriction fragments from labeled PCR-amplified SSU rRNA sequences (3). Given two primer sequences and a set of restriction sequences, the length from the beginning of the most probable binding site for the labeled primer (5′ primer) to the first restriction site is calculated. If no restriction site is encountered before the most probable binding site of the second primer is reached, the length from primer to primer is given. The set of 5′ restriction fragment lengths for the different mycobacterial SSU rRNAs with different restriction enzymes were computed to provide reference values for comparison with observed values determined from our analysis.

TABLE 1. Observed and expected 5′-terminal restriction fragment lengths for 13 species of mycobacteria

Taxon	Observed (expected)[a] fragment length (bp)				
	*Dpn*II	*Hae*III	*Hha*I	*Msp*I	*Rsa*I
M. simiae	477 (477)	26 (24)	142 (142)	123 (123)	38 (37)
M. kansasii	477 (477)	181 (182)	142 (142)	92 (92)	480 (477)
M. avium	477 (477)	31 (29)	329 (329)	123 (123)	41 (41)
M. gordonae	475 (477)	31 (29)	475 (477)	238 (237)	480 (477)
M. intracellulare	477 (477)	28 (29)	329 (329)	123 (123)	41 (39)
M. marinum	75 (75)	182 (182)	329 (329)	123 (123)	480 (477)
M. terrae	76 (75)	31 (29)	330 (330)	123 (123)	36 (37)
M. tuberculosis	76 (75)	190 (190)	164 (164)	123 (123)	480 (477)
M. xenopi	480 (477)	38 (38)	145 (145)	125 (123)	480 (477)
M. scrofulaceum	450 (449)	175 (175)	135 (135)	123 (123)	38 (36)
M. fortuitum	71 (71)	27 (24)	132 (131)	233 (233)	410 (408)
M. bovis	76 (75)	182 (182)	157 (157)	123 (123)	480 (477)
M. chelonae	71 (71)	26 (24)	321 (321)	123 (123)	36 (35)

[a] Expected lengths, shown in parentheses, were calculated from computer simulations.

RESULTS AND DISCUSSION

The SSU rRNAs from 13 different mycobacterial isolates were individually amplified by PCR with SSU rRNA-specific primers. The set of PCR primers used is capable of efficiently amplifying the SSU rRNA sequences from each isolate, yielding a PCR product that is about 480 bp in length. The lengths of the PCR products were determined by analysis of agarose gel electrophoresis results (products were compared with markers of known sizes), by computer analysis of primer hybridization locations relative to the published Ribosomal Database Project sequences (3, 9), and by analysis of the uncut fragments with an automated DNA sequence analyzer and GENESCAN.

Figure 1 shows the display of an automated DNA sequencer gel. The SSU rRNA PCR products from two *M. avium* samples (lanes 1 to 5 and 6 to 10) and one *M. tuberculosis* sample (lanes 11 to 15) were digested with five different restriction enzymes and are displayed in adjacent lanes (*Dpn*II, lanes 1, 6, and 11; *Hae*III, lanes 2, 7, and 12; *Hha*I, lanes 3, 8, and 13; *Msp*I, lanes 4, 9, and 14; and *Rsa*I, lanes 5, 10, and 15). The unique 5′ proximal restriction fragment for each species can be clearly observed as a yellow fluorescent band in each lane on the gel. Each combination of mycobacterial species and restriction enzyme produces a characteristic band. The similarity between the two *M. avium* samples (lanes 1 and 6, 2 and 7, 3 and 8, 4 and 9, 5 and 10) and their difference from the *M. tuberculosis* sample (lanes 11 to 15) is readily apparent from the gel display. Each mycobacterial species has a unique and reproducible pattern of restriction fragments when analyzed with a set of restriction enzymes.

The red fluorescent bands are internal size standards which allow accurate sizing of the restriction fragments (with accuracy to the base-pair level). The intense fluorescent band at the bottom of each lane of the gel is residual labeled primer used for the PCR amplification and manufacturing of the internal size standard (about 22 bp in length). In lanes 2 and 7, the *M. avium* restriction fragments are 29 bp in length and appear just above the intense fluorescent band at the bottom.

Table 1 summarizes the 5′-end-labeled restriction fragment lengths (rounded off to whole integers) that were observed following restriction digestion of the SSU rRNA PCR products from 13 different mycobacterial taxa (two to five different samples for each taxon) with five different restriction enzymes. Table 1 also shows the expected 5′ restriction fragment length for each combination of mycobacterial species and restriction enzyme. These expected 5′ restriction fragment lengths were calculated from the SSU rRNA sequences of the mycobacteria in the Ribosomal Database Project database (9) by using our computer algorithm (3). In over half of the comparisons, the observed and expected restriction fragment lengths are identical. The greatest difference between observed and expected restriction fragment lengths is a mere 3 bp. This is indicative of the remarkable resolution of this technique. The observed 5′ restriction fragment lengths were used as a reference library for the identification of unknown mycobacterial species by this PCR protocol.

A set of 20 samples from the Mycobacteriology Research Laboratory at Olive View-UCLA Medical Center were ana-

TABLE 2. Identification of 20 unknown samples by digestion of their 5′-end-labeled PCR-amplified SSU rRNA genes

Sample no.	Fragment length (bp)					Identification determined by:	
	*Dpn*II	*Hae*III	*Hha*I	*Msp*I	*Rsa*I	Conventional methods	Our method
8	477	26	142	123	38	*M. simiae*	*M. simiae*
90	477	26	142	123	38	*M. simiae*	*M. simiae*
91	477	26	142	123	38	*M. simiae*	*M. simiae*
26	477	181	142	92	480	*M. kansasii*	*M. kansasii*
101	477	31	329	123	41	*M. avium*	*M. avium*
108	477	31	329	123	41	*M. avium*	*M. avium*
109	477	31	329	123	41	*M. avium*	*M. avium*
120	475	31	475	238	480	*M. gordonae*	*M. gordonae*
111	477	28	329	123	41	*M. intracellulare*	*M. intracellulare*
106	477	28	329	123	41	*M. intracellulare*	*M. intracellulare*
119	75	182	329	123	480	*M. marinum*	*M. marinum*
115	76	31	330	123	36	*M. terrae*	*M. terrae*
110	76	190	164	123	480	*M. tuberculosis*	*M. tuberculosis*
105	480	38	145	125	480	*M. xenopi*	*M. xenopi*
E-9L	450	175	135	123	38	*M. scrofulaceum*	*M. scrofulaceum*
E25-4	450	175	135	123	38	*M. scrofulaceum*	*M. scrofulaceum*
E-3	71	27	132	233	410	*M. fortuitum*	*M. fortuitum*
5681	76	182	157	123	480	*M. bovis*	*M. bovis*
W364	76	182	157	123	480	*M. bovis*	*M. bovis*
CW51	71	26	321	123	36	*M. chelonae*	*M. chelonae*

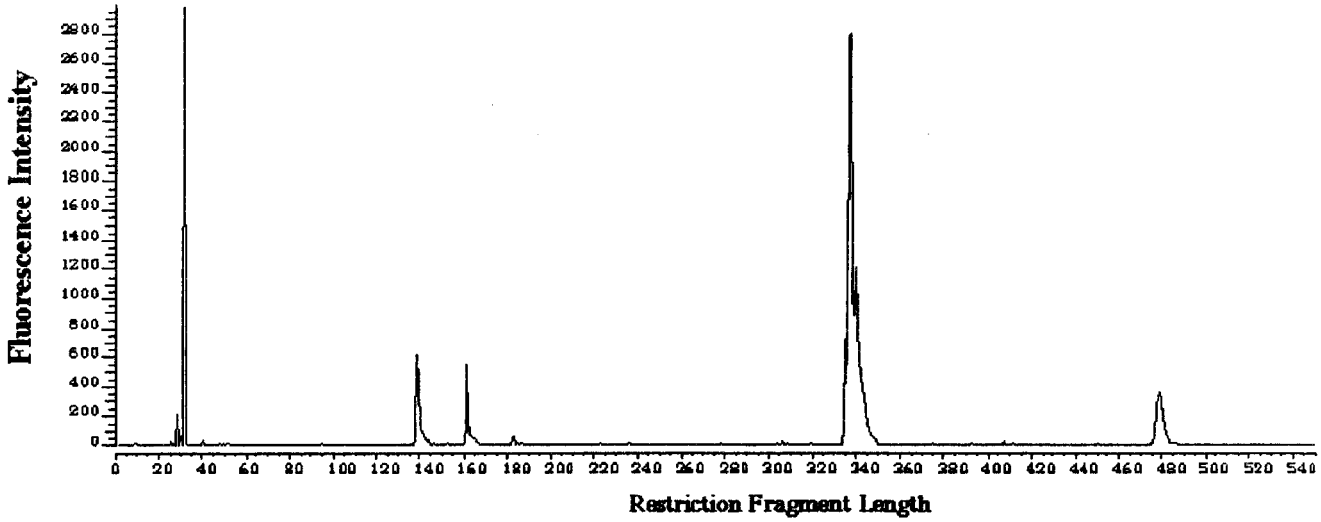

FIG. 2. Electropherogram produced from *Hha*I digestion of the SSU rRNA PCR product of a mixture of four mycobacterial genomic DNAs. Each peak is representative of a species present in the mixture. The contributions of the individual mycobacterial species to this pattern of bands are as follows: *M. kansasii* fragment length, 142 bp; *M. tuberculosis* fragment length, 164 bp; *M. avium* fragment length, 329 bp; *M. gordonae* fragment length, 475 bp. The leftmost peak (22 bp) on the electropherogram is produced by residual labeled primer from PCR. The fluorescence intensity of each peak is proportional to the amount of genomic DNA present for each species in the mixture. The electrophoresis was conducted for 6 h on an ABI 373 automated sequencer with limits at 800 V and 30 mA. The size and intensity of each band were determined by using GENESCAN software.

lyzed. Each of these samples had been previously identified by conventional techniques in that laboratory. The samples were analyzed as a numbered set of unknowns. Table 2 shows the lengths of the labeled restriction fragments produced by our analysis of these samples with five different restriction enzymes. By comparing the pattern of 5′-end-labeled restriction fragment lengths produced by each enzyme shown in Table 2 with the 5′ restriction fragment length library shown in Table 1, the unknowns can be readily identified. For one pair of the mycobacterial species, the pattern of 5′-end-labeled restriction fragments (for this set of five restriction endonucleases) is so similar that unambiguous identification of these species may not always be possible. *M. avium* and *M. intracellulare* differ only by *Hae*III digestion, with *M. avium* and *M. intracellulare* yielding fragments of 31 and 28 bp, respectively. In all other pairwise combinations of these 13 species, the restriction fragment length patterns unambiguously identify the species. A total of 78 pairwise comparisons for the 13 mycobacterial species are shown in Table 1. The 5′ restriction fragment length patterns for these pairwise comparisons differ by two or more enzymes for 65 of the 78 comparisons (83%). Thus, this analysis provides a robust identification of these mycobacterial species. The close agreement between diagnoses by conventional methods and our identifications of mycobacterial species suggests that our protocol is a potentially powerful tool for use in medical microbiology laboratories.

The ability to accurately identify more than one organism in a sample is an advantage provided by this technique. Normally a patient is infected with only one organism, but on occasion, especially with immunocompromised patients, it is possible to have multiple organism infections. To demonstrate the ability of this protocol to identify more than one organism in a sample, DNAs from four different mycobacterial species (*M. kansasii*, *M. tuberculosis*, *M. avium*, and *M. gordonae*) were mixed into a single sample. The PCR product was digested with *Hha*I and analyzed. Figure 2 shows the resulting electropherogram. The contributions of the individual mycobacterial species to this pattern of bands are as follows: *M. kansasii* fragment length, 142 bp (12%); *M. tuberculosis* fragment length, 164 bp (9%); *M. avium* fragment length, 329 bp (62%); *M. gordonae* fragment length, 475 bp (16%). At the extreme left of the electropherogram is a peak produced by residual labeled primer left over from PCR. The presence of several organisms in a single sample can be readily detected even when the amount of each organism varies considerably. The peak area is a reflection of the concentration of each organism in the mixture.

A major advantage of this protocol is that it offers a potential for rapid turnaround time (36 h) from a clinical sample to an accurate diagnosis. Conventional methods take a minimum of 3 to 8 weeks for accurate identification because of the long generation time of mycobacteria. Hybridization probe methods, which have become common in mycobacterial identification, require a minimum 2-week growth period to produce the required DNA and have the limitation that they can identify only a few species. Other methods under development, such as PCR-based methods which are rapid, still lack the ability to identify a broad spectrum of mycobacteria. None of the methods have the ability to identify more than one organism in a single sample.

A potential drawback in utilization of this protocol for identification of mycobacteria may be the availability of automated DNA sequencers in clinical laboratories. Although automated sequencers are relatively expensive, their performance, accuracy, and utility have been well documented and they are becoming common equipment in research and some clinical laboratories. Considering the speed and accuracy provided for the identification of mycobacteria, the cost associated with an automated DNA sequencer is not excessive.

This study demonstrates the feasibility of using this method for fast, sensitive, and accurate identification of mycobacteria. This method could circumvent problems associated with long culturing times and can be used for a broad spectrum of mycobacterial species. Studies to further evaluate the use of this method with clinical specimens are in progress.

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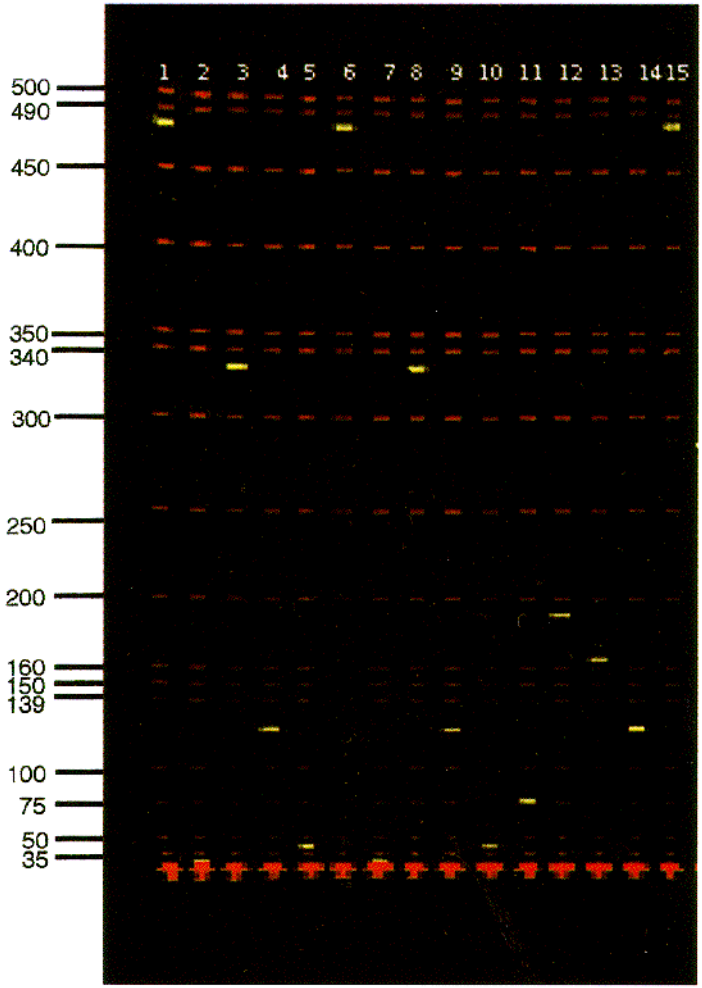
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