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Identification of mycobacteria to the species level by automated restriction enzyme fragment length polymorphism analysis

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Abstract An automated method for the restriction fragment length polymorphism (RFLP) analysis for the differentiation of mycobacteria to the species level is described. After polymerase chain reaction (PCR) amplification of a sequence of the gene encoding the 65-kDa surface antigen common to all mycobacteria the product was investigated by RFLP analysis. For accurate determination of fragment sizes the asymmetrically fluorescein-labelled PCR product was partially digested with restriction site enzymes *Bst*EII and *Hae*III. The fragments obtained were analysed electrophoretically using an automated laser fluorescence DNA sequencer. Determination of fragment sizes revealed a deviation of ±1 base pair (bp; 0.6%) when compared to expected sizes. The validity of this approach was confirmed by analysing mycobacterial DNA obtained from pure cultures of *Mycobacterium* (M.) *tuberculosis* and alcohol-fixed smears as well as paraffin-embedded sputa of patients with culture-proven tuberculosis. Additionally a diagnostic algorithm was established by investigation of cultured *M. bovis, M. bovis* bacille Calmette-Guérin, *M. avium, M. intracellulare* and *M. fortuitum*. The method allows the identification of restriction enzyme sites which are only 40 bp apart. Partial restriction enzyme digestion of asymmetrically fluorescence-labelled PCR products will presumably lead to the discovery of new restriction enzyme sites.

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Introduction

In the routine laboratory setting differentiation of mycobacteria is performed by determination of phenotypic and biochemical characteristics [11]. Identification of an unknown organism can take 4–8 weeks due to the long generation times of organisms. Thus various methods have been developed for the rapid identification of mycobacteria. Today DNA- or RNA-based hybridization probes are available which are able to identify *Mycobacterium (M.) tuberculosis, M. avium* and *M. intracellulare* [12, 16]. Furthermore the identification of mycobacterial species can be performed by high-performance liquid chromatography patterns of mycolic acids [2]. However, both methods require a large number of bacilli which have to be obtained by isolation of pure culture. Recently two studies were published describing the identification of mycobacterial species in uncultured clinical samples [18, 23]. Since the amount of mycobacterial DNA present in clinical samples is usually insufficient [17], a two-step assay had to be performed. These assays combined the amplification of a sequence common to all mycobacteria with subsequent restriction fragment length polymorphism (RFLP) analysis of unique restriction sites characteristic for most of the mycobacterial species. Respective RFLP patterns are recognized visually. However, problems may occur if sizes of fragments obtained differ by only a few base pairs (bp). Due to this fact samples are run in parallel with stored digested reference myobacteria strain DNA or computers converting running distance on electrophoresis to molecular size of fragments are used [19].

Using an automated laser fluorescence DNA sequencer we describe a rapid and reliable automated method for size determination of DNA fragments ob-

tained by a modified restriction site enzyme digestion. In order to evaluate this method strains of cultured *M. tuberculosis, M. bovis, M. bovis* bacille Calmette-Guérin (BCG), *M. avium, M. intracellulare* and *M. fortuitum* were examined according to a previously published study by Telenti et al. [23] using the two restriction enzymes *Bst*EII and *Hae*III. Thus, a new reference algorithm for diagnostic application of the automated identification of mycobacteria to the species level was generated. Finally, the practicability of this method for the pathologist was demonstrated by investigation of alcohol-fixed as well as paraffin-embedded clinical specimens.

Materials and methods

Strains of *M. tuberculosis* complex (*M. tuberculosis, M. bovis, M. bovis* BCG), *M. avium, M. intracellulare* and *M. fortuitum* were obtained from culture collection at the Federal Public Health Laboratory of the University of Innsbruck, Austria. Initial identification of mycobacterial strains was performed by conventional biochemical methods [11]. Additionally ten alcohol-fixed auramin-positive sputum smears and ten paraffin-embedded sputum samples of patients with culture-proven tuberculosis were investigated to demonstrate the practicability of this method on clinical specimens. Acid-fast bacilli (AFB) had been microscopically demonstrated by Ziehl-Neelson and auramin staining in five cases each. Presence of amplifiable mycobacterial DNA had been demonstrated in all specimens; specificity of the polymerase chain reaction (PCR) product had been evaluated by direct sequence analysis as published previously [24].

All strains were grown on solid medium (Löwenstein-Jensen medium) using standard methods. Colony growth from each strain was mixed in 0.5 ml of sterile water in a bijou bottle containing five sterile glass beads until a suspension equivalent to no. 0.5 MacFarland opacity standard (Difco) was obtained. A 1:10 dilution in sterile water was heated to 94° C for 20 min and the samples were stored frozen at –20° C until tested.

Alcohol-fixed and auramin-positive sputum smears mounted on glass slides were subjected to four changes in a decreasing series of alcohols (100% to 50%) followed by rehydration of samples in distilled water. The material was removed from glass slides with 100 µl digestion buffer containing proteinase K (100 mM TRIS-hydrochloric acid (HCl), pH 8.0, 25 mM ethylenediaminetetaacetic acid, 0.5% sodium dodecyl sulphate, 0.01% proteinase K). Proteinase K digestion was performed overnight at 55° C. Material was used as template without any further purification. From the paraffin-embedded sputum samples 5-µm-thick sections were cut and mounted on glass slides. Subsequent to dewaxing of samples in xylene the same procedure was performed as described above.

PCR was performed according to the standard protocol of Mullis and Faloona [15]. A final volume of 20 µl contained 0.2 pmol/µl of primers 5'-ACCAACGATGGTGTGTCCAT-3' (sense) and fluorescein labelled 5'-Fluor-CTTGTCGAACCGCATACCCT-3' (antisense), 200 µM of each dNTP, 10 mM TRIS-HCl (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 U Ampli-*Taq* DNA-polymerase (Perkin-Elmer Corporation, Norwalk, Conn., USA), and 1 µl template containing 5–20 AFB. The reaction was subjected to 30 cycles of amplification on an automated thermocycler (Biomed, Bachofer, Germany). Following a first denaturation step at 94° C for 4 min, subsequent cycling consisted of denaturation at 94° C for 30 s, annealing for 60 s starting at 57° C and a stepwise decrease of 1° C after each third cycle to finally 54° C, and extension at 72° C for 90 s.

Two microlitres of the reaction mixtures were analysed on native 12% polyacrylamide gels (acrylamide/piperazinpolyacrylamide, T (total concentration of acrylamid)=30%, C (crosslinking factor)=3.3%) without further purification. Polyacrylamide gel electrophoresis was carried out on a horizontal electrophoresis system (Multiphor, Pharmacia, Sweden) with ultrathin gels baked on Gelbond Pag (FMC, Rockland, Me., USA) using a discontinuous buffer system as described previously [4, 24]. The gel was run for 45 min at 1000 V, 40 mA, 5 W and 15° C. Bands were visualized by silver staining according to standard protocols [6].

Partial restriction enzyme digestion of amplification products was performed according to a standard protocol [11] which was modified as follows: 5 µl of PCR product were added to a mixture containing 0.25 U enzyme (*Bst*EII or *Hae*III; Boehringer Mannheim, Germany), 1.25 µl of respective restriction buffer (5X buffer B or M) and 6.0 µl of water. *Bst*EII digestion was performed at 60° C, *Hae*III digestion at 37° C for 30 min each.

One microlitre of partially digested PCR product was mixed with 5 µl formamide solution (95% formamide and 5 mg/ml dextran blue). After denaturation samples were loaded on a 6.6% (19:1) acrylamide-bisacrylamide denaturing (7 M urea) sequencing gel. Electrophoresis was carried out by an automated laser fluorescence sequencer (A.L.F. Sequencer, Pharmacia Biotechnology, Sweden). The gel was run for 4 h at 1600 V, 38 mA, 45 W and 45° C. Fragments were analysed with the Fragment Manager software (Pharmacia Biotechnology) utilizing Microsoft Windows 3.1.

External size markers consisted on double-stranded DNA from PCR products which were labelled with fluorescein on one 5' end. The sizes of these products were 123 bp, 268 bp, 383 bp and 439 bp, respectively. The undigested part of the 439 bp PCR product and the fluorescein-labelled (unbound) 20 bp primer served as internal standards.

Sequence analysis was performed by solid-phase sequencing of single-stranded PCR products as described previously [4]. PCR fragments were obtained by amplification with the primers described above in which one primer was biotinylated. Oligonucleotide primers 5'-GAGATCGAGCTGGAGGATCC-3' (sense) and 5'-AGCTGCAGCCCAAAGGTGTT-3' (antisense) [10] were used as internal sequencing primers. Using fluorescein-15-dATP as internal label and T7 polymerase (AutoRead Kit, Pharmacia Biotechnology) DNA sequences of PCR products were determined by dideoxy sequencing. Gel electrophoresis, data collection and analysis were performed on the A.L.F. Sequencer.

Results

Identification of myobacteria to the species level by means of RFLP analysis is shown in Fig. 1. Using a laser fluorescence sequencer for the electrophoresis and automated measurement of fragments, detection was restricted to the fragment containing the 5' end fluorescein-labelled primer (Fig. 1A). Therefore, an incomplete restriction enzyme digestion of the asymmetrically fluorescein-labelled PCR product was performed. Consecutively, several (fluorescence-labelled) fragments, the size of which depended on the distance between restriction site and fluorescein-labelled primer, were found (Fig. 1B). The automated laser fluorescence sequencer detection of these PCR fragments, obtained by *Bst*EII digestion of the 439 bp product of *M. tuberculosis*, is shown in Fig. 2A. Subsequently, sizes of these fragments were evaluated by comparison with external and internal standards (Fig. 2B). This was performed by use of Fragment Manager software. In eight of ten investigated alcohol-fixed smears and seven of ten paraffin-embedded sputa containing unknown amounts of mycobacterial DNA, detectable fragments were obtained by the procedure described above. In the remaining five cases a modification concerning the time period required for digestion (15–45

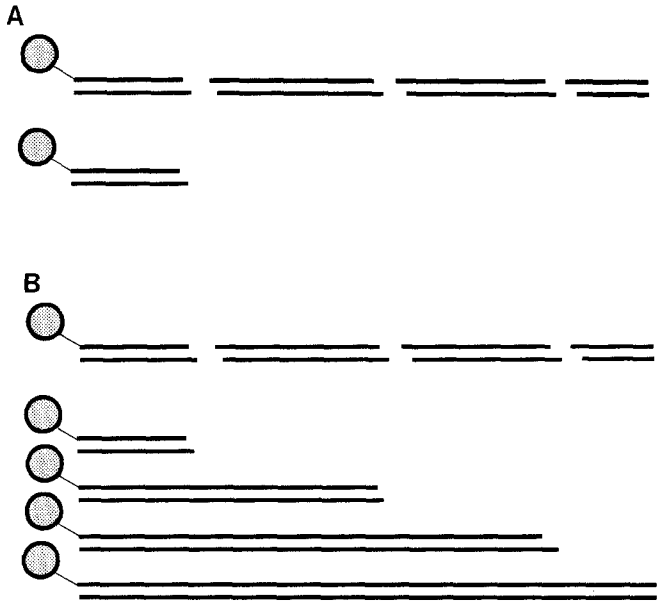

Fig. 1 **A** A complete digest of an asymmetrically labelled polymerase chain reaction (PCR) product is shown resulting in a single detectable fragment. **B** A partial digest of an asymmetrically labelled PCR product results in fragments reflecting the precise distance of the cutting site from the label

min) was necessary. No differences in RFLP patterns of *M. tuberculosis*-DNA obtained from pure culture, auramin-positive smears or paraffin-embedded sputa were observed.

In order to examine the value of this automated RFLP analysis we compared the evaluated fragment sizes with sizes expected by the known nucleotide sequence. This was done by means of *M. tuberculosis* [21] revealing a maximal deviation of the observed fragment size to the expected fragment size consistently smaller than 1 bp (Table 1). Accordingly the size evaluation exceeded 99.4%.

Investigating *M. bovis, M. bovis* BCG, *M. avium, M. intracellulare* and *M. fortuitum* by this method no differences in mycobacterial strains of the *M. tuberculosis* complex could be found. However, *M. avium, M. intracellulare* and *M. fortuitum* showed distinct patterns of fragments. The algorithm of the RFLP patterns of all

Fig. 2 Detection of PCR fragments obtained by *Bst*EII digestion of the 439 base pair (bp) product by the automated laser fluorescence sequencer is shown in **A**. On the *x*-axis it can be seen at which time (min) fragments were detected. In tracing 36, fragments with unknown sizes, and in tracing 40 fragments of known size, are shown. Unknown fragment sizes are evaluated by comparison with external and internal standards (**B**). Consecutively determined length of all fragments can be seen on the *x*-axis (bp). ▼ Peaks of internal standards (20 bp, 439 bp), ▽ peaks of external standards (123 bp, 268 bp, 383 bp) □ detected fragments with unknown sizes

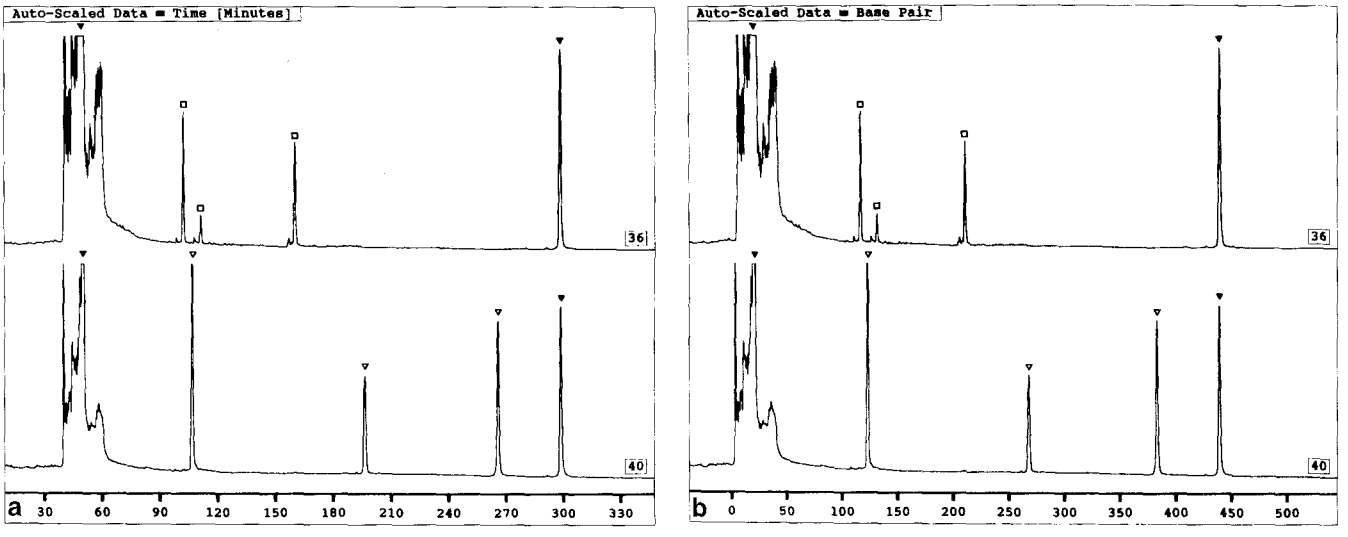

Table 1 Determination of fragment sizes of *Mycobacterium tuberculosis* by partial restriction enzyme digestion

Restriction enzyme	Observed fragment size (bp)	Expected size (bp)	Deviation (bp)	Accuracy (%)
*Bst*EII	116.7	116	0,7	99.40
	131.5	132	0,5	99.62
	210.6	211	0,4	99.81
*Hae*III	153.2	153	0,2	99.87
	221.9	222	0,1	99.95
	264.1	264	0,1	99.96
	280.8	281	0,2	99.93
	302.8	303	0,2	99.93
	314.7	315	0,3	100

Table 2 Algorithm for the differentiation of mycobacteria to the species level

	*Bst*EII	*Hae*III
M. tuberculosis	116/131/210 bp	153/222/264/281/303/315 bp[a]
M. bovis	116/131/210 bp	153/222/264/281/303/315 bp[a]
M. bovis BCG	116/131/210 bp	153/222/264/281/303/315 bp[a]
M. avium	211 bp	40[c]/142/184/221/263/280/315 bp[b]
M. intracellulare	116/ /211 bp	40[c]/ /185/221/ /280/314 bp[b]
M. fortuitum	116/131/210 bp	40[c]/ 97/ /221/244/296 bp[b]

[a] Fragment sizes determined by nucleotide sequence
[b] Fragment sizes determined by automated detection of restriction fragments
[c] Discovered restriction site yielding a fragment of 40 nucleotides in length

mycobacterial strains investigated is shown in Table 2. In direct sequence analysis performed to confirm that the obtained PCR products were specific a complete homology to the known 383 bp sequences was found [10].

Discussion

RFLP analysis by means of an automated laser fluorescence sequencer was described for the first time by Voss and coworkers [26]. Subsequently several labelling methods of PCR products were introduced to detect all fragments obtained by restriction enzyme digestion. The results of these attempts, however, were mainly disappointing [8, 13, 14]. Brenner and Livak [1] described a method of size separation of restriction fragments by labelling fragments at 5' overhangs using dideoxynucleotides tagged with fluorescent dyes. Analysis was performed by PAGE and detection of fluorescent emissions by a DNA sequencer. An alternative method to RFLP was introduced by Cawkwell et al. [3] by amplification of sequences containing microsatellites using one fluorescence-labelled primer. Detection of PCR products was performed by an automated DNA sequencer. Thus our method represents a modification of existing techniques. We were able to demonstrate that the identification of mycobacteria to the species level can also be performed by RFLP analysis of partially digested PCR products. In comparison with the studies of Plikaytis et al. [18] and Telenti et al. [23] this method offers an essential improvement by the introduction of a laser fluorescence DNA sequencer for the automated detection and measurement of fluorescence-labelled fragments [27]. Subsequent accurate sizing of these fragments can be performed by comparison of detected fragments with external and internal standards (Fig. 2). Thus, in contrast to common methods using completely digested PCR products and estimation of fragment sizes varying within a range of ±5 bp [23], we were able to evaluate the size of the respective fragments with a deviation less than 1 bp (99.4% accuracy). Accuracy of fragment sizing is not necessary in our study; however, it may be of importance investigating short tandem repeats by RFLP [9].

Another advantage of our method is the possibility of data storage. By the use of a computer programm (Fragment Manager software) it has become possible to compare two DNA samples at different times and on different gels with both excellent reproducibility and accuracy. Thus, the use of various DNA references which have to be run in parallel with the samples investigated can be avoided in most cases.

Furthermore the partial restriction enzyme digestion has led to the discovery of a new *Hae*III site yielding a fragment 40 nucleotides in size (Table 2). Due to the fact that complete digestion of a PCR product, containing restriction sites close to each other, will result in small fragments which escape notice or are indistinguishable from primer-dimers, Telenti and co-workers [23] were unable to recognize this restriction enzyme site characteristic for *M. avium, M. intracellulare* and *M. fortuitum*. Similar observations were made by Smith and Birnstiel [22] describing in 1976 the partial restriction enzyme digestion of asymmetrical radioactively labelled DNA. It can be presumed that the partial restriction enzyme digestion technique will provide further information in a variety of studies by the detection of (additional) restriction enzyme sites which are only 20–50 bp apart.

In contrast to Telenti and co-workers [23] no inhibition of restriction enzymes by PCR products obtained from smear-positive sputa was found. Thus, the algorithm of RFLP patterns shown in Table 2 offers the opportunity to identify most common mycobacteria in clinical specimens. However, investigation of further culture-defined myobacterial strains will be necessary to increase the value of this method.

Theoretically, restriction fragment analysis can be performed in all cases with amplifiable (mycobacterial) DNA. Our results demonstrate that the detection and identification of mycobacteria to the species level by this method can be performed on alcohol-fixed or paraffin-embedded material, even on archival tissue samples [25]. Restriction digest of PCR products, however, depends on conditions and times used as well as the amount of amplification product (varying from 0.5 fmol to 0.5 pmol/µl). Therefore, samples have to be run at least in triplicate. Nevertheless, the approximate turnaround time from receiving samples to sending out reports and the costs for such an investigation are less in comparison to traditional methods (such as Löwenstein-Jensen medium).

Up to now identification of atypical mycobacteria in fixed specimens could only be performed by sequencing [20]. Partial degradation of DNA [7] or the loss of RNA

[5], however, will prevent sequence analysis and the consecutive search for homologue sequences in many cases. Thus, RFLP analysis offers the pathologist the possibility of retrospective identification of mycobacteria to the species level on paraffin-embedded specimens.

Automated identification of mycobacteria by RFLP analysis in combination with the use of an automated laser fluorescence sequencer is a simple, rapid, highly reliable and accurate method.

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