MOLECULAR TAXONOMY AND EPIDEMIOLOGY

Comparison of three restriction endonucleases in IS1245-based RFLP typing of *Mycobacterium avium*

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IS1245-based restriction fragment length polymorphism (RFLP) analysis has been proposed recently for molecular typing of *Mycobacterium avium* isolates. As there is no standardised method with respect to the optimal restriction enzyme, three restriction endonucleases were tested for analysis of 17 human isolates. The restriction endonucleases, selected on the basis of the physical maps of IS1245 and of the highly homologous IS1311, were *Bsa*AI, that cleaves IS1245, *Pvu*II, that cleaves IS1311, and *Nru*I, that cleaves both IS1245 and IS1311. All the restriction endonucleases yielded polymorphic and complex RFLP patterns. However, *Bsa*AI- and *Nru*I-generated bands were more evenly distributed and easier to detect than *Pvu*II-generated bands, most of which clustered in a narrow zone of the fingerprint. In some cases, DNA digestion with *Bsa*AI or *Nru*I yielded probe-specific restriction fragments of molecular size lower than expected. Moreover, digestion with *Nru*I, which was expected to generate the highest numbers of bands in all the isolates, yielded fewer bands than were obtained with *Bsa*AI or *Pvu*II in 14 and 5 isolates, respectively. These findings might suggest the existence of unidentified IS1245-related insertion element(s) in *M. avium* isolates. Computer analysis of the IS1245-based RFLP patterns of *M. avium* isolates showed that the restriction endonucleases were capable, although with minor differences, of defining distinct banding patterns and clusters of identical or highly related isolates, thus confirming IS1245-based RFLP analysis as a useful technique for epidemiological studies.

Introduction

Mycobacterium avium, long recognised as a primary pathogen of birds, behaves as an opportunist human pathogen that can be isolated from environmental, animal and human sources [1]. In immunocompetent patients, it causes pulmonary infections and cervical lymphadenitis [2] and, occasionally, soft tissue infections [3]. However, in a high proportion (25–50%) of patients with the acquired immunodeficiency syndrome (AIDS), *M. avium* causes severe disseminated infections [4, 5].

The sources of human infection by *M. avium* and routes of transmission are not completely clear. Therefore, the availability of *M. avium* strain-specific markers might provide a basis for epidemiological studies which could define the environmental sources and the routes of transmission and, possibly, identify clones virulent for man. A number of methods for

typing *M. avium* isolates have been reported, including serotyping [6], multi-locus enzyme electrophoresis [7, 8], pulsed-field gel electrophoresis for analysis of large restriction fragments of genomic DNA [9], and the polymerase chain reaction (PCR) [10]. Recently, methods based on restriction fragment length polymorphism (RFLP) have been proposed [11, 12] resulting from the variability of the copy number and the chromosomal location of mycobacterial insertion sequences (IS).

Several IS have been reported in *M. avium*, although not all are useful for RFLP analysis. For example, IS901 and IS902 share 98% homology at the DNA level [13, 14], and are of limited utility for differentiation of *M. avium* isolates because of a very low degree of mobility and limited polymorphism [12, 15, 16]. IS1110, a highly mobile insertion element of the IS900 family, shows c. 60% homology with IS901 and IS902, and is uncommon in *M. avium* isolates because of its recent acquisition [15]. As a consequence, it is not useful for epidemiological studies. On the other hand, the recently identified IS1245, a 1414-bp element belonging to the IS256 family of IS, seems

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to represent a suitable candidate for IS-based RFLP of *M. avium*, in that it is limited to *M. avium* and is not found in *M. intracellulare* or in other mycobacterial species. Furthermore, it is present in a high copy number in *M. avium* strains isolated from human and animal sources [11]. Very recently, another insertion element, named IS1311, which shows 85% homology at the DNA level with IS1245, has been detected in *M. avium* [11, 12].

IS1245-based RFLP analysis of *M. avium* entails the growth of the organism, extraction of DNA, restriction endonuclease digestion, Southern blotting, and probing for the IS element. As there is no standardisation yet with respect to the optimal choice of restriction endonuclease for IS1245-based RFLP analysis, three restriction endonucleases were tested for an optimal discrimination of IS1245-based RFLP patterns in the typing of *M. avium*.

Materials and methods

Bacterial strains

M. avium strains were isolated from clinical specimens by the radiometric Bactec system (Becton Dickinson) according to standard procedures and identified by molecular probes (Gen-Probe, San Diego, CA, USA). A total of 17 isolates was included in the present study; 15 isolates were from HIV-positive patients (13 from blood, one from urine, one from sputum); two isolates were from sputum of HIV-negative individuals.

Genomic DNA isolation

M. avium strains were grown on liquid Middlebrook medium (Becton Dickinson); c. 1.5 ml of the bacterial cultures were collected into a microcentrifuge tube and heated at 80°C for 20 min to kill the bacteria. The bacterial pellet was then collected by centrifugation for 5 min at 12 000 g and suspended in 400 µl of TE buffer (0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA). Lysozyme was added to a final concentration of 1.25 mg/ml and the tube was incubated at 37°C. After overnight incubation, 70 µl of SDS 10% and 5 µl of proteinase K (10 mg/ml) were added and the tube was incubated for 12 min at 65°C. To remove cell wall debris, denatured protein and polysaccharides, 100 µl of 5 M NaCl and 100 µl of N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) (100 mg/ml in 0.7 M NaCl) were added; after 10 min at 65°C, 750 µl of chloroform isoamyl alcohol (24:1 v:v) were added and the mixture was vortex mixed for 15–20 s. After centrifugation for 7 min at 12 000 g, DNA was precipitated by adding 1 volume of isopropanol to the aqueous supernate. After 30 min at –20°C the mixture was centrifuged for 15 min at 12 000 g, the pellet was washed once with ethanol 70%, air-dried and finally dissolved in TE buffer. DNA concentration was estimated by reading the optical density at 260 and

280 nm in a Gene Quant II spectrophotometer (Pharmacia Biotech).

Chromosomal DNA digestion and Southern blotting

Chromosomal DNA (4.5 µg) of each strain was digested overnight at 37°C with 6 U of the restriction endonuclease *Bsa*AI (BioLabs, USA), 10 U of the restriction endonuclease *Nru*I (Amersham), or 12 U of the restriction endonuclease *Pvu*II (Boehringer Mannheim, Germany) in a final volume of 20 µl. The DNA fragments generated were separated electrophoretically on an agarose 0.8% gel and stained with ethidium bromide 0.5 µg/ml. A mixture of *Pvu*II-digested supercoiled DNA ladder (Gibco BRL) and *Hae*III-digested ΦX174 DNA (Boehringer Mannheim) was run in two lanes of each gel and served as molecular size markers ranging from 16.2 to 0.603 kb. Electrophoresis was at 3.2 V/cm (100 V) for 10 min and at 0.8 V/cm (25 V) overnight in a GNA 200 gel apparatus (LKB-Pharmacia, Sweden). DNA fragments were denatured with 0.25 M HCl for 10 min and, after two washes with 0.4 M NaOH for 20 min, blotted on to a nylon filter (Hybond N-plus membrane; Amersham) by vacuum for 180 min.

IS1245 probe

A 427-bp probing sequence for IS1245 fragment was prepared from DNA of an *M. avium* isolate by PCR, as described previously [11], with oligonucleotides P1 5'GCCGCCGAAACGATCTAC and P2 5'AGGTGGC-GTCGAGGAAGAC as primers. Briefly, PCR was performed in 0.5-ml microcentrifuge reaction tubes in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, Triton X-100 0.1%, 25 µM primers P1 and P2, 200 µM deoxynucleoside triphosphates, 1.25 U of *Taq* polymerase (Dynazyme) and 15 ng of *M. avium* DNA. The mixture was overlaid with 75 µl of mineral oil to prevent evaporation. The reaction was subjected to 30 cycles of amplification (1 min at 94°C, 1 min at 65°C, 1 min at 72°C) and one final 10-min incubation at 72°C in an OmniGene temperature cycler (Hybaid). The 427-bp amplifcon was purified by Sephadex G-50 chromatography column, precipitated with ethanol 96% and solubilised with TE buffer.

Labelling of probes

IS1245 probe and molecular size standard probes, i.e., *Pvu*II-digested supercoiled ladder DNA and *Hae*III-digested ΦX174 DNA, were covalently labelled with horseradish peroxidase (HRP) by glutaraldehyde according to the procedure of the ECL Direct System (Amersham).

Hybridisation and detection

After one wash in buffer $2 \times \text{SSC}$ (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), nylon filters were pre-hybridised by incubating in 10–12 ml of hybridisation buffer (ECL Direct System, Amersham) for 15 min at 42°C in roller bottles in a hybridisation oven (Hybaid). The formulation of the buffer supplied in the ECL kit includes 6 M urea which is equivalent to 50% formamide in reducing the T_m of hybridisation. Filters were then hybridised by the addition of *c.* 200 ng of IS1245 probe and 25–40 ng of each molecular size marker probe and incubation overnight at a temperature not exceeding 42°C in roller bottles at 6 rpm. Filters were then washed twice for 20 min at 42°C with a primary wash buffer (6 M urea, SDS 0.4%, $0.5 \times \text{SSC}$) in the roller bottles and then with a secondary wash buffer ($2 \times \text{SSC}$) for no more than 15 min at room temperature on a shaking platform. Hybridisation was then detected on autoradiographic films by the enhanced chemiluminescence gene detection system (Hyperfilm-ECL; Amersham).

Computer-assisted analysis of fingerprints

The RFLP patterns were scanned with an Epson GT 8000 scanner at 200 dots/inch and *M. avium* fingerprints were compared by 3.1 GelCompar software (Applied Maths, Belgium; Windows version) in an IBM-compatible computer. The lanes were corrected for angle distortion and the RFLP patterns were normalised by equating external molecular size markers between the films. The Dice co-efficients of similarity [17] of all pairwise comparisons of patterns were

calculated and a dendrogram of pattern relatedness constructed by UPGMA clustering, following the instructions of the GelCompar manufacturer. Similarity coefficients of fingerprints of a reference *M. avium* isolate run in different gels ranged between 85 and 95%; accordingly, any two *M. avium* isolates with similarity coefficients > 85% were considered identical.

Results and discussion

For IS1245-based RFLP analysis of *M. avium* isolates, the selection of the restriction endonuclease and the DNA probe are critical. Ideally, the use of a restriction endonuclease which cleaves the IS only once and a DNA probe which does not overlap the endonuclease site would result in fragments defining both the copy number and the chromosomal positions of the IS, i.e., the basis of the DNA polymorphism among the isolates. Fig. 1 shows the physical maps of IS1245 and the highly homologous IS1311 [11, 12], as well as the cleavage sites of restriction enzymes which generate blunt end fragments. For the present study, restriction endonucleases *Bsa*AI and *Pvu*II, that cleave IS1245 and IS1311, respectively, once and *Nru*I, that cleaves both IS1245 and IS1311 once were selected.

A 427-bp DNA sequence was used as a probe, prepared by PCR with IS1245-specific primers, as described by Guerrero *et al.* [11]. The probe sequence is 80% homologous to the target sequence on IS1311. Although the hybridisation conditions in the present

Fig. 1. Physical maps of the *M. avium* insertion elements IS1245 and IS1311 (GeneBank accession numbers L33879 and U16276, respectively). The cleavage sites of restriction enzymes generating blunt end fragments (defined by computer program RESTRI, Pc/Gene, IntelliGenetics, CA, USA) are depicted. The light grey vertical bars represent the inverted repeats bordering IS DNA. The horizontal dark grey bar represents the 427-bp IS probe. The lines to the left and right denote chromosomal DNA. *Bsa*AI cleaves IS1245 at base pair 982 (CAC⁺GTC); *Pvu*II cleaves IS1311 at base pair 737 (CAG⁺CTG); *Nru*I cleaves both IS1245 and IS1311 at base pair 116 and 54 (TCG⁺CGA), respectively.

study include a relatively low T_m temperature, i.e., 42°C, which was necessary to prevent loss of peroxidase activity of the probe, and the formulation of the buffer ensured stringent conditions under which the DNA probe could also hybridise with the 80% homologous target sequence on IS1311.

The 427-bp probe does not overlap the cleavage sites of the selected enzymes (see Fig. 1). This indicates that only one of the two fragments into which each IS is cleaved by endonuclease digestion will hybridise with the probe. The minimal sizes of IS1245 probe-

hybridising restriction fragments generated by *Bsa*AI, *Nru*I, or *Pvu*II, as determined by the distance between the restriction site on the insertion element and a hypothetical chromosome restriction site located just outside the insertion element are approximately 0.98, 1.26 and 0.74 kb, respectively. Furthermore, the restriction enzyme *Nru*I, which cleaves both IS1245 and IS1311, would be expected to generate in all isolates a number of IS1245 probe-specific restriction fragments that is the sum of the copy number of the two insertion elements, each fragment containing one copy of either IS1245 or IS1311 fragments. On the

Fig. 2. DNA RFLP patterns of 17 *M. avium* isolates. Chromosomal DNA of each isolate, numbered at the bottom, was digested with restriction endonucleases *Bsa*AI, *Nru*I, or *Pvu*II (indicated at the top of each panel). A 427-bp PCR-amplified fragment of IS1245 was used as DNA probe. Lane M shows the marker fragments of DNA size (kb).

other hand, *Bsa*AI or *Pvu*II endonucleases will generate probe-positive restriction fragments that may contain more than one IS; for example, digestion of genomic DNA with *Bsa*AI may generate, depending on the location of *Bsa*AI restriction sites on the chromosome, probe-positive restriction fragments containing: (i) one copy of *IS1245*, (ii) one copy of *IS1245* and one or more copies of *IS1311*, (iii) one or more copies of *IS1311* and no *IS1245* copy.

Fig. 2 shows the fingerprints of 17 *M. avium* isolates obtained by digestion of genomic DNA with the selected restriction endonucleases and by probing with the *IS1245*-derived 427-bp DNA sequence. The *IS1245*-based RFLP patterns were polymorphic and complex with all the restriction endonucleases tested. Under these electrophoresis conditions, *Pvu*II, which was the enzyme used for RFLP analysis of *M. avium* isolates in previously published reports [10–12], was the least satisfactory, as most of the *Pvu*II-generated bands clustered in a narrow zone of the fingerprints and several faint bands appeared, especially in the low molecular size range. In contrast, *Bsa*AI- and *Nru*I-generated bands were more evenly distributed and easier to detect. In particular, similar to the results for strains studied by other authors [10, 11], *Pvu*II digestion of genomic DNA yielded 6–20 (average 14.6) probe-specific restriction fragments; *Bsa*AI yielded 9–25 (average 18.6) restriction fragments of molecular size ranging approximately from 12.1 to 0.9 kb; and *Nru*I gave 7–27 (average 16.0) bands ranging approximately from 10.1 to 1.1 kb.

Three *M. avium* isolates yielded one probe-specific

Table 1. Number of *IS1245* probe-specific bands generated by digestion with three restriction endonucleases

Isolate no.	Number of bands*		
	<i>Bsa</i> AI	<i>Nru</i> I	<i>Pvu</i> II
1	16	15	15
2	23	17	14
3	15	14	15
4	24	19	12
5	15	14	14
6	24	18	16
7	23	16	16
8	17	15	15
9	18	14	17
10	17	13	19
11	17	18	20
12	21	13	15
13	25	22	17
14	21	22	14
15	10	8	8
16	9	7	6
17	21	27	15

*Bands were detected automatically by GelCompar software.

restriction fragment of molecular size lower than expected on the basis of the physical maps of *IS1245* and *IS1311*; isolates 2 and 14 showed one band of *c.* 0.9 kb following DNA digestion with *Bsa*AI and isolate 15 showed one band of *c.* 1.1 kb following *Nru*I digestion. These findings might be due to the presence of new restriction sites within some copies of the IS or to the deletion of part of the IS element. However, the existence in *M. avium* DNA of a novel *IS1245*-related element(s) can also be postulated. This hypothesis is further supported by the finding that the numbers of *Nru*I-generated bands (see Table 1) were lower than those obtained with *Bsa*AI and *Pvu*II in

Fig. 3. *Bsa*AI-generated DNA RFLP patterns of 17 *M. avium* isolates. Banding patterns are ordered by similarity. Corresponding dendrograms are on the left. The position of bands in each lane has been normalised so that band positions of all strains are mutually comparable. The top scale depicts similarity coefficients. Numbers at the bottom indicate sizes (kb) of standard DNA fragments; a, b, c, and d indicate clusters of identical clones.

14 and 5 of the 17 *M. avium* isolates, respectively, which is not consistent with the existence in these isolates of only IS1245 and IS1311. In fact, if the isolates contained only these two insertion elements, *Nru*I, which can cleave both IS1245 and IS1311 once, should have generated in all the isolates the highest number of IS1245 probe-specific restriction fragments, i.e., the sum of the copy number of the two insertion elements. The existence of unidentified insertion elements in *M. avium* has also been suggested by Roiz *et al.* [12].

To test whether the IS1245-based polymorphism generated by the different restriction endonucleases

might represent a tool for the study of *M. avium* strain relatedness, the banding patterns obtained with each enzyme were studied for similarity by computer analysis and dendrograms were constructed to show the degree of relatedness among strains according to a previously described algorithm [18]. Results are shown in Figs. 3, 4 and 5 for *Bsa*AI, *Nru*I, and *Pvu*II, respectively. In *Bsa*AI-generated RFLP analysis (Fig. 3), 11 distinct banding patterns were found, as four banding patterns were shared by more than one isolate (i.e., by a cluster of isolates). Each cluster, indicated in Fig. 3 as *a*, *b*, *c*, and *d*, included fingerprints with > 85% similarity coefficient. One cluster (*d*) contained four isolates (isolates 4, 7, 14, 2) and three

Fig. 4. *Nru*I-generated DNA RFLP patterns of 17 *M. avium* isolates. See legend to Fig. 3 for further details.

Fig. 5. *Pvu*II-generated DNA RFLP patterns of 17 *M. avium* isolates. See legend to Fig. 3 for further details.

clusters each contained two isolates (clusters *a*, *b*, and *c* included isolates 3 and 9, 1 and 8, and 6 and 12, respectively). In *Nru*I-generated RFLP analysis (Fig. 4), 13 distinct banding patterns were found. Again, isolates 4, 7, 14, 2, and isolates 1 and 8 occurred in two clusters (indicated as clusters *d* and *b*, respectively). Isolates 3 and 9 and isolates 6 and 12, (included in clusters *a* and *c*, respectively, according to *Bsa*AI analysis), showed a similarity coefficient < 85%, as they differed by at least one band. In *Pvu*II-generated RFLP analysis (Fig. 5), 11 distinct banding patterns were found. The clusters *a* and *b* again included isolates 3 and 9 and isolates 1 and 8, respectively; cluster *c* included three isolates (isolates 6, 12, and 17). Isolates 2, 14, 4 and 7, constituting a cluster of four isolates by *Bsa*AI and *Nru*I analysis, were grouped in two highly related clusters with 84% similarity coefficient (clusters including isolates 2, 14 and 4, 7, respectively).

Thus, the different restriction endonucleases tested in the present study for the IS1245-based RFLP analysis of *M. avium* isolates are capable, although with minor differences, of defining distinct banding patterns and clusters of identical or highly related isolates. IS1245-based RFLP analysis employing *Bsa*AI and *Nru*I restriction endonucleases is used in our laboratory for molecular typing of *M. avium* isolates and is capable of differentiating new from recurrent infections, as well as monoclonal from polyclonal infections, in HIV-positive patients (Garzelli *et al.* unpublished observations). IS1245-based RFLP analysis appears to be a suitable technique for the definition of genetic relatedness between *M. avium* isolates. However, it should be tested on numerous isolates in epidemiological studies for further validation. Standardisation of the technique, especially in respect of the choice of restriction endonuclease and probing sequence, is needed to permit comparison of results between different laboratories.

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