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Molecular typing of *Mycobacterium avium* isolates by sequencing of the 16S–23S rDNA internal transcribed spacer and comparison with IS1245-based fingerprinting

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The nucleotide sequence of the variable 16S–23S rDNA internal transcribed spacer (ITS) was determined in 32 strains of *Mycobacterium avium*, including 29 clinical isolates, two environmental isolates and the reference strain *M. avium* ATCC 35712. The results were compared with those obtained by the IS1245-based restriction fragment length polymorphism (RFLP) assay. The strains belonged to three distinct ITS sequevars, Mav-A, Mav-B and Mav-C. Sixteen of 17 isolates of the Mav-B sequevar were from HIV-positive patients; the Mav-A sequevar included six and five isolates from HIV-positive and HIV-negative individuals, respectively, as well as the two environmental isolates and the *M. avium* reference strain ATCC 35712; only one isolate, from a HIV-infected patient, belonged to the Mav-C sequevar. IS1245-RFLP analysis of *M. avium* isolates of sequevars Mav-A and Mav-B showed that isolates occurring in clusters of identical or highly related RFLP patterns generally belong to the same sequevar, and that *M. avium* strains belonging to the same sequevar may present distinct and unrelated IS1245-RFLP patterns. The question of the molecular markers specific for *M. avium* clones pathogenic for man is discussed.

Introduction

Mycobacterium avium, long recognised as a primary pathogen of birds, behaves as an opportunist human pathogen. In immunocompetent patients, the organism causes pulmonary infections and cervical lymphadenitis and, occasionally, soft tissue infections. Before the recent introduction of antiretroviral therapy, *M. avium* caused severe disseminated infections in a high proportion (25–50%) of patients with the acquired immunodeficiency syndrome (AIDS) [1].

The epidemiology of *M. avium* infections has not been completely defined. *M. avium* can be isolated from environmental, animal and human sources. Tap water is regarded as the main reservoir of the organism, at least for human infections [2], although recently other vehicles, such as pigs and food, have been suggested

[3, 4]. Analysis of restriction fragment length polymorphism (RFLP), based on the insertion sequence (IS) IS1245, has been proposed as a suitable technique to type *M. avium* isolates for epidemiological studies [5–8]. By this approach, *M. avium* strains isolated from human patients almost invariably show highly polymorphic, multi-banded IS1245-RFLP patterns that share a high degree of similarity with a significant proportion of isolates from pigs [3, 5, 6, 9]. In contrast, the IS1245 banding patterns of isolates from a wide variety of bird species are characterised by a very low number of the IS [5–7, 9]; the ‘bird pattern’ *M. avium* strains are rarely found among human or pig isolates [5, 7, 9].

Typing of *M. avium* isolates has been also performed by sequencing the 16S–23S rDNA internal transcribed spacer (ITS) [10]. Because this region of the rRNA operon is transcribed but does not code for a final product, it allows more sequence variation than the 16S rDNA, and, therefore, is suitable for subspecies classification. According to this typing system, strains with a given ITS sequence are grouped into a specific ‘sequevar’. Five *M. avium* sequevars (named Mav-A to

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Mav-E), differing by one or two base pair substitutions and one insertion, have been identified [10, 11].

The purpose of the present study was to type a number of *M. avium* strains, mostly isolated from clinical specimens, by 16S–23S rDNA ITS sequence analysis and to compare the results with those obtained by the IS1245-based RFLP assay.

Materials and methods

Bacterial strains

Thirty-two *M. avium* strains, including 29 clinical isolates, two environmental isolates and the avian *M. avium* reference strain ATCC 35712, were studied. The clinical and environmental isolates were identified by molecular probe (Gen-Probe, San Diego, CA, USA). Mycobacterial colonies were selected from Middlebrook medium plates (Becton Dickinson), grown in liquid Middlebrook medium and then typed by 16S–23S rDNA ITS nucleotide sequencing as described below.

16S–23S rDNA ITS nucleotide sequencing

For DNA extraction, bacterial cells were suspended in 300 µl of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, NaN₃ 0.1% containing Chelex 100 (BioRad) 20%. Samples were held for 30 min at 56°C, vortex mixed and then kept at 100°C for 12 min. After centrifuging at 12 000 rpm for 3 min, the supernates were collected and the samples were stored at 4°C until sequence analysis was performed.

The 16S–23S rDNA ITS region was sequenced as described by Frothingham and Wilson [10], with slight modifications. In particular, the ITS region was amplified with oligonucleotides Ec16S.1390p (5' TTGTACAACACCGCCCGTCA 3') and Mb23S.44n (5' TCTCGATGCCAAGGCATCCACC 3') as primers. Primer Ec16S.1390p begins at position 1390 of the 16S rDNA gene of *Escherichia coli*, is homologous to the sense strand and is highly conserved among bacteria. Primer Mb23S.44n is homologous to the antisense strand of 23S rDNA of both *M. bovis* BCG and *M. leprae* and it is also well conserved among gram-positive bacteria. PCR was performed in 0.5-ml microcentrifuge reaction tubes in a final volume of 50 µl containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, Triton X-100 0.1%, 0.1 µM each primer, 10 mM deoxynucleoside triphosphates, Taq polymerase (DynaZyme) 2 U and bacterial lysate 12.5 µl. The mixture was overlaid with mineral oil to prevent evaporation. PCR amplification was performed with an OmniGene temperature cycler (Hybaid, UK) set for one 3-min cycle at 94°C and 30 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. The PCR products were visualised on agarose 2% gels stained with ethidium bromide.

Sequencing of the PCR products was performed with an automated apparatus (ALFexpress DNA sequencer, Pharmacia Biotech) with the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech). The sequencing primers included one of the PCR primers, Mb23S.44n, and a conserved internal primer, Ec16S.1492p (5' AAGTCGTAACAA-GGTA 3'). The sequence reaction was performed in a thermal cycler (Hybaid). The temperature was cycled to 95°C for 1 min, then to 65°C (primer Mb23S.44n) or to 40°C (primer Ec16S.1492p) for 1 min, and then to 72°C for 2 min for a total of 30 cycles.

IS1245-based RFLP assay

The IS1245-based RFLP assay was run as described previously [7]. Briefly, the bacterial pellet from cultures in liquid Middlebrook medium was suspended in saline and heated at 80°C for 20 min. Genomic DNA, extracted by the N-cetyl-N,N,N-trimethyl ammonium bromide method, was digested with restriction endonuclease *Nru*I and electrophoresed on an agarose 0.8% gel. DNA fragments were blotted on to a nylon filter and hybridised by addition of a PCR-generated, peroxidase-labelled IS1245 probe. Hybridisation was detected on autoradiographic films by the enhanced chemiluminescence gene detection system (Hyperfilm-ECL, Amersham). The RFLP patterns were scanned and fingerprints were compared by GelCompar software version 4.1 (Applied Maths, Belgium).

Results and discussion

The nucleotide sequence of the 16S–23S rDNA ITS was determined in 32 *M. avium* strains, including 29 clinical isolates, two environmental isolates and the reference strain *M. avium* ATCC 35712. PCR amplification with primers Ec16S.1390p and Mb23S.44n resulted in the detection of a single band of *c.* 480 bp in all the strains. The 16S–23S ITS sequence was obtained with primers that included one of the PCR primers, Mb23S.44n, and a conserved internal primer, Ec16S.1492p. The sequenced strains yielded three highly related ITS sequences, shown in Fig. 1, that differed by only one or two nucleotides and defined three distinct *M. avium* sequevars, Mav-A, Mav-B and Mav-C [10]. The association of the *M. avium* ITS sequevar with each isolate is shown in Table 1. Only one isolate (from the blood of an HIV-positive patient) belonged to sequevar Mav-C; 17 isolates, all but one from HIV-positive individuals, belonged to sequevar Mav-B (14 from blood, 3 from respiratory specimens). Sequevar Mav-A included six isolates from five different clinical specimens of HIV-positive patients and five isolates from HIV-negative individuals, mostly from respiratory specimens, as well as the two environmental isolates and the avian reference strain ATCC 35712.

Sequevar

Mav-A	AAGGAGCACC	ACGAAAAGCA	CCCCAACTGG	TGGGGTGCGA	GCCGTGAGGG	GTTCCCGTCT	GTAGTGGACG	GGGGCCGGGT	80
Mav-B									80
Mav-C									80
Mav-A	GCGCAACAGC	AAATGATTGC	CAGACACACT	ATTGGGCCCT	GAGACAACAC	TCGGTCCGTC	CGTGTGGAGT	CCCTCCATCT	160
Mav-B									160
Mav-C									160
Mav-A	TGGTGGTGGG	GTGTGGTGTT	TGAGTATTGG	ATAGTGGTTG	CGAGCATCTA	GATGAGCGCA	TGGTCTTCGT	GGCCGGCGTT	240
Mav-B							G..		240
Mav-C							C..		240
Mav-A	CATCGAAATG	TGTAATTTCT	TTTTTAATCT	TTGTGTGT	278				
Mav-B		T			278				
Mav-C		C			278				

Fig. 1. Nucleotide sequences of the 16S–23S rDNA ITS, defining sequevars Mav-A, Mav-B and Mav-C, detected among 31 *M. avium* isolates. Dots indicate nucleotide identity.

Table 1. Characteristics of *M. avium* isolates sequenced

Isolate no.	Source	Specimen	Patient's HIV serology	ITS sequevar
207	Human	Blood	+	Mav-C
486A	Human	Blood	+	Mav-B
485A	Human	Blood	+	Mav-B
906	Human	Blood	+	Mav-B
926B	Human	Blood	+	Mav-B
927A	Human	Blood	+	Mav-B
496B	Human	Bronchial wash	+	Mav-B
71	Human	Sputum	–	Mav-B
932B	Human	Sputum	+	Mav-B
280A	Human	Blood	+	Mav-B
912A	Human	Blood	+	Mav-B
387	Human	Blood	+	Mav-B
935B	Human	Blood	+	Mav-B
911B	Human	Blood	+	Mav-B
38	Human	Blood	+	Mav-B
84	Human	Blood	+	Mav-B
165	Human	Blood	+	Mav-B
291	Human	Blood	+	Mav-B
101	Human	Blood	+	Mav-A
903B	Human	Blood	+	Mav-A
302	Human	Pleural fluid	–	Mav-A
89B	Human	Sputum	+	Mav-A
128	Human	Sputum	–	Mav-A
295	Human	Sputum	–	Mav-A
301	Human	CSF	+	Mav-A
303A	Human	Urine	–	Mav-A
304	Human	Sputum	–	Mav-A
297	Human	Urine	+	Mav-A
343	Human	Stools	+	Mav-A
17497	Environment	Water	NA	Mav-A
9297	Environment	Water	NA	Mav-A
ATCC 35712*	Chicken	NA	NA	Mav-A

ITS, 16S–23S rDNA internal transcribed spacer; CSF, cerebrospinal fluid; NA, not applicable.

**M. avium* reference strain.

The ITS sequence data show that *M. avium* strains belonging to sequevar Mav-A and Mav-B were isolated from the majority of human infections and further support previous reports indicating the predominance of strains of sequevar Mav-B in severe infections, particularly disseminated infections in AIDS patients [11, 12]. Furthermore, these data underscore the heterogeneity of sources from which Mav-A organisms

can be isolated, which include human patients, either HIV-positive or -negative, animals and environmental sources.

To evaluate whether any relationship might exist between the 16S–23S rDNA ITS sequence-defined sequevar and the IS1245-based RFLP fingerprinting, 12 and 11 *M. avium* isolates belonging, respectively, to

sequevars Mav-B and Mav-A were typed by a previously described IS1245-based RFLP assay [7] and a dendrogram of pattern relatedness among the strains was constructed. As shown in Fig. 2 (panel A),

the IS1245-based RFLP patterns of the isolates were, in general, polymorphic and complex, as reported in other studies [5–7, 9], with the exception of two strains, one of which was the reference strain *M. avium* ATCC

Fig. 2. Relationships between IS1245-based RFLP fingerprints and *M. avium* sequevars of clinical isolates. The IS1245-banding patterns are ordered by similarity and the corresponding dendrogram is shown on the left of each panel. The position of bands in each lane has been normalised so that band positions of all strains are mutually comparable. Scale at the top depicts similarity coefficients. Numbers at the bottom indicate sizes (in kb) of standard DNA fragments. The strain numbers and the *M. avium* sequevar are shown to the right of the panels. In panel A, parentheses depict the clusters of identical or highly related isolates; the similarity coefficients of the clustered isolates are shown on the right.

35712, that showed the typical 'bird-type' RFLP pattern. Assuming that those isolates yielding banding patterns with a similarity coefficient >85% were essentially identical or highly related, five clusters of strains were detected. Two clusters of six isolates, as well as two smaller clusters of two isolates, included isolates belonging, respectively, to sequevars Mav-A and Mav-B; isolates of different sequevar were detected only in one cluster of two elements. These data, although obtained from a limited number of isolates, indicate that *M. avium* strains occurring in clusters of identical or highly related RFLP patterns generally belong to the same sequevar. However, as shown in panels B and C of Fig. 2 – where the fingerprints of Mav-B and Mav-A isolates, respectively, are reported – not all the isolates within the same sequevar showed identical or highly related IS1245-RFLP patterns, as expected.

In spite of many efforts, the several methods that have been employed so far to type *M. avium* strains have failed to define specific markers for pathogenic clones, or to definitely identify the source of infection and the route(s) of transmission for man. Several studies, including the present one, suggest a predominance of sequevar Mav-B organisms in disseminated disease in AIDS patients [11, 12]; in another study, predominance of one ribotype among isolates from AIDS patients was similarly reported [13]. On the other hand, the IS1245-based RFLP analysis, which possesses a discriminatory power between the isolates up to the clonal level, has demonstrated in several studies that AIDS patients are infected by unrelated, highly variable strains [5–7, 9]. However, in studies performed in Italy and Denmark, the IS1245-based RFLP analysis showed genetic relatedness among isolates from distinct geographic areas [7, 14], which also suggests the possibility of the existence of predominant clones in *M. avium* disease in HIV-infected patients. Taken together, the data point to the conclusion that a large variety of *M. avium* clones can produce infection in man, although clones with certain molecular characteristics seem predominant. It is likely that predominance of *M. avium* clones in human infections, either belonging to a particular sequevar or ribotype taxon or showing genetically related IS1245-RFLP patterns, may reflect their selection or adaptation to a particular environmental niche that facilitates transmission to man.

The search for *M. avium* determinants directly involved in pathogenicity, and the development of the relevant

molecular typing assay, will probably contribute to the clarification of many aspects of the epidemiology of *M. avium* infections.

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