JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1997, p. 2503–2510 Vol. 35, No. 10
0095-1137/97/$04.00+0

Use of Different Molecular Typing Techniques for Bacteriological Follow-Up in a Clinical Trial with AIDS Patients with *Mycobacterium avium* Bacteremia

M. PICARDEAU,[1] A. VARNEROT,[1] T. LECOMPTE,[2]† F. BREL,[2]†
T. MAY,[2]† AND V. VINCENT[1]†*

Laboratoire de Référence des Mycobactéries, Institut Pasteur, 75724 Paris Cedex 15,[1] and Service des Maladies Infectieuses et Tropicales, Hôpitaux de Brabois, Nancy,[2] France

Received 16 April 1997/Returned for modification 31 May 1997/Accepted 12 July 1997

One hundred ninety-six *Mycobacterium avium* isolates from blood samples recovered from 93 AIDS patients for several months were typed by serotyping, by IS*1245* restriction fragment length polymorphism (RFLP) analysis and in some cases RFLP analysis with plasmids pVT2 and pLR7 as probes, and by pulsed-field gel electrophoresis (PFGE). PCR typing of single colonies was also used to detect polyclonal infections. Strains belonged mainly to serotypes 1, 4, and 8. pVT2- and pLR7-related plasmids were detected in strains from 49% of the patients. The IS*1245* RFLP and PFGE analyses showed a 96.8% diversity of the *M. avium* strains from the 93 patients. The vast majority (95.2%) of infections were monoclonal, indicating that recent infection is unlikely, even at an advanced stage of AIDS. For one patient, sequential isolates gave divergent patterns of sensitivity and resistance to clarithromycin, but all were identified as the initial clone. RFLP analysis and PCR typing of single colonies allowed for the detection of three polyclonal infections during the bacteriological follow-up. Among strains from patients whose samples were positive by culture after treatment for 2 to 15 months, 97.4% were the same as the initial strain. In conclusion, relapses and failures were mostly due to the initial strain. These relapses and failures resulted either from the selection of resistant mutants or the reappearance of sensitive strains, suggesting the persistence of nonsterilized tissue reservoirs.

In developed countries where the incidence of tuberculosis is low, the clinical importance of mycobacteria other than tubercle bacilli is increasing. This trend is due to the growing population of immunocompromised individuals as a result of increasing life expectancy, the more widespread use of sophisticated surgery and immunosuppressive chemotherapy of cancer or other diseases, and the AIDS pandemic. Mycobacterial infections can indicate the onset of clinical AIDS; this occurs among 4% of the AIDS patients in France, a rate which has not changed since 1993, as shown by the latest statistics established in 1995 (21). The proportion of patients with mycobacterial infections is very much higher among those with advanced stages of AIDS than among those with early stages of AIDS. In the United States and European countries, 25 to 50% of patients then develop a mycobacterial disease (17). Among immunocompetent patients *Mycobacterium avium*, *M. kansasii*, *M. xenopi*, or *M. malmoense* is the most prevalent mycobacterium causing disease, depending on the country (9, 30). Among AIDS patients, *M. avium* infection is largely the most prevalent in all geographic regions (17). *M. avium* infection is the most frequent systemic opportunistic disease and the third most common disease among AIDS patients, with only *Pneumocystis carinii* pneumonia and Kaposi's sarcoma being more frequent (16).

Unlike tuberculosis, other mycobacterial diseases are not transmissible and are thought to be acquired from organisms in the environment, from which the causative agents are frequently isolated (9, 30). Diverse mycobacterial species, including *M. avium*, can be isolated from healthy individuals, and colonization may be common (30). However, it is not known whether *M. avium* dissemination in AIDS patients results from a new infection acquired once immunodeficiency becomes substantial or is due to the onset of disease from a previous infection or colonization.

Several typing techniques were used to determine whether isolates from AIDS patients represent a particular subset of *M. avium* strains. Serotyping studies with isolates from AIDS and non-AIDS patients from diverse geographic regions showed different distributions of the prevalent serovars (2, 6, 8, 15, 19, 37, 45). Hybridization studies with plasmids pVT2 and pLR7 showed that plasmids related to both of these plasmids are more frequently detected in strains isolated from AIDS patients than in strains isolated from patients without AIDS (5, 18). Other studies report a weak association between the plasmid content and the human immunodeficiency virus infection status (14, 26). The internal transcribed spacer (ITS) between the 16S and the 23S rRNA genes can be used to define *M. avium* sequevars; one sequevar predominates in isolates from AIDS patients and children with lymphadenitis (7, 10). The results of *M. avium* genomic restriction fragment length polymorphism (RFLP) analyses with the probes pMB22 isolated from *M. avium* subsp. *paratuberculosis* or the insertion sequences (ISs) IS*1245* and IS*1311* have been contradictory. The pMB22 probe, containing IS*900*, demonstrated a single highly conserved strain of *M. avium* in AIDS patients distinct from the patterns of the majority of strains from non-AIDS patients (12). IS*1245* and IS*1311* RFLP and pulsed-field gel electrophoresis (PFGE) patterns were highly polymorphic and did not correlate with human immunodeficiency virus serological status (32, 33). PFGE has recently been used to show that disseminated polyclonal infections are frequent in AIDS patients (1, 33). An *M. avium* strain isolated from hospital tap water has been isolated in AIDS patients hospitalized in the same ward

* Corresponding author. Phone: (33) 1 45 68 83 60. Fax: (33) 1 40 61 31 18. E-mail: vvincent@pasteur.fr.

† Investigator representing the Curavium Group. Other group members are listed in the appendix.

(41). These results suggest the occurrence of concurrent or successive, recently acquired, possibly nosocomial infections. However, the evidence remains limited and mostly depends on retrospective analysis of sporadic isolates from patients in the same hospital diagnosed with *M. avium* infection.

We investigated *M. avium* isolates recovered from blood during the Curavium therapeutic trial (22). Diverse typing techniques were used, including serotyping, plasmid hybridization, and genotypic pattern determination by IS*1245* RFLP analysis, PFGE, and PCR typing. The use of several techniques relying on independent molecular markers allowed for a comparison and evaluation of the typing methods.

The aim of the study was (i) to characterize the *M. avium* strains isolated from the blood of AIDS patients from all parts of France (the Curavium trial was multicentric and involved clinical centers throughout France); (ii) to use different, independent molecular markers to determine if relapses or failures were due to a particular subset of *M. avium* strains; (iii) to ascertain the frequency of polyclonal and monoclonal infections; and (iv) to identify strains responsible for relapses and treatment failures and establish whether these strains corresponded to recurrent or new infections.

(This work is part of a doctoral thesis in microbiology presented by M.P.)

MATERIALS AND METHODS

***M. avium* strains.** The 196 strains included in the present study were all isolated from the blood of the first 93 patients enrolled in the Curavium trial (22). Among the 196 isolates were single isolates from 54 patients from whom only the initial strain was available and 142 sequential isolates from 39 patients. The sequential isolates were recovered from 25 patients with relapses and 10 patients who were considered to have failed treatment according to the criteria used in Curavium trial (Tables 1 and 2). Patients with a negative blood culture at month 2 and a subsequent positive culture were considered to have had a relapse; treatment failure was defined as the persistence of an infecting strain in month 2 of treatment. The four remaining patients had positive blood cultures before month 2 only.

Strains were isolated from blood samples by the Dupont Isolator technique with plating on Löwenstein-Jensen medium. Strains were identified as *M. avium* by using a DT6-specific probe (35). After isolation, strains were subcultured on Middlebrook 7H10 agar medium. The isolated colonies were studied by PCR techniques, as described below.

The MICs of clarithromycin were determined by the BACTEC method (13). The MIC was defined as the lowest concentration that inhibited 99% of bacterial growth. The breakpoint for sensitivity was set at an MIC of 8 μg per ml.

Serotyping. Serotypes were determined by thin-layer chromatography and detection of specific glycopeptidolipids after mild saponification of lipid extracts as described by Brennan et al. (3).

RFLP analysis. Genomic DNAs were prepared and digested with *Pvu*II as described previously (28); DNA fragments were then resolved by overnight electrophoresis on a 1% (wt/vol) agarose gel at 1.5 V/cm and were transferred to a nylon membrane (Hybond-N^+; Amersham, Amersham International, Buckinghamshire, United Kingdom).

An IS*1245* probe was prepared by PCR with primers P1 and P2 as described previously (11). The 427-bp probe within IS*1245* was purified from the agarose gel by the GeneClean procedure (Bio 101, Inc., La Jolla, Calif.) and was then labelled with horseradish peroxidase by using ECL (Amersham). Hybridization and washing steps were performed as described previously (28).

Cluster analysis of RFLP patterns. RFLP patterns were scanned and analyzed by using the Taxotron package (Taxolab; Institut Pasteur Paris, France). This package comprises the RestrictoScan, RestrictoTyper, Adanson, and Dendrograph programs. Lanes and bands were detected with the RestrictoScan program. Fragment lengths were interpolated by the Spline algorithm implemented by the RestrictoTyper software. The similarity index was calculated by the RestrictoTyper program after setting the fragment length error tolerance to 5%. The single linkage was computed by the Adanson program, and the Dendrograph program drew the dendrogram.

PFGE. *M. avium* isolates were cultivated in 5 ml of Middlebrook 7H9 broth. The cultures were diluted in 40 ml of fresh medium to an optical density at 650 nm of 0.08 and were incubated at 37°C until they reached an optical density of 0.2. Plugs were prepared as described previously (28) and were digested with 30 U of *Dra*I. Large restriction fragments were separated by zero-integrated-field electrophoresis with the AutoBase system (Vysis, Les Ulis, France) for 65 h at room temperature with the 8- to 500-kb ROM card.

TABLE 1. RFLP analysis of strains from patients with relapses[a]

Patient[b]	Susceptibility to clarithromycin on the following blood culture date[c]:								
	D0	M3	M4	M5	M6	M7	M8	M9	M>10
01/01	S[d]	0	R[d]	R[d]	R[d]				
09/02	S[d]	0	0	R[d]	R[d]	R[d]	0	0	0
09/04	S[d]	R[d]	+	R[d]	R[d]	R[d]			
10/02	S[d]	R[d]	R[d]	R[d]	R[d]	0	R[d]	†	
11/03	S[d]	0	0	0	0	0	0	R[d]	†
14/01	S[d]	0	0	R[d]	R[d]	†			
14/04	S[d]	0	R[d]	R[d]	R[d]	0			
17/02	S[d]	0	0	+	R[d]			†	
20/02	S[d]	S[d]	R[d]	R[d]	R[d]	0	†		
21/02	S[d]	ND	R[d]	ND	†				
21/04	S[d]	R[d]	R[d]	R[d]	R[d]	0	0	0	0
22/03	S[d]	I[d]	+	[d]	S[d]	S/R[d]	+	R[d]	R[d]
23/01	S[d]	R[d]	+	+	+	+	+	R[d]	†
32/02	S[d]	0	+	+	+	R[d]	†		
42/01	S[d]	0	0	R[d]	R[d]	+	R[e]		
47/02	S[d]	0	R[d]	†					
49/02	S[d]	ND	0	0	0	0	R[d]	R[d]	+
51/01	S[d]	0	R[d]	R[d]	R[d]	†			
55/02	S[d]	R[d]	+	+	R[d]		R[d]	†	
10/06	S[d]	S[d]	S[d]	0	0	0	0	R[d]	†
14/02	S[d]	0	0	0	0	R[d]	R[d]	R[d]	R[d]
22/02	S[d]	S[d]	+	S[d]	S[d]	S[d]	+	0	R[d]
36/07	S[d]	0	0	0	+	+	+	R[d]	
47/01	S[d]	0	+	0	0	0	0	0	R(M13)[e], R(M15)[d]
47/04	S[d]	+	S[d]	0	0	0	0	0	†

[a] *M. avium* isolates of 25 patients with relapses (negative blood culture at month 2 and a subsequently positive blood culture) were studied by IS*1245* RFLP analysis.

[b] Patients 01/01 to 55/02 were treated with clarithromycin and clofazimine). Patients 10/06 to 47/04 were treated with clarithromycin, rifabutin, and ethambutol.

[c] Day 0 (D0) was the day of inclusion in the study. M, month; S, susceptible; I, intermediate; R, resistant +, positive blood culture (strain not typed); 0, negative blood culture; ND, no data on blood culture; †, death of patient.

[d] The strain was typed by IS*1245* RFLP analysis.

[e] The RFLP pattern was different from the baseline (day 0) pattern.

PCR. The PCR typing method described previously (29) was modified for isolated colonies. Briefly, one colony of *M. avium* was taken from Middlebrook 7H10 plates, suspended in 20 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8]) containing 1% Triton X-100, and incubated for 30 min at 100°C; dimethyl sulfoxide was included in the PCR mixture to improve the specificity of amplification of a G+C-rich genome and avoid nonspecific bands (38).

Plasmid hybridization. Plasmids pJC20 (pBR322:pLR7) and pVT101 (pUC19:pVT2) were kindly provided by J. T. Crawford and J. O. Falkinham, respectively (5, 18). Plasmids were radiolabelled with [α-^{32}P]dCTP by using a commercially available kit (Megaprime; Amersham). The nylon membranes previously used for IS*1245* RFLP analysis were stripped and reprobed with the two plasmid probes.

RESULTS

Serotype analysis and plasmid content. The serotype of each isolate obtained on day 0 from the blood of the first 93 patients enrolled in the trial was determined. Only 4% of the isolates were nontypeable, and 3% did not produce detectable amounts of antigenic glycopeptidolipids. The majority (63%) of typeable isolates belonged to three serotypes, serotypes 1, 4, and 8. The minor serotypes detected were serotypes 2, 3, 9, 10, and 21.

M. avium isolates from 51 randomly selected patients were probed for hybridization with *M. avium* plasmids pLR7 and pVT2 to evaluate the value of these plasmids as epidemiological markers. The pLR7 probe gave signals with *M. avium*

TABLE 2. RFLP analysis of treatment failures[a]

Patient[b]	Susceptibility to clarithromycin on the following blood culture date[c]:								
	D0	M2	M3	M4	M5	M6	M7	M8	M>9
10/04	S[d]	S[d]	S[d]	R[d]	R[d]	†			
30/01	S[d]	R[d]	0	ND	ND	0			
36/11	S[d]	+	R[d]	0	0				
39/01	S[d]	R[d]	R[d]	0	0	0	0	0	+
50/05	S[d]	S[d]	S[d]	S[d]	†				
11/02	S[d]	S[d]	ND	ND	ND	S[d]	ND	ND	ND
27/02	S[d]	S[d]	0	+	†				
39/02	S[d]	S[d]	S[e]	S[d]	S[e]	S[e]	†		
42/03	S[d]	+	S[d]	ND	†				
43/02	S[d]	+	S[d]	0	0	S[d]	+	S[d]	

[a] *M. avium* isolates from 10 patients with treatment failures (persistence of a positive blood culture at month 2) were studied by IS*1245* RFLP analysis.

[b] Patients 10/04 to 50/05 were treated with clarithromycin and clofazimine. Patients 11/02 to 43/02 were treated with clarithromycin, rifabutin, and ethambutol.

[c] Day 0 (D0) was the day of inclusion in the study. M, month; S, susceptible; R, resistant; +, positive blood culture (strain not typed); 0, negative blood culture; ND, no data on blood culture; †, death of patient.

[d] The strain was typed by IS*1245* RFLP analysis.

[e] The RFLP pattern was different from the baseline (day 0) pattern (see Fig. 4).

isolates from 23 patients (46%), and the pVT2 probe hybridized with *M. avium* isolates from 4 patients (8%). Strains from two patients hybridized with both probes. Strains harboring plasmids (from 49% of the patients) presented polymorphic RFLP patterns. Plasmid profiles were highly stable during the bacteriological follow-up, which was for up to 12 months (data not shown).

We found no association between serotype and plasmid hybridization group.

RFLP analysis. A dendrogram of the IS*1245* RFLP patterns of the strains isolated on day 0 from the 93 patients was drawn (Fig. 1). The patterns were polymorphic and complex. The average IS copy number per strain was 17, and the range was 1 to 27 copies. The high degree of diversity of the patterns reflects the heterogeneity of the *M. avium* species, as reported previously in studies based on RFLP analysis or PFGE (1, 11, 29, 32, 33). However, two patterns (strains from patients 20/01 and 11/01) were identical, and three other pairs of strains were closely related (Fig. 2A).

These eight strains were analyzed by other molecular biology-based techniques, including PFGE and RFLP analysis with the pLR7 probe, to assess whether the similarities of the patterns were due to the typing limits of the IS*1245* RFLP technique. According to the interpretation criteria for PFGE patterns defined by Tenover et al. (34), differences involving more than three bands are necessary to establish that strains are different. Only strains from patients 21/07 and 40/01 could be differentiated; the other strain pairs showed PFGE patterns which did not differ by three bands or more (Fig. 2B). These observations were confirmed with the pLR7 probe, which successfully differentiated strains from patients 21/07 and 40/01. Isolates from patients 10/01 and 20/01 did not carry any pLR7-related plasmid, and the other strain pairs gave identical plasmid profiles (Fig. 2C).

The trial database was investigated to try to identify any epidemiological link between patients with identical isolates. Neither the place of residence nor the hospital attended was the same for any pair. The blood cultures had been received in

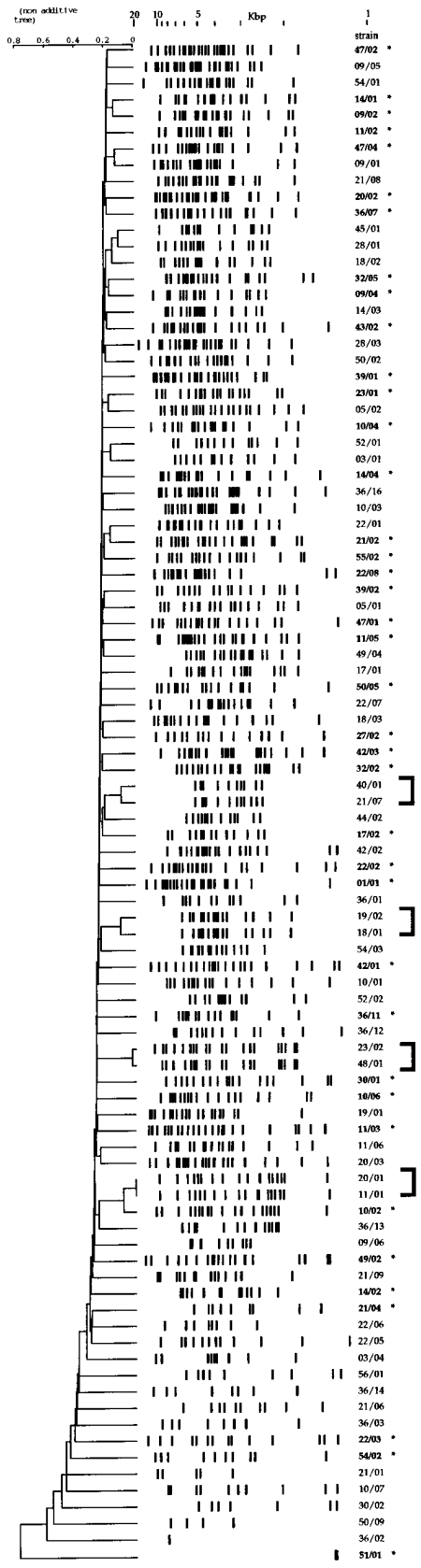

FIG. 1. Dendrogram of IS*1245* RFLP patterns of 93 *M. avium* strains isolated upon inclusion of the patients in the study. Asterisks indicate patients from whom sequential isolates were available. Brackets indicate highly related patterns.

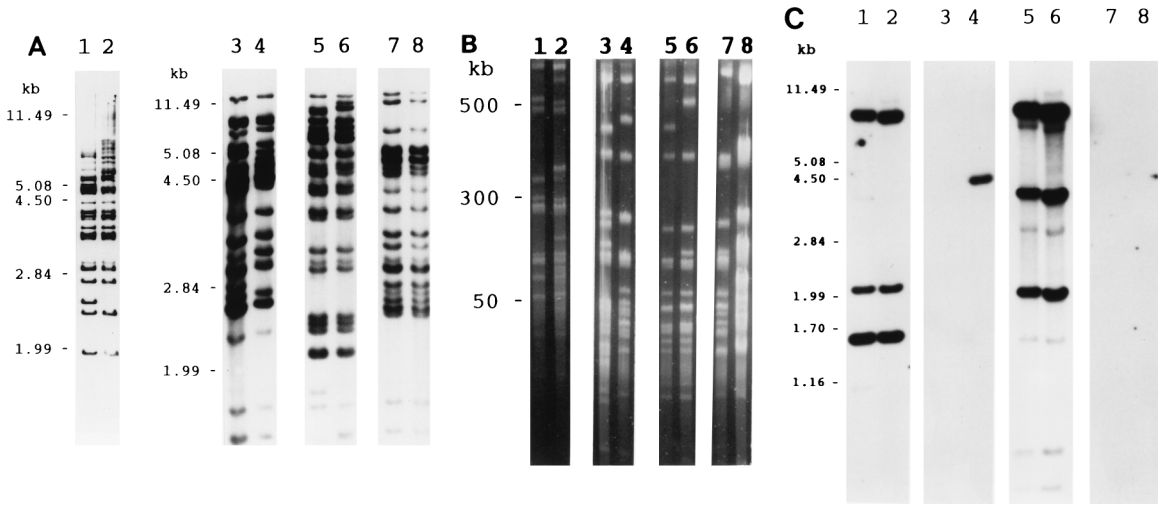

FIG. 2. Comparison of PFGE and plasmid hybridization patterns for highly related IS*1245* RFLP patterns. (A) Southern blot analysis of *Pvu*II-digested genomic DNA probed with IS*1245*. (B) PFGE separation of *Dra*I-digested genomic DNA. (C) Southern blot analysis of *Pvu*II-digested genomic DNA probed with pLR7. Lanes 1, patient 18/01; lanes 2, patient 19/02; lanes 3, patient 21/07; lanes 4, patient 40/01; lanes 5, patient 48/01; lanes 6, patient 23/02; lanes 7, patient 10/01; lanes 8, patient 20/01. The positions of molecular size markers (in kilobase pairs) are indicated on the left.

our laboratory at different times of the year, excluding laboratory contamination as a likely explanation.

For the 39 patients from whom sequential isolates were available, the average number of strains studied per patient was 3.7 (range, 2 to 12 strains), and the interval between the first and last positive *M. avium* culture was between 2 and 15 months (median, 6.8 months). RFLP typing showed identical patterns for strains isolated throughout bacteriological follow-up for 37 patients, indicating monoclonal infections. Variation of one copy was observed between patterns for strains from a single patient (Fig. 3). This may be due to either the mobility of the IS or IS-mediated genetic rearrangements and was not considered to reflect the acquisition of a different strain, as has been similarly established in epidemiological studies of tuberculosis based on typing by IS*6110* RFLP analysis (4). Faint bands in the IS*1245* RFLP patterns were commonly encountered. They were due to cross hybridization with IS*1311*, which shares 85% nucleotide sequence identity with IS*1245*. However, the presence of faint bands with different molecular weights detected in sequential isolates suggests polyclonal infections, a possibility which may be checked by PCR typing of isolated colonies.

For two patients, strains isolated during follow-up presented RFLP patterns different from those of the initial isolate (Fig. 4). For patient 47/01, blood cultures remained negative for 7 months, but were positive during month 11, and subsequently, the pattern of the strain obtained during month 11 was mixed, corresponding to the baseline pattern plus a different pattern produced by a strain isolated in month 13 (Fig. 4). The isolate obtained during month 15 gave the baseline pattern, suggesting a transient mixed infection between months 11 and 15. Strains isolated after the long period of negative blood culture were resistant to clarithromycin (Tables 1 and 2). For patient 39/02, the sequentially isolated strains gave three different patterns and were all sensitive to clarithromycin (Table 2). The initial pattern was observed during months 2 and 4, whereas a different pattern was observed during months 5 and 6. The pattern of the strain obtained during month 3 included faint bands suggesting the simultaneous presence of the two patterns (Fig. 4).

PCR typing. To check for the presence of polyclonal cultures, we typed several single colonies from the same culture by a PCR technique (29).

On Middlebrook 7H10 strains grew as three distinct morphological variants, as described previously (31): smooth, domed, and opaque; smooth, flat, and transparent (SmT); and

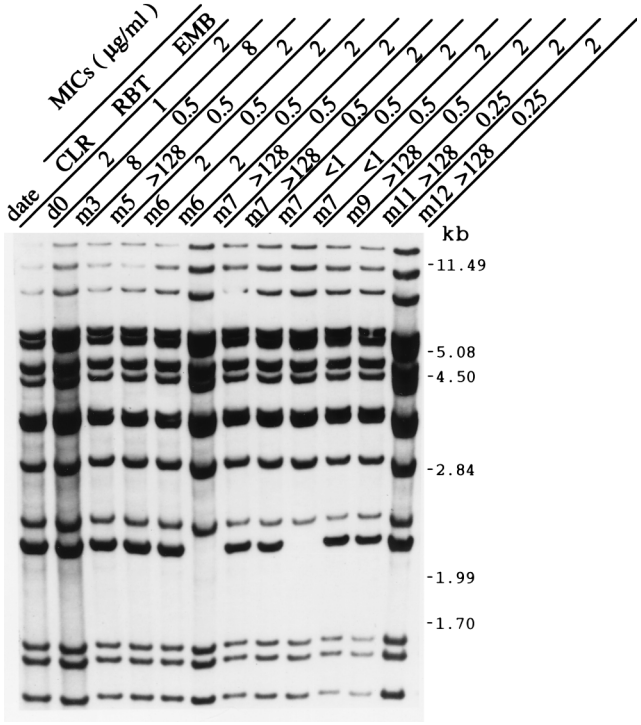

FIG. 3. IS*1245* RFLP patterns of sequential isolates from patient 22/03. For each isolate, MICs of clarithromycin (CLR; resistant, MIC > 8 μg/ml), rifabutin (RBT; resistant, MIC > 1 μg/ml), and ethambutol (EMB; resistant, MIC > 8 μg/ml) are indicated. The positions of molecular size markers (in kilobase pairs) are indicated on the right. d, day; m, month.

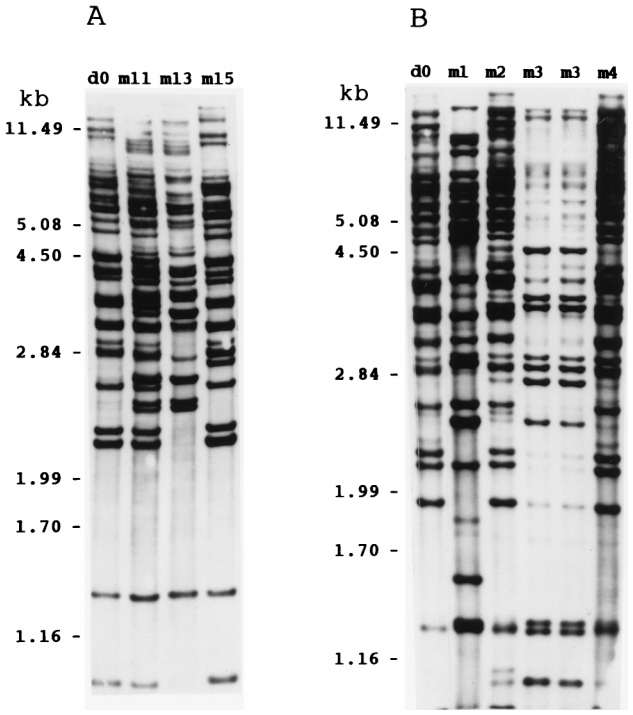

FIG. 4. IS*1245* RFLP patterns of sequential isolates from two patients. (A) Patient 47/01; (B) patient 39/02. The dates that blood samples were obtained for culture are indicated as follows: d0, inclusion; m1, month 1; m2, month 2; m3, month 3; m4, month 4; m11, month 11; m13, month 13; m15, month 15. The positions of molecular size markers (in kilobase pairs) are indicated on the left.

rough, flat, and opaque. Some SmT colonies were pigmented yellow to orange (SmTp). Among our isolates, the SmT and SmTp forms were the most frequent.

Nine independent colonies from the first culture (day 0) of blood from each of 30 randomly selected patients were typed by PCR; six of these patients were able to provide sequential isolates and 24 provided only a baseline isolate. We defined the infection as monoclonal if all the colonies from the same culture presented a single pattern and as polyclonal if different PCR types were detected. Most infections were monoclonal, despite the diversity of morphotypes observed (Fig. 5). Two different PCR patterns corresponding to two morphotypes were found for only one patient (patient 42/01; Fig. 5G). These data suggest that the patient had a polyclonal infection upon inclusion in the clinical trial. Indeed, reanalysis of the IS*1245* RFLP patterns revealed faint bands with different molecular weights between the sequential isolates, possibly indicating an RFLP pattern of a different strain (data not shown).

We also typed colonies of strains from the two patients (39/02 and 47/01) who presented different RFLP patterns during follow-up (Fig. 4). For both patients, PCR typing of colonies isolated from the baseline blood culture did not reveal the presence of initial polyclonal infections. However, for patient 47/01, PCR typing confirmed the presence of a mixed population in the month 11 culture (data not shown), as observed by RFLP analysis (Fig. 4A). For patient 39/02, PCR revealed two different patterns during month 3. RFLP analysis performed with cultures from colonies with distinct PCR patterns showed, on the one hand, the baseline pattern as faint bands in the RFLP pattern (Fig. 4B) and, on the other hand, a distinct pattern corresponding to an additional strain (data not shown).

Clarithromycin-resistant strains. No strain isolated on day 0 was resistant to clarithromycin. As assessed by IS*1245* RFLP analysis the emergence of clarithromycin resistance was due to the selection of mutants from the initial clone (Tables 1 and 2). For an isolate from one patient, patient 22/03, we observed reversion to sensitivity, while the IS*1245* RFLP pattern remained unchanged (Fig. 3). For this patient, blood isolates were sensitive from the day of inclusion in the study up to 3 months of treatment, the month 5 isolate was resistant, and surprisingly, the month 6 isolate was sensitive. Isolates obtained from month 7 to month 12, the last month of the bacteriological follow-up, were resistant (Fig. 3; Table 1). Individual colonies from the culture of blood sampled at month 7 were tested independently for clarithromycin resistance. Both resistant and sensitive colonies were detected and isolated (25). The 23S rRNA sequences of the resistant and sensitive clones were determined. The A-2058 mutation conferring resistance was detected only in clarithromycin-resistant strains (data not shown). These data suggest a monoclonal infection, despite variations in clarithromycin susceptibilities during treatment.

DISCUSSION

The present bacteriological study was based on the Curavium therapeutic trial, which focused on disseminated *M. avium* infections in AIDS patients. It compared the efficacies of two-drug combinations, clarithromycin-rifabutin-ethambutol, and clarithromycin-clofazimine (22).

The distributions of serotypes and plasmid contents were similar for baseline strains and for strains associated with relapses or failures, indicating that the latter strains did not represent a particular subset of *M. avium* strains. Our study indicates that serotyping and plasmid content analysis provide only limited epidemiological information. The prevalence of particular plasmids and serotypes in our sample may simply reflect their prevalence in the immediate environment, as previously suggested in other studies (17, 45).

The IS*1245* RFLP patterns of *M. avium* strains from AIDS patients were highly diverse. These results contradict the homogeneity reported by Hampson et al. (12) but agree with other reports based on PFGE and IS*1245* and IS*1311* RFLP analyses (1, 11, 29, 32, 33, 41). Baseline strains from 93 patients gave 89 different RFLP patterns, which corresponds to 95.7% diversity. The diversity increases to 96.8% if an additional technique, PFGE or plasmid hybridization, is also used. No common infection source or contamination event could be suspected except for six patients whose strains grouped in three clusters (Fig. 1 and 2). Due to the paucity of epidemiological information available in the Curavium database, direct transmission from patient to patient could not be assessed. The identities of the strains from these three pairs of patients may reflect an unidentified common source of infection.

The rapidity of PCR makes PCR typing appropriate for preliminary screening and especially for investigation of several colonies from a single strain. If polyclonal infections are suspected from RFLP patterns or divergent antibiotic susceptibility profiles, PCR typing allows for rapid confirmation. Our data also confirm the presence of multiple, isogenic morphotypes originating from the same clone (43).

Bacteriological characteristics of *M. avium* infection. The epidemiological markers allowed for the detection among the 93 patients in the study of three patients (i.e., 4.3%) with polyclonal and/or subsequent infections. In previous reports, the percentage was higher, up to 31% (1, 23, 33, 40). However, 60% of our patients had a low bacillus load, with less than 10

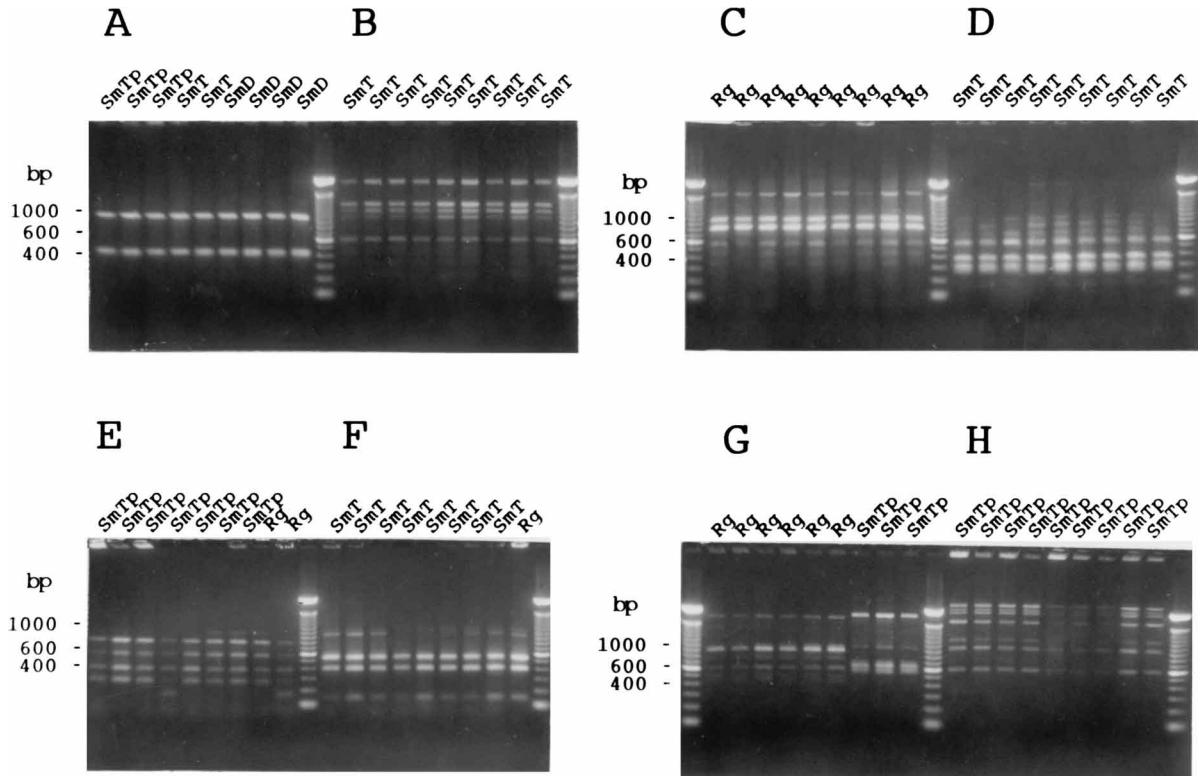

FIG. 5. PCR typing of isolated colonies from different single cultures. (A) Patient 36/02; (B) patient 22/01; (C) patient 49/01; (D) patient 01/01; (E) patient 21/09; (F) patient 36/13; (G) patient 42/01; (H) patient 25/01. All patterns correspond to those for the baseline isolate (day 0). The morphotype of the typed colony is indicated for each lane, as follows: SmD, smooth, domed, and opaque; SmT, smooth, flat, and translucent; SmTp, smooth, flat, and pigmented yellow to orange; and Rg, rough, flat, and opaque. The positions of molecular size markers (in base pairs) are indicated on the left.

CFU per ml of blood (data not shown). Few colonies were thus recovered from each blood sample, and therefore, only the major strain component of the bacillus population may have been detected. Blood samples were plated on solid medium (Isolator technique), which is theoretically the best technique for detecting polyclonal infections, because in broth faster-growing strains can overgrow other strains that are present. The low percentage of polyclonal infections detected could not be explained by the sensitivity or the discriminatory power of the PCR typing method.

The question of whether disseminated *M. avium* infection in AIDS patients is mostly due to the acquisition of a recent infection or reactivation of a previous latent infection is still unresolved. Contradictory evidence has been reported (30, 39, 42, 44). Our results suggest that recent infection due to a strain other than the initial isolate is rare, even at an advanced stage of AIDS (patients enrolled in the Curavium trial had CD4 lymphocyte counts under 100 per ml). However, we screened for polyclonal *M. avium* infection by analysis of sequential isolates only from patients receiving treatment, which may prevent the acquisition of new strains.

We showed that *M. avium* bacteremia could be intermittent, with a positive blood culture on enrollment followed by negative and then positive blood cultures due to the same strain. Some patients had no detectable bacteremia for up to 6 or 8 months after 1 month of therapy; many others had transiently negative blood cultures for shorter periods (Tables 1 and 2). The concentrations of the antibiotic in blood and tissue are not necessarily correlated during the early stages of therapy (20, 36). Torriani et al. (36) found *M. avium* in the bone marrow and other organs of abacteremic AIDS patients. *M. avium* may establish an infection in tissue which then gives rise to the release of small numbers of organisms into the blood (20). Intermittently positive blood cultures may reflect relatively light burdens of *M. avium* in tissues, and the tissue reservoir may respond to treatment more slowly than blood. This indicates the difficulty of distinguishing between treatment failures, defined as blood cultures remaining positive after 2 months of treatment, and relapses, defined as infections in patients with negative blood cultures after 2 months of treatment who turn positive again during the last phase of the therapeutic trial. For all patients with failures or relapses, the strains recovered during treatment were the same as the initial isolates (Tables 1 and 2). For two patients (patients 47/01 and 39/02), although one or more strains were present, the initial isolate was detected throughout the follow-up. Thus, the response to treatment was not sustained and should therefore be prolonged. Moreover, a single negative blood culture is not evidence of the absence of a disseminated *M. avium* infection, and a close bacteriological follow-up is necessary to detect these systemic infections reliably.

Successives episodes of bacteremia were due to the persistence of a single strain, as assessed from the IS*1245* RFLP patterns. The emergence of clarithromycin resistance was due to the selection of resistant mutants from the initial bacterial population exposed to antibiotics. However, one patient (patients 22/03) presented strains for which MICs were high but that then became susceptible and then again became resistant. All strains from this patient had identical RFLP patterns, indicating a monoclonal infection (Fig. 3). The primary mechanism of macrolide resistance in *M. avium* results from a mutation in domain V of the 23S rRNA gene, involving A-2058 or

A-2059 (or both) (24, 27). *M. avium* and other slowly growing mycobacteria have a single rRNA operon, and normally, recessive mutations exert a dominant phenotype. A reverse mutation at the same nucleotide target is a highly unlikely event. The simultaneous maintenance of sensitive and resistant clones for a prolonged period of time in patient 22/03 may therefore have been due to the poor treatment compliance of the patient or to the presence of the *M. avium* strain in tissue reservoirs less accessible to the antibiotic. Patients with disseminated *M. avium* disease who respond to therapy and who become abacteremic do not become *M. avium*-free. During therapy, clarithromycin-resistant and -sensitive isolates are probably always present somewhere in the body. The apparent variations in antibiotic susceptibility have previously been reported for strains isolated from the same patient (40). If the treatment is stopped, either because of patient noncompliance or because of adsorption problems, then the resistant organisms will no longer have a selective advantage and will be outgrown by wild-type organisms: clarithromycin-resistant *M. avium* isolates survive less well in vivo than wild-type sensitive isolates.

In conclusion, disseminated *M. avium* infections in our AIDS patients were mostly monoclonal. Persistent positive blood cultures and relapses after a prolonged therapeutic period were due to the initial strain. These observations are somewhat surprising because the source of *M. avium* infection is the environment and exposure to diverse *M. avium* strains is likely to be frequent. Moreover, the vast majority of patients carried unrelated *M. avium* strains. Despite marked differences between the transmission and physiopathology of *M. avium* infection and tuberculosis, the use of molecular epidemiological markers established similar conclusions in terms of the diversity of strains from nonepidemiologically related patients: infections are monoclonal and relapses and treatment failures are due to the initial infectious strain.

APPENDIX

The following principal investigators and institutions (all of which are located in France) participated in the ANRS 033 Curavium trial: C. Beuscart, Centre d'Etudes et de Recherche en Informatique Médicale, Faculté de Médecine, Lille; A. P. Blanc, T. Allegre, H. Chardon, and O. Bellon, CHG, Aix en Provence; J. Bru, J. P. Gaillat, D. Charvier, and A. Sedaillan, CHG, Annecy; L. Guillevin, B. Jarrousse, O. Lortholary, D. De Zeeow, M. Scavizzi, and M. Soilleux, Hôpital Avicenne, Bobigny; C. Bazin, P. Cren, D. Hazera, M. Six, C. Morel, B. Malbruny, and M. Goubin, CHU, Caen; J. Dormont, F. Boue, D. Livartowsky, A. Dulioust, J. Pillot, L. Lebrun, and M. Mole, Hôpital Béclère, Clamart; P. Vinceneux, M. F. Borie, C. Michon, and M. Boussougant, Hôpital L. Mourier, Colombes; H. Portier, P. Chavanet, D. Grappin, A. Kazmierczak, and J. M. Duez, Hôpital du Bocage, Dijon; C. Perronne, P. De Truchis, C. Risbourg, C. Nauciel, E. Ronco, and P. Matsiota, Hôpital R. Poincaré, Garches; C. Champetier De Ribes, G. Force, and P. Conzi, Hôpital Perpétuel Secours, Levallois; C. Trepo, L. Cotte, D. Carré, G. Panteix, and H. De Montclos, Hôtel-Dieu, Lyon; T. Saint-Marc, J. L. Touraine, H. Makloufi, J. Fleurette, and G. Zambardi, Hôpital E. Herriot, Lyon; H. Gallais, I. Ravaux, D. Di Costanzo, P. De Micco, and C. Bollet, Hôpital de la Conception, Marseille; F. Janbon, J. Reynes, A. Atoui, M. Vidal, C. Boulanger, C. Perez, and F. Fabre, Centre Gui De Chauliac, Montpellier; P. Canton, B. Hoen, C. Amiel, C. Rabaud, A. Le Faou, and M. Dailloux, CHU Brabois, Nancy; J. Y. Grolleau, M. Litoux, F. Raffi, V. Reliquet, M. Charonnat, and D. Moinard, Hôtel-Dieu, Nantes; J. P. Cassuto, D. Faraut, B. Reboulot, P. Lefebvre, and T. Fosse, Hôpital de Cimiez, Nice; P. Balmes, J. M. Mauboussin, M. Ramuz, and A. Gouby, CHU, Nîmes; F. Vachon, E. Bouvet, I. Hamidi, E. Bergogne, D. Nouhouayi, J. L. Vildé, P. Longuet, and L. Gérard, Hôpital Bichat, Paris; D. Sicard, D. Salmon, R. Chapuis, O. Zak Dit Zbar, and P. Nevot, Hôpital Cochin, Paris; W. Rozenbaum, C. Jacomet, M. Hadacek, P. M. Girard, J. Nicolas, and A. Bure, Hôpital Rothschild, Paris; B. Dautzenberg, J. Grosset, M. Gentilini, C. Katlama, D. Mouthon, C. Olivier, and C. Truffot, Hôpital Pitié Salpêtrière, Paris; B. Becq-Giraudon, J. P. Breux, G. Agius, and A. Bourgoin, Hôpital J. Bernard, Poitiers; J. Deville, G. Remy, C. Rouger, I. Beguinot, C. G. Chippaux, and O. Bajolet, Hôpital R. Debré, Reims; F. Cartier, C. Michelet, A. Andrieux, J. L. Avril, and F. Autuly, CHU Pontchaillou, Rennes; G. Humbert, F. Borsa Lebas, I. Gueit, P. Debab, and J. F. Lemeland, Hôpital C. Nicolle, Rouen; F. Lucht, D. Berthelot, A. Fresard, and G. Dorche, Hôpital de Bellevue, St. Etienne; J. M. Lang, P. Fraisse, M. Kempf, H. Monteil, and Y. Piemont, Hôpitaux Universitaires, Strasbourg; A. Lafeuillade, P. Pellegrino, E. Delbeke, and M. J. Gevaudan, Hôpital Chalucet, Toulon; D. Jaubert, Y. Muzellec, and P. Brisou, Hôpital d'Instruction des Armées, Toulon Naval; M. Duffaut, E. Bonnet, M. Bicart, M. J. Larreng, and R. Bauriaud, Hôpital de Rangueil, Toulouse; J. C. Auvergnat and J. Trille, Hôpital Purpan, Toulouse; P. Choutet, J. M. Besnier, A. Goudeau, and B. Cattier, Hôpital Bretonneau, Tours; M. Micoud, P. Leclercq, M. Ruiz, P. Le Noc, and D. Duborgel, CHU, Grenoble; Y. Mouton, D. Vallet, M. Caillaux, CHU, Tourcoing; J. C. Imbert, O. Picard, L. Blum, G. Vaudre, J. C. Petit, and V. Lalande, Hôpital Saint Antoine, Paris; M. Thomas, V. Jeantils, J. C. Torlotin, and P. Cruaud, Hôpital J. Verdier, Bondy; J. M. Estavoyer, M. Vanlemmens, M. Roche, Y. Michel-Briand, and P. Plesiat, CHU, Besançon; J. P. Faller, CHG, Belfort; G. Lepeu and M. Brun, Hôpital H. Duffaut, Avignon; A. Bourgeade, J. Moreau, P. Brouqui, and A. Levraud, Hôpital Houphouët Boigny, Marseille; and C. Caquet, J. F. Delfraissy, C. Goujard, and M. Ranoux, Hôpital Bicêtre, Kremlin Bicêtre.

ACKNOWLEDGMENTS

M.P. is the recipient of a fellowship from Agence Nationale de Recherches sur le SIDA (ANRS).

We thank K. Nash for helpful advice. We gratefully thank J. P. Aboulker, B. Carbonelle, and D. Vittecoq for their active participation in the independent review committee of the Curavium trial.

REFERENCES

1. **Arbeit, R. D., A. Slutsky, T. W. Barber, J. N. Maslow, S. Niemczyk, J. O. Falkinham III, G. T. O'Connor, and C. F. von Reyn.** 1993. Genetic diversity among strains of *Mycobacterium avium* causing monoclonal and polyclonal bacteremia in patients with AIDS. J. Infect. Dis. **167:**1384–1390.
2. **Askgaard, D. S., S. B. Giese, S. Thybo, A. Lerche, and J. Bennedsen.** 1994. Serovars of *Mycobacterium avium* complex isolated from patients in Denmark. J. Clin. Microbiol. **32:**2880–2882.
3. **Brennan, P. J., L. Heifets, and B. P. Ullom.** 1982. Thin-layer chromatography of lipid antigens as a means of identifying nontuberculous mycobacteria. J. Clin. Microbiol. **15:**447–455.
4. **Cave, M. D., K. D. Eisenach, G. Templeton, M. Salfinger, G. Mazurek, J. H. Bates, and J. T. Crawford.** 1994. Stability of DNA fingerprint pattern produced with IS*6110* in strains of *Mycobacterium tuberculosis*. J. Clin. Microbiol. **32:**262–266.
5. **Crawford, J. T., and J. H. Bates.** 1986. Analysis of plasmids in *Mycobacterium avium-intracellulare* isolates from persons with acquired immunodeficiency syndrome. Am. Rev. Respir. Dis. **134:**659–661.
6. **Dawson, D. J.** 1990. Infection with *Mycobacterium avium* complex in Australian patients with AIDS. Med. J. Aust. **153:**466–468.
7. **De Smet, K. A. L., I. N. Brown, M. Yates, and J. Ivanyi.** 1995. Ribosomal internal transcribed spacer sequences are identical among *Mycobacterium avium-intracellulare* complex isolates from AIDS patients, but vary among isolates from elderly pulmonary disease patients. Microbiology **141:**2739–2747.
8. **De Smet, K. A. L., T. J. Hellyer, A. W. Khan, I. N. Brown, and J. Ivanyi.** 1996. Genetic and serovar typing of clinical isolates of the *Mycobacterium avium-intracellulare* complex. Tubercle Lung Dis. **77:**71–76.
9. **Falkinham, J. O., III.** 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev. **9:**177–215.
10. **Frothingham, R., and K. H. Wilson.** 1994. Molecular phylogeny of the *Mycobacterium avium* complex demonstrates clinically meaningful divisions. J. Infect. Dis. **169:**305–312.
11. **Guerrero, C., C. Bernasconi, D. Burki, T. Bodmer, and A. Telenti.** 1994. IS*1245*: a novel insertion element from *Mycobacterium avium* is a specific marker for analysis of strain relatedness. J. Clin. Microbiol. **33:**304–307.
12. **Hampson, S. J., J. Thompson, M. T. Moss, F. Portaels, E. P. Green, J. Hermon-Taylor, and J. J. McFadden.** 1989. DNA probes demonstrate a single highly conserved strain of *Mycobacterium avium* infecting AIDS patients. Lancet **i:**65–68.
13. **Heifets, L. B.** 1996. Drug susceptibility testing. *In* G. Clauser (ed.), Clinics in laboratory medicine. The W. B. Saunders Company, Philadelphia, Pa.

14. **Hellyer, T. J., I. N. Brown, J. W. Dale, and C. S. F. Easmon.** 1991. Plasmid analysis of *Mycobacterium avium-intracellulare* (MAI) isolated in the United Kingdom from patients with and without AIDS. J. Med. Microbiol. **34:**225–231.
15. **Horsburg, C. R., Jr., D. L. Cohn, R. B. Roberts, H. Masur, R. A. Miller, A. Y. Tsang, and M. D. Iseman.** 1986. *Mycobacterium avium-M. intracellulare* isolates from patients with or without acquired immunodeficiency syndrome. Antimicrob. Agents Chemother. **30:**955–957.
16. **Horsburg, C. R., Jr.** 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:**1332–1338.
17. **Inderlied, C. I., C. A. Kemper, and L. E. M. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.
18. **Jucker, M. T., and J. O. Falkinham III.** 1990. Epidemiology of infection by nontuberculous mycobacteria. IX. Evidence for two DNA homology groups among small plasmids in *Mycobacterium avium*, *Mycobacterium intracellulare* and *Mycobacterium scrofulaceum*. Am. Rev. Respir. Dis. **142:**858–862.
19. **Julander, I., S. Hoffner, B. Petrini, and L. Ostlund.** 1996. Multiple serovars of *Mycobacterium avium* complex in patients with AIDS. APMIS **104:**318–320.
20. **Kemper, C. A., D. Havlir, A. E. Bartok, C. Kane, B. Camp, N. Lane, and S. C. Derenski.** 1994. Transient bacteremia due to *Mycobacterium avium* complex in patients with AIDS. J. Infect. Dis. **170:**488–493.
21. **Lot, F., J. Pillonel, R. Pinget, F. Cazein, P. Gouezel, and A. Laporte.** 1997. Diminution brutale du nombre de cas de SIDA, rôle des nouvelles stratégies thérapeutiques? Bull. Epidemiol. Hebd. **11:**43–49.
22. **May, T., F. Brel, C. Beuscart, V. Vincent, C. Perrone, T. Doco-Lecompte, T. Saint-Marc, B. Dautzenberg, J. Grosset, and the Curavium Group.** A comparison of combination therapy in the treatment of HIV-infected patients with disseminated *Mycobacterium avium* bacteremia. Clin. Infect. Dis., in press.
23. **Meier, A., L. Heifets, R. J. Wallace, Jr., Y. Zhang, B. A. Brown, P. Sander, and E. C. Böttger.** 1996. Molecular mechanisms of clarithromycin resistance in Mycobacterium avium: observation of multiple 23S rDNA mutations in a clonal population. J. Infect. Dis. **174:**354–360.
24. **Meier, A., P. Kirschner, B. Springer, V. A. Steingrube, B. A. Brown, R. J. Wallace, Jr., and E. C. Böttger.** 1994. Identification of mutations in 23S rRNA gene of clarithromycin-resistant *Mycobacterium intracellulare*. Antimicrob. Agents Chemother. **38:**381–384.
25. **Moinard, D., and P. Melchior.** Personal communication.
26. **Morris, S. L., D. A. Rouse, A. Malik, S. D. Chaparas, and F. G. Witebsky.** 1990. Characterization of plasmids extracted from AIDS-associated *Mycobacterium avium* isolates. Tubercle **71:**181–185.
27. **Nash, K. A., and C. B. Inderlied.** 1995. Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. Antimicrob. Agents Chemother. **39:**2625–2630.
28. **Picardeau, M., A. Varnerot, J. Rauzier, B. Gicquel, and V. Vincent.** 1996. *Mycobacterium xenopi* IS*1395*, a novel insertion sequence expanding the IS*256* family. Microbiology **142:**2453–2461.
29. **Picardeau, M., and V. Vincent.** 1996. PCR typing of *Mycobacterium avium* isolates. J. Clin. Microbiol. **34:**389–392.
30. **Portaels, F.** 1995. Epidemiology of mycobacterial diseases. Clin. Dermat. **13:**207–222.
31. **Prinzis, S., B. Rivoire, and P. J. Brennan.** 1994. Search for the molecular basis of morphological variation in *Mycobacterium avium*. Infect. Immun. **62:**1946–1951.
32. **Roiz, M. P., E. Palenque, C. Guerrero, and M. J. Garcia.** 1995. Use of restriction fragment length polymorphism as a genetic marker for typing *Mycobacterium avium* strains. J. Clin. Microbiol. **33:**1389–1391.
33. **Slutsky, A. M., R. D. Arbeit, T. W. Barber, J. Rich, C. F. von Reyn, W. Pieciak, M. A. Barlow, and J. N. Maslow.** 1994. Polyclonal infections due to *Mycobacterium avium* complex in patients with AIDS detected by pulsed-field gel electrophoresis of sequential clinical isolates. J. Clin. Microbiol. **32:**1773–1778.
34. **Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan.** 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. **33:**2233–2239.
35. **Thierry, D., V. Vincent, F. Clément, and J. L. Guesdon.** 1993. Isolation of specific DNA fragments of *Mycobacterium avium* and their possible use in diagnosis. J. Clin. Microbiol. **31:**1048–1054.
36. **Torriani, F. J., C. A. Behling, J. A. McCutchan, R. H. Haubrich, and D. V. Havlir.** 1996. Disseminated *Mycobacterium avium* complex: correlation between blood and tissue burden. J. Infect. Dis. **173:**942–943.
37. **Tsang, A. Y., J. C. Denner, P. J. Brennan, and J. K. McClatchy.** 1992. Clinical and epidemiological importance of typing of *Mycobacterium avium* complex isolates. J. Clin. Microbiol. **30:**479–484.
38. **Varadaraj, K., and D. M. Skinner.** 1994. Denaturants or cosolvents improve the specificity of PCR amplification of a G+C-rich DNA using genetically engineered DNA polymerases. Gene **140:**1–5.
39. **von Reyn, C. F., T. W. Barber, R. D. Arbeit, C. H. Sox, G. T. O'Connor, R. J. Brindle, C. F. Gilks, K. Hakkarainen, A. Ranki, C. Bartholomew, J. Edwards, A. N. A. Tosteson, and M. Magnusson.** 1993. Evidence of previous infection with *Mycobacterium avium-Mycobacterium intracellulare* complex among healthy subjects: an international study of dominant mycobacterial skin test reactions. J. Infect. Dis. **168:**1553–1558.
40. **von Reyn, C. F., N. J. Jacobs, R. D. Arbeit, J. N. Maslow, and S. Niemczyck.** 1995. Polyclonal *Mycobacterium avium* infections in patients with AIDS: variations in antimicrobial susceptibilities of different strains of *M. avium* isolated from the same patient. J. Clin. Microbiol. **33:**1008–1010.
41. **von Reyn, C. F., J. N. Maslow, T. W. Barber, J. O. Falkinham III, and R. D. Arbeit.** 1994. Persistent colonisation of potable water as a source of *Mycobacterium avium* infection in AIDS. Lancet **343:**1137–1141.
42. **Wayne, L. G., L. S. Young, and M. Bertram.** 1986. Absence of mycobacterial antibody in patients with acquired immune deficiency syndrome. Eur. J. Clin. Microbiol. Infect. Dis. **5:**363–365.
43. **Wright, E. L., S. Zywno-van Ginkel, N. Rastogi, and W. W. Barrow.** 1996. Monoclonal infections involving *Mycobacterium avium* presenting with three distinct colony morphotypes. J. Clin. Microbiol. **34:**2475–2478.
44. **Yajko, D. M., D. P. Chin, P. C. Gonzalez, P. S. Nassos, P. C. Hopewell, A. L. Reingold, C. R. Horsburg, Jr., M. A. Yakrus, S. M. Ostroff, and W. K. Hadley.** 1994. *Mycobacterium avium* complex in water, food, and soil samples collected from the environment of HIV-infected individuals. J. AIDS Hum. Retrovirol. **9:**176–182.
45. **Yakrus, M. A., and R. C. Good.** 1990. Geographic distribution, frequency, and specimen source of *Mycobacterium avium* complex serotypes isolated from patients with acquired immunodeficiency syndrome. J. Clin. Microbiol. **28:**926–929.