7-1994

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Alexander M. Slutsky
VA Medical Center

Robert D. Arbeit
Boston University

Thomas W. Barber
Boston University

Josiah Rich
Harvard University

C Fordham von Reyn
Dartmouth College

JOURNAL OF CLINICAL MICROBIOLOGY, July 1994, p. 1773–1778
0095-1137/94/$04.00+0

Vol. 32, No. 7

Polyclonal Infections Due to *Mycobacterium avium* Complex in Patients with AIDS Detected by Pulsed-Field Gel Electrophoresis of Sequential Clinical Isolates

ALEXANDER M. SLUTSKY,[1]† ROBERT D. ARBEIT,[1,2]* THOMAS W. BARBER,[2,3] JOSIAH RICH,[4] C. FORDHAM VON REYN,[5] WALTER PIECIAK,[4] MAUREEN A. BARLOW,[1] AND JOEL N. MASLOW[1,2]

Infectious Diseases Section, Medical Service, VA Medical Center,[1] Department of Medicine, Boston University School of Medicine,[2] Maxwell Finland Laboratory for Infectious Diseases,[3] and Infectious Diseases Section, Brigham and Women's Hospital, and Department of Medicine, Harvard School of Medicine,[4] Boston, Massachusetts, and Infectious Disease Section, Dartmouth-Hitchcock Medical Center and Department of Medicine, Dartmouth Medical School, Hanover, New Hampshire[5]

Received 7 March 1994/Returned for modification 6 April 1994/Accepted 25 April 1994

Invasive infection with organisms of the *Mycobacterium avium* complex (MAC) is common among patients with advanced human immunodeficiency virus infection. In previous studies, we analyzed multiple individual colonies of MAC isolated from specimens obtained at the same time and observed that 14 to 20% of patients are simultaneously infected with more than one strain. In this study, we examined sequential isolates from 12 patients with AIDS who had two or more MAC isolates available from clinical specimens collected more than 1 week apart; the intervals between the first and last specimens ranged from 8 to 192 (median, 46) days. For each isolate, restriction digests of genomic DNA were analyzed by pulsed-field gel electrophoresis; DNA was prepared by using a protocol, described here in detail, which had been optimized for conditions of bacterial growth and lysis. The pulsed-field gel electrophoresis analysis identified four patients (33%) infected with two different MAC strains. Both *M. avium* and *M. intracellulare* were cultured from blood specimens from two patients. In each of the four patients, the second strain was identified from a culture taken within 14 days of the initial study isolate, and in three of these patients, the first strain was detected again in a subsequent culture. These observations suggest that the presence of two different strains among isolates from sequential cultures may reflect ongoing polyclonal infection. We conclude that polyclonal infection with MAC is common among patients with AIDS. The identification of such infections may be critical in the development of effective treatments.

Infections with organisms of the *Mycobacterium avium* complex (MAC) occur in up to 40% of patients with advanced human immunodeficiency virus (HIV) infection (14); ≥95% of the isolates represent *M. avium* strains (19). Precise epidemiologic studies of these and other nontuberculous mycobacterial infections require a reproducible and highly discriminatory method for differentiating individual strains (9). We and other investigators have described the use of pulsed-field gel electrophoresis (PFGE) to resolve MAC, *M. paratuberculosis*, *M. fortuitum*, and *M. tuberculosis* strains (1, 2, 7, 12, 20). On the basis of a detailed examination of the conditions for bacterial growth and lysis reported here, we have developed a protocol for consistent and efficient isolation of mycobacterial genomic DNA suitable for PFGE studies. We describe here the use of this protocol to perform PFGE analyses of 55 MAC isolates from 12 patients, from each of whom two or more positive cultures were obtained at least 1 week apart. These studies identified four patients (33%) infected with two different MAC strains isolated from sequential cultures, including two patients infected with *M. avium* and *M. intracellulare* isolates.

* Corresponding author. Mailing address: Research Service (151), VA Medical Center, 150 So. Huntington Avenue, Boston, MA 02130. Phone: (617) 278-4416. Fax: (617) 739-6394. Electronic mail address: rarbeit@bu.edu.

† Present address: Department of Clinical Microbiology, Strong Memorial Hospital, Rochester, NY 14642.

MATERIALS AND METHODS

Bacterial isolates. ATCC 35718 (TMC 702, kindly provided by J. O. Falkinham), an *M. avium* serovar 3 strain cultured from a cervical lymph node of a child, was used for the studies to optimize the isolation of genomic DNA. Patients with cultures yielding MAC from sterile sites (blood, bone marrow, and liver) were identified from clinical microbiology laboratory records or from an international study of MAC epidemiology (1); isolates were retrieved for those patients from whom two or more positive cultures were obtained at least 1 week apart. Clinical isolates were obtained from the Mycobacteriology Division, Massachusetts State Laboratory (n = 21) and the clinical microbiology laboratory of the Brigham and Women's Hospital (n = 12) and as part of the MAC epidemiology study (n = 22).

Preparation of DNA in situ in agarose. Bacteria were inoculated from slants into 10 ml of modified Middlebrook 7H9 broth prewarmed to 37°C; this broth is composed of 4.7 g of Middlebrook 7H9 powder (BBL Microbiology Systems, Cockeysville, Md.) per ml; 0.5 M sucrose, 0.05% (wt/vol) Tween 80, 0.2% (wt/vol) D-glucose, and 10% (vol/vol) oleic acid-albumin complex (BBL). The solutions of 2× Middlebrook powder–0.1% Tween 80, 1 M sucrose, and 4 g of D-glucose per liter were autoclaved separately. After cooling, these solutions were mixed with 10% (vol/vol) oleic acid-albumin complex under aseptic conditions, aliquoted into 15-ml glass screw-cap tubes, and stored at 4°C.

The broth cultures were incubated at 37°C with rotation (20 to 30 rpm) for 4 to 14 days to an optical density at 600 nm (OD_{600}) of 0.20 (~1×10^8 to 2×10^8 CFU/ml). One milliliter of 10× ACT solution (1 mg of ampicillin per ml, 10 mg of D-cycloserine per ml, 10 mg of D-threonine per ml) was then added, and the cultures were reincubated for an additional 12 to 18 h. The cultures were centrifuged at 1,000 × *g* (calculated at the pellet) for 20 min at 4°C, the media were aspirated, and the bacteria were resuspended in 1/50 of the culture volume in TS buffer (50 mM Tris, 0.5 M sucrose, pH 7.6). The suspension was carefully transferred to a microcentrifuge tube, immediately frozen in dry ice-ethanol, and then either thawed on wet ice for subsequent processing or stored at −70°C.

After thawing, the cell suspension was immediately mixed with an equal volume of 1.3% molten 60°C InCert agarose (FMC Bioproducts, Rockland, Maine) in TEN buffer (50 mM Tris, 250 mM EDTA, 200 mM NaCl, pH 7.6). To prevent crystallization of 250 mM EDTA that occurred with boiling, 2× TEN buffer was warmed to 60°C and mixed with an equal volume of 2.6% molten agarose previously dissolved in water in a boiling water bath. The cell-agarose mixture was quickly dispensed into plug molds and allowed to solidify at 4°C. Subsequent treatment with lysozyme and proteinase K was performed as previously described (10).

PFGE analysis. Plugs were restriction digested overnight with 20 U of *Ase*I (New England Biolabs, Beverly, Mass.) as previously described (10), and the restriction fragments were separated in a 1% agarose gel (Seakem GTG; FMC) on a CHEF DRII apparatus (Bio-Rad, Richmond, Calif.) at 15°C for 22 h with pulse times of 1 to 40 s ramped linearly.

RESULTS

Optimal isolation of mycobacterial genomic DNA for PFGE. Preparation of mycobacterial DNA in agarose is complicated primarily by the long bacterial generation time and difficulty in lysing the cell wall; the latter step is critical for isolation of DNA suitable for restriction digestion. Mycobacteria grown in liquid media typically clump because of the hydrophobic lipoarabinomannan layer, which also protects the underlying peptidoglycan layer from cell wall-lysing enzymes (13). Clumping was decreased in media supplemented with Tween 80 at 0.05% and further limited by Tween 80 at 0.1%, although the higher concentration resulted in slightly decreased mycobacterial growth rates. It is of note that mycobacteria grown in liquid medium, frozen at −70°C in 80% modified Middlebrook 7H9 broth–20% glycerol, and then subcultured into modified Middlebrook 7H9 broth with either concentration of Tween 80 demonstrated minimal clumping.

Treatment of growing mycobacteria with ampicillin and D-cycloserine has been reported to increase their susceptibility to lysing agents (7, 20); we also included D-threonine, another inhibitor of cell wall synthesis, prior to cell lysis. Several hours after the addition of ACT, bacterial growth ceased (as assessed spectrophotometrically) and cellular debris accumulated in the tubes. Increasing the duration and/or concentration of ACT treatment resulted in increased debris and decreased DNA yields. This suggested that the mycobacterial subpopulation affected by ACT was osmotically unstable and susceptible to premature lysis and DNA degradation. To protect the osmotically fragile cells, we tested sucrose, sodium succinate, sorbitol, and glycerol as osmotic stabilizers in the resuspension buffer (data not shown). Only 0.5 M sucrose consistently protected the cells from premature lysis without inhibiting bacterial growth.

Multiple parameters were varied to determine the optimal conditions for bacterial lysis and DNA yield; several protocol modifications are summarized in Table 1 and illustrated in Fig. 1. The best results were obtained by adding 0.5 M sucrose to both the growth medium and the resuspension buffer (Fig. 1, lanes 4 through 7); resuspension of mycobacteria in the Tris-sodium chloride buffer routinely used for other organisms (10) resulted in premature lysis and poorer DNA yields (Fig. 1, lane 5 versus lane 8).

A freeze-thaw cycle, used to prepare protoplasts of gram-positive bacilli (15), has been incorporated into other protocols for the isolation of mycobacterial DNA to synchronize the

TABLE 1. Modifications of the cell growth and lysis protocol used to isolate mycobacterial DNA

Protocol modification[a]	Use for Fig. 1, lane:							
	1	2	3	4	5	6	7	8
ACT added to growth media	+	+	+	+	+	+	+	+
Sucrose in growth media	−	−	−	+	+	+	+	+
Sucrose in resuspension buffer	+	+	+	+	+	+	+	−
Freezing-thawing prior to enzymatic lysis	−	−	+	−	+	+	−	+
Zymolase in lysis solution	−	+	−	−	−	+	+	−
Lysozyme in lysis solution	+	+	+	+	+	−	−	+

[a] Protocol modifications are described in Materials and Methods. A plus sign indicates that the reagent or procedure was included in preparing the DNA demonstrated in the corresponding lane in Fig. 1, and a minus sign indicates that it was omitted.

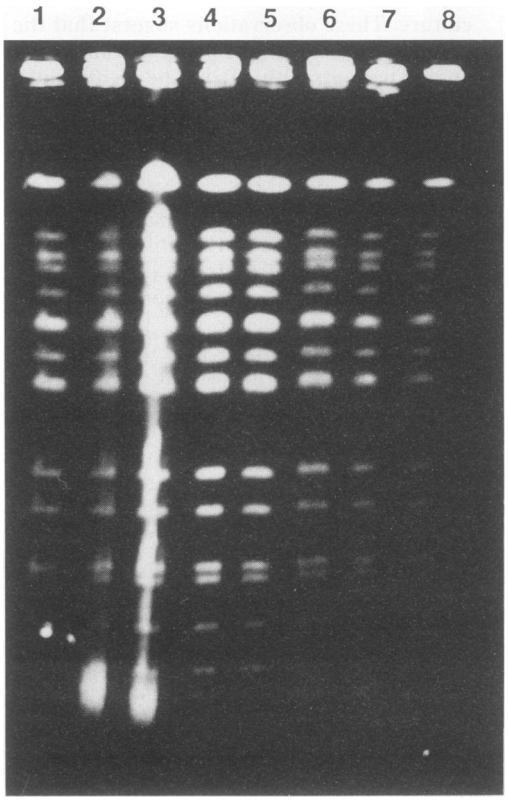

FIG. 1. PFGE of *Ase*I restriction digests of whole chromosomal DNA of *M. avium* ATCC 35718 (serovar 3). The lane numbers correspond to the protocol modifications indicated in the corresponding columns of Table 1. See the text for details.

processing of bacteria with different growth rates (7, 18). In our experiments, freezing-thawing increased the quantity of DNA (Fig. 1, lanes 3, 5, and 6 versus lanes 1, 4, and 7, respectively); however, the effect was least apparent for cells grown in the presence of sucrose (Fig. 1, lane 5 versus lane 4). Additional experiments demonstrated that for cultures grown and resuspended in sucrose and subsequently treated with lysozyme, the DNA yields obtained by pretreatment with freezing-thawing alone were comparable to those obtained by ACT treatment alone, with no consistent benefit from using both treatments (data not shown). Thus, the cell population(s) susceptible to freezing-thawing may be similar to those susceptible to ACT treatment.

To minimize the activity of endogenous endonucleases that would degrade the DNA, the bacterial suspension was maintained at 4°C after thawing and mixed as soon as possible with molten agarose in EDTA buffer. Several enzymes, including zymolase, lysostaphin, subtilisin, and lysozyme, were tested for the ability to disrupt the mycobacterial cell wall (data not shown). The combination of lysozyme and zymolase has been used for mycobacterial lysis (7); however, we observed that DNA yields obtained with lysozyme treatment alone were better than or comparable to those obtained with either zymolase alone (Fig. 1, lanes 4 and 5 versus lanes 7 and 6, respectively) or zymolase and lysozyme (Fig. 1, lane 1 versus lane 2) (additional data not shown). Note that efforts to increase cell lysis (by freezing-thawing or by treatment with multiple enzymes) without providing osmotic protection for the organisms during growth and ACT treatment resulted in increased DNA yields but with marked streaking of the DNA digests during electrophoresis (Fig. 1, lanes 1 and 3 versus lanes 4 and 5, respectively). We speculate that in the absence of osmotic stabilization, a greater fraction of the cells harvested are resistant to lysis and that such intact cells interfere with the migration of the DNA that is released and digested.

In summary, as detailed in Materials and Methods, we observed that the optimal protocol for obtaining mycobacterial DNA in agarose for subsequent PFGE analysis included treatment of growing cultures with ACT, addition of 0.5 M sucrose to both the broth medium and resuspension buffer, freezing-thawing after resuspension, and treatment of cells in agarose with lysozyme.

Analysis of sequential isolates from HIV-infected patients with disseminated MAC. We used the above-described protocol to analyze 55 isolates from 12 HIV-infected individuals who had disseminated MAC and from whom two or more isolates were available from specimens obtained at least 1 week apart (Table 2). There were two to five (median, three) positive culture specimens per patient. Only one colony was available from most of the primary cultures; for eight cultures from four patients, multiple colonies were collected as part of the MAC epidemiology study (1). (These four patients are included in this report because additional isolates were available from clinical cultures taken outside of the epidemiology study.) There were 2 to 10 (median, 4) isolates per patient. The intervals from the first to the last available isolates ranged from 8 to 192 (median, 46) days.

Four patients (33%) were infected with two different MAC strains (Table 2 and Fig. 2). For each of these patients, the two different strains were identified among isolates from the first two cultures yielding MAC; the time spans between the first available isolate and the first isolate representing the second strain ranged from 4 to 14 (median, 6) days. For three of these patients, the first strain was again detected in a subsequent culture. Only four patients were receiving antimicrobial agents expected to have significant activity against MAC (i.e., clarithromycin, ethambutol, clofazimine, amikacin, ciprofloxacin, or rifampin) at the time any of the specimens were obtained. Only one of these, patient 1049, had documented polyclonal disease; the two different strains were both cultured prior to the institution of antimycobacterial therapy. Patients 1235 and 1236 were infected with the same MAC strain, as defined by PFGE (Fig. 2); both patients received care at the same institution. Each of the other study patients was infected with one or more unique strains. For patients 1235 and 1237, infected with two different MAC strains, one of the strains was identified as *M. intracellulare* (Table 2); all of the other isolates in this study were identified as *M. avium*.

DISCUSSION

This report describes an efficient and reliable methodology for isolating mycobacterial genomic DNA in agarose suitable for examination by PFGE. We have used this protocol to analyze genomic DNAs from several mycobacterial species, including *M. avium*, *M. intracellulare*, *M. gordonae*, *M. bovis*, and *M. bovis* BCG (1, 5). Thus, the technique described here is generally applicable both to nontuberculosis species and to those of the *M. tuberculosis* complex. Several other investigators have described the use of PFGE to analyze strains of mycobacteria (2, 4, 7, 12, 20); however, these reports have not described the basis for specific steps in their protocols.

We observed two factors that were critical for the isolation of suitable DNA. (i) The yield of DNA was increased by adding 0.5 M sucrose to the growth medium and to the buffers in which the cells were resuspended prior to being embedded in agarose. We believe that the sucrose serves to protect cells that will be easily lysed in situ in agarose against spontaneous osmotic lysis in solution. (ii) A freeze-thaw cycle, which has been included in most protocols to isolate DNA from mycobacteria (7, 12, 18, 20), was explicitly confirmed to facilitate lysis. When processing MAC isolates, we routinely add ACT to the culture 12 to 18 h prior to harvesting; the cost and effort of this step are nominal, and although it is not strictly critical for some strains, it appears to facilitate lysis for others. However, addition of ACT resulted in poorer DNA yields for *M. bovis* and *M. bovis* BCG (5). Although an early report described the use of zymolase and lysozyme to lyse organisms cast in agarose (7), we found that when the cells were suitably prepared (by ACT pretreatment or freezing-thawing), lysozyme alone provided sufficient lysis for isolation of high-quality DNA. Consequently, we omit zymolase, which is considerably more expensive than lysozyme and technically more difficult to use because it tends to precipitate in EDTA solutions.

By using this protocol, we examined 55 isolates from 12 HIV-infected patients for whom two or more MAC isolates were available with an interval of at least 1 week between the first and last isolates. We detected two distinct MAC strains in four patients (33%). In each patient, both strains were identified in cultures $\leq$14 days apart, and in three of the patients, the index strain was identified in a subsequent culture. These observations suggest that the presence of two different strains among isolates from sequential cultures may reflect ongoing polyclonal infection. For two of the patients infected with two different MAC strains, the isolates were identified as *M. avium* and *M. intracellulare*. Antimicrobial therapy did not appear to be a contributing factor in the development of acute polyclonal infection. However, patient 1049, from whom two different MAC strains were cultured over 8 days, had a clinical recurrence 17 months later while being treated with four antimycobacterial drugs. At that time, he had recurrent MAC bacteremia; PFGE analysis of a single isolate identified a third distinct

TABLE 2. Microbiologic data and concurrent antimicrobial treatments for 12 AIDS patients with multiple MAC isolates cultured over time

Patient no.[a]	No. of days after initial culture	Source(s) of culture(s)	Patient isolate PFGE pattern(s)[b]	Antimicrobial treatment at time culture obtained[c]
1009	0	Blood	A	RIF, EMB
	6	Liver	A	RIF, EMB
	104	Blood	A A A	RIF, EMB, AMK, CIP
1045	0	Bone marrow	A	INH, RIF, PZA
	32	Blood, feces	A A A, A A A	INH, RIF, PZA
	123	Bone marrow	A	INH, RIF, PZA, CLA
	130	Blood	A A	INH, RIF, PZA, CLA
1049	0	Blood	A	None
	6	Urine	B	None
	8	Bone marrow	A	None
1052	0	Blood, feces, sputum	A A A, A A A, A	None
	14	Blood	A	None
1060	0	Sputum, feces	A A, A A A	None
	192	Bone marrow	A	None
1212	0	Bone marrow	A	None
	26	Blood	A	None
1213	0	Blood	A	None
	14	Sputum	A	None
	15	Bone marrow	A	None
1217	0	Sputum	A	INH
	88	Pleural fluid	A	INH
	112	Blood	A	INH
1228	0	Sputum	A	None
	36	Blood	A	None
	44	Bone marrow	A	None
1235	0	Blood	A[d]	None
	6	Blood	B B	None
	13	Blood	B	None
	49	Blood	A[d]	CLA
1236	0	Blood	A	None
	4	Blood	B	None
	11	Blood	B	None
1237	0	Blood	A	None
	14	Blood	B[d]	None
	56	Blood	A	None
	121	Blood	A	None

[a] Patients 1009 to 1060 were enrolled in the international study of MAC epidemiology (1); the isolates obtained from patient 1009 on day 104 were included in a previous report (1).

[b] The PFGE patterns are listed for each of the isolates analyzed for each patient; when multiple colonies were saved from the primary culture and analyzed separately, the result obtained for each colony is given. The first pattern observed for each patient was designated A; if a different pattern was detected among subsequent isolates from that patient, it was designated B.

[c] Antimicrobial agents: RIF, rifampin; EMB, ethambutol; AMK, amikacin; CIP, ciprofloxacin; INH, isoniazid; PZA, pyrazinamide; CLA, clarithromycin.

[d] The isolates cultured from patient 1235 on days 0 and 49 and from patient 1237 on day 14 were *M. intracellulare*; all other isolates were *M. avium*.

strain (data not shown). It is unclear whether this episode of bacteremia represents a relapse with a strain not detected among the isolates initially available or infection with a new strain.

We have previously reported detection of concurrent bacteremia with two distinct *M. avium* strains in 2 (14%) of the first 14 patients enrolled in an international study of MAC epidemiology (1); those observations reflected a PFGE analysis of three colonies from a single culture. Among the first 25 patients enrolled in that study for whom multiple colonies have been examined, we have detected a total of 5 patients (25%) simultaneously infected with more than one MAC strain (unpublished data). As noted, the patients reported here included five patients from the epidemiology study for whom additional nonstudy isolates were available. These patients had monoclonal infection on the basis of an analysis of the multiple colonies available from their study cultures; for each patient, the same infecting strain was detected among their nonstudy isolates. Overall, we have identified 9 patients (24%) with polyclonal infection among 37 patients for whom multiple isolates have been analyzed.

Mazurek et al. analyzed MAC isolates from nine patients for

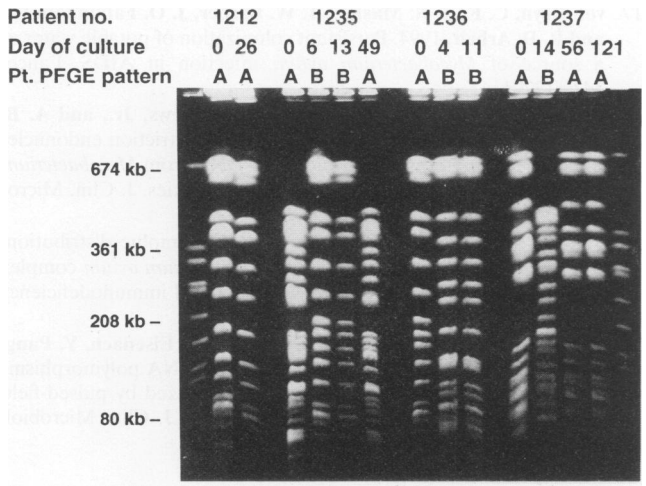

FIG. 2. PFGE of *Ase*I restriction digests prepared from sequential MAC isolates cultured from patients 1212, 1235, 1236, and 1237. For each patient, the day of isolation and the PFGE pattern designations appear above each isolate. The first pattern observed for each patient was designated A; if a different pattern was detected among subsequent isolates from that patient, it was designated B. Patients 1235, 1236, and 1237 were infected with two distinct strains, as resolved by PFGE; for patient 1212, only one strain was detected. The isolates obtained from patient 1235 on days 6 and 13 demonstrated a PFGE pattern (designated 1235B) indistinguishable from that of the isolate obtained from patient 1236 on day 0 (designated 1236A); these isolates were considered to represent the same strain. All of the remaining patterns were distinctly different and were considered to represent unique MAC strains. The first and last lanes contained *Sma*I restriction digests of *Staphylococcus aureus* ATCC 8325, included as molecular size markers. Pt., patient.

whom two or more isolates were available (12). The presence or absence of HIV infection in these patients was not explicitly stated. For six patients, only respiratory isolates (spanning 1 day to 2 years) were available. From three patients, at least one isolate was cultured from blood or bone marrow; isolates from these patients spanned 1 day to 27 months. Each of the nine patients was infected with a single unique MAC strain, as determined by PFGE. AIDS patients infected with two mycobacterial species have been previously reported (6, 8, 11, 16), including one patient with a simultaneous bloodstream infection due to *M. avium* and *M. intracellulare* (3).

The observation that two patients were infected with the same *M. avium* strain as resolved by PFGE, suggests a possible common-source exposure. Although a detailed epidemiologic investigation was not performed, both patients are known to have been treated at the same hospital prior to the onset of invasive MAC disease, suggesting possible nosocomial acquisition. In the course of our prospective studies, we have recently identified similar clusters of patients infected with the same *M. avium* strain (17). For those patients, the infecting strain was recovered from the hot water system of the hospital at which they were treated. We are currently examining the water supply of the hospital to which patients 1235 and 1237 of this report were both admitted.

We conclude that patients with advanced AIDS may commonly be infected with multiple MAC strains. Such polyclonal infection can be detected by using PFGE to analyze either multiple colonies from individual cultures or single colonies from multiple separate cultures obtained over time. However, the true prevalence of polyclonal infection among patients with AIDS has not been accurately determined nor has the impact of these infections on the response to antimycobacterial treatment been defined. The possibility that patients with AIDS are infected with more than one MAC strain, either concurrently or over time, must be considered in designing and evaluating treatment studies.

ACKNOWLEDGMENTS

We thank J. O. Falkinham, Kurt Stottmeier, and Gale Newman for providing mycobacterial isolates and for many helpful discussions; Irene George and the Mycobacteriology Division, Massachusetts State Laboratory, for providing mycobacterial isolates; Sandy Niemczyk, Nuria Gine, and Richard Waddell for data collection; and Marie Scurti for assistance in preparing the manuscript.

Financial support was provided by the Public Health Service (grant AI-30373 [A.M.S., R.D.A., T.W.B., C.F.V., and J.N.M.]) and the Medical Research Program, Department of Veterans Affairs, Washington, D.C. (R.D.A. and J.N.M.).

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