Variable-Number Tandem-Repeat Analysis of Respiratory and Household Water Biofilm Isolates of “*Mycobacterium avium* subsp. *hominissuis*” with Establishment of a PCR Database

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“*Mycobacterium avium* subsp. *hominissuis*” is an important cause of pulmonary disease. It is acquired from environmental sources, but there is no methodology for large population studies. We evaluated the potential of variable-number tandem-repeat (VNTR) analysis. Clinical and household biofilm *M. avium* isolates underwent molecular identification. Testing for IS901 was done to separate *M. avium* subsp. *avium* from *M. avium* subsp. *hominissuis*. VNTR types were defined using VNTR loci, and subtyping was performed using 3′ *hsp65* and internal transcribed spacer (ITS) sequencing. Forty-nine VNTR types and eight subtypes of *M. avium* subsp. *hominissuis* (IS901 negative) were identified among 416 isolates of *M. avium* from 121 patients and 80 biofilm sites. Of those types, 67% were found only among patient isolates, 11% only among household water isolates, and 23% among both. Of 13 VNTR types that included ≥4 patients, the majority (61.5%) represented geographic clustering (same city). Most VNTR types with multiple patients belonged to the same 3′ *hsp65* sequence code (sequevar). A total of 44 isolates belonging to four *M. avium* subsp. *hominissuis* VNTR types (8%), including three with the rare Mav-F ITS sequence and 0/8 subspecies, produced amplicons with IS901 PCR primers. By sequencing, all 44 amplicons were not IS901 but ISMav6, which was recently observed in Japan but had not been previously described among U.S. isolates. VNTR analysis of *M. avium* subsp. *hominissuis* isolates is easier and faster than pulsed-field gel electrophoresis. Seven VNTR loci separated 417 isolates into 49 types. No isolates of *M. avium* subsp. *avium* were identified. The distributions of the VNTR copy numbers, the allelic diversity, and the low prevalence of ISMav6 differed from the findings for respiratory isolates reported from Japan.

The species “*Mycobacterium avium* subsp. *hominissuis*” is an important cause of chronic lung disease in the setting of bronchiectasis and disseminated disease in patients with AIDS. The species is acquired from environmental sources, with recent studies focusing on household water. It is present worldwide, with all areas of the United States (especially the southern United States) being considered high-risk areas for the disease.

Efforts to compare isolates of *M. avium* have been difficult. Three subspecies are currently recognized, namely, *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum*. A fourth subspecies, *M. avium* subsp. *hominissuis* (including human isolates), has also been proposed (1, 2). IS901 is present in all isolates of *M. avium* subsp. *avium* but is not present in isolates of *M. avium* subsp. *hominissuis* (1); this difference has been used to separate the two subspecies (1, 2). Strain comparisons within *M. avium* (as well as with other members of the *Mycobacterium avium* complex [MAC], especially *Mycobacterium intracellulare*) have most frequently utilized pulsed-field gel electrophoresis (PFGE) (3, 4), which is expensive, technically difficult, time-consuming, and useful only for comparing small numbers of isolates. Other methods, such as repetitive sequence-based PCR (rep-PCR) (5), random amplified polymorphic DNA PCR (RAPD-PCR) (6), gene sequencing (2), and sequencing of the 16S-23S internal transcribed spacer (ITS) region (1, 2), have also been employed for *M. avium* complex strain comparisons.

Genome sequences of several strains and subspecies of *M. avium* (including the type strain of *M. avium* subsp. *avium*, ATCC

25291) have been made available (7). A number of variable-number tandem repeats (VNTRs) of the minisatellite class (50 to 60 bp) have been identified and used in preliminary studies. Such studies include the use of mycobacterial interspersed repetitive unit (MIRU) loci (8) and *M. avium* tandem-repeat (MATR) loci (some of which recognize the same tandem repeat) (9). VNTR analysis has been used for strain comparisons and population studies of other species of mycobacteria, including *M. intracellulare*, *Mycobacterium ulcerans*, and *Mycobacterium tuberculosis* (8–18). Studies on the use of VNTR analysis for *M. intracellulare*

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isolates were reported from Japan and France in 2009 and 2010 (11, 13), respectively, and from the United States in 2013 (12). Similar studies on the use of VNTR analysis for *M. avium* isolates have also been reported, but none of those studies evaluated isolates from the United States (9, 15, 18). Here we describe the use of *M. avium* VNTR analysis for comparison of environmental water and respiratory disease isolates from the United States and utilization of the composite data to create a database of isolates.

MATERIALS AND METHODS

Study design. There currently is no established, rapid, simple, discriminatory method for *M. avium* strain comparisons or population studies. Studies have shown the ability of VNTR analysis to meet the criteria for discrimination from the closely related species *M. intracellulare* (11–13), and preliminary studies have shown VNTR analysis to be feasible with *M. avium* (8, 9). Thus, we undertook a study on the use of VNTR analysis in the clinical setting of U.S. patients with nodular bronchiectasis and *M. avium*. Isolates studied for microbiological relapse of MAC-related lung disease were compared with PFGE and VNTR analysis, and VNTR types with two or more patients were compared by 5' *hsp65* sequencing. VNTR typing results for select groups were compared with results from pulsed-field gel electrophoresis (PFGE) and 3' *hsp65* sequencing.

Patients. Patients were selected from bronchiectasis and nontuberculous mycobacteria (NTM) clinics at the University of Texas Health Science Center at Tyler (UTHSCT) and the Pulmonary and Critical Care Division, Lankenau Medical Center (Wynnewood, PA). Patient isolates were candidates for study if the patients had nodular bronchiectasis. Most patients met current American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) 2007 guidelines for clinical disease (19). Some patients and their isolates were included in previous or current clinical studies from the UTHSCT and the Lankenau Medical Center (20). Clinical information on the presence of bronchiectasis and the home address (city) were extracted from medical records. This study was approved by the UTHSCT and Lankenau Medical Center institutional review boards (IRBs).

Isolates. Patients who had nodular bronchiectasis and whose MAC isolates were submitted for strain comparisons because of microbiological relapse during or after drug treatment of *M. avium* subsp. *hominissuis* or for comparisons with household water/biofilm isolates or who had a request for sputum MAC species identification and were found to have *M. avium* were chosen for study; the latter two groups consisted of only a single *M. avium* isolate. For patients with suspected microbiological relapse, current isolates were compared to previous isolates stored at -70°C or stored in their original broth culture vials at room temperature following primary isolation. Sputum cultures from patients had been submitted to and processed by the clinical mycobacteriology laboratory as described previously (3, 4, 12). Sputum isolates from three recent studies (20; R. J. Wallace, Jr., unpublished data) comparing patient and household water MAC isolates (5) were also included. Isolates were from Texas, Oklahoma, Louisiana, Arkansas, and Pennsylvania. These studies were also approved by the UTHSCT and Lankenau Medical Center IRBs.

Environmental isolates from household water and biofilms from multiple sites and states were also collected and underwent processing and culture as described previously (5). These isolates were from showerhead filters, shower pipes, bathroom and kitchen faucet filters, bathroom and kitchen pipes, bathtub inlets, and (when available) hot tub filters. Isolates were obtained from Texas, Louisiana, Oklahoma, Maryland, and Pennsylvania.

Species and subspecies identification. Isolates for study initially were identified as MAC using a commercial hybridization assay (AccuProbe MAC probe; Hologic Inc.) and then were identified as *M. avium*, *M. intracellulare*/*Mycobacterium chimaera*, or MAC X using the multiplex 16S rRNA gene PCR technique described by Wilton and Cousins (21) or *hsp65* PCR-restriction analysis (PRA) (22). To confirm the species iden-

tification, sequencing of the internal transcribed spacer (ITS) region was performed (23, 24).

IS901 has been shown to be present in all isolates of *M. avium* subsp. *avium* and *M. avium* subsp. *silvaticum* but absent in isolates of *M. avium* subsp. *hominissuis* (1, 2). Therefore, one or more isolates from each patient underwent PCR for IS901, as described previously (2). Clinical isolates with negative results for IS901 by PCR were considered to belong to *M. avium* subsp. *hominissuis*. Isolates that produced an amplicon the size of IS901 underwent sequencing of the PCR product, to determine whether the product was indeed IS901. Three reference strains of *M. avium* subsp. *avium*, i.e., the type strain ATCC 25291T (TMC 724; chicken, serotype 2), ATCC 35718 (TMC 721; child's lymph node, serotype 3), and ATCC 35712^T (TMC 701; chicken, serotype 2), were included as positive controls. Two human reference strains of *M. avium* subsp. *hominissuis* (which has no designated type strain), i.e., ATCC 700898 (strain MAC 101) and MAC 104 (both from the blood of patients with AIDS), were included as negative controls.

Pulsed-field gel electrophoresis. PFGE was performed as described previously, using the restriction enzymes XbaI and AseI (3, 4, 12). Isolates from the same patient were compared and classified as indistinguishable, closely related, possibly related, or unrelated on the basis of differences in band number and position, using methods originally described by Tenover et al. for bacterial outbreaks and then modified for MAC (4, 12, 25). Strains in the first three categories were considered clonal. Different (unrelated) genotypes from the same patient were given letter designations; numbers were added to the letter designations (e.g., A2) if minor band differences were observed.

VNTR analysis. All isolates with different genotypes (unrelated PFGE patterns) from individual patients with multiple isolates, as well as single isolates that did not undergo PFGE, underwent VNTR analysis. For some patients, multiple isolates of the same PFGE genotype also underwent typing. One MIRU locus (MIRU locus 3) described by Bull et al. (8) and six MATR loci described by Inagaki et al. (9) (MATR-1, MATR-2, MATR-3, MATR-7, MATR-13, and MATR-14) were used; those six loci had the greatest allelic diversity of 15 loci studied by Inagaki et al. (9). One of the MATR loci amplified was the same locus as a MIRU tandem repeat (MIRU tandem repeat 292 = MATR-2) (9). PCR products were sized with 2% agarose gel electrophoresis. Allele designations for each amplicon reflected the number of complete tandem repeats.

DNA fingerprinting of isolates with the same VNTR types, using PFGE and 3' *hsp65* gene sequencing. *M. avium* isolates from five or more different patients that were subsequently shown to belong to the same VNTR type were compared to each other by using PFGE for selected VNTR types. Isolates from two or more different patients also underwent comparisons by using 3' *hsp65* gene sequencing, as described previously (2, 26).

PCR and DNA sequencing of ITS, VNTR alleles of different sizes, and IS901. Sequencing of the ITS region (263 bp) was performed as described previously (23, 24). Results were compared to ITS sequences in GenBank, including the Mav-A (GenBank accession no. EF521901.1), Mav-B (GenBank accession no. CP000479.1), Mav-G (GenBank accession no. AF315839.1), and Mav-F (GenBank accession no. AF315838) sequences. Examples of each of the alleles of different sizes for each of the seven primers were also sequenced, to determine the length (size) of each tandem repeat.

As noted previously, isolates that were PCR positive for IS901 underwent amplicon sequencing. The sequences then underwent BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) using the GenBank database.

Comparison of VNTR copy numbers and allelic diversity with isolates from Japan. A comparison of the VNTR copy numbers and allelic diversity for each of the MATR-VNTR loci used to separate the VNTR types from the United States in the current study was made with a prior study by Inagaki et al. of respiratory isolates from Nagoyi, Aichi, Japan (9). The allelic diversity among the U.S. isolates was calculated using the formula of Selander et al. (27).

FIG 1 PCR analysis of clinical isolates of *M. avium* subsp. *hominissuis* using IS901 primers. (A) Analysis of clinical isolates. Lane 1, 100-bp ladder; lane 2, *M. avium* subsp. *avium* type strain ATCC 25291; lanes 3 to 12, clinical isolates of *M. avium* subsp. *hominissuis* from different VNTR groups; lane 13, PCR water control. (B) Analysis of Mav-F isolates. Lane 1, 100-bp ladder; lane 2, ATCC 25291^T; lanes 3 and 4, VNTR type 40 isolates; lanes 5 to 8, VNTR type 51 isolates from four patients; lane 9, PCR water control. Numbers on the left, marker sizes (in base pairs). As expected, ATCC 25291^T produced an amplicon with these primers (panels A and B, lanes 2), consistent with the presence of IS901. One isolate from VNTR type 40 and one isolate from VNTR type 51 also generated bands with sizes similar to that of the IS901 amplicon (panel A, lanes 8 and 12, respectively). These results were confirmed using additional isolates from the two VNTR types (panel B, lanes 3 to 8). With sequencing, the PCR products from all isolates except for the *M. avium* subsp. *avium* type strain ATCC 25291 showed 100% sequence identity to ISMAV6 but not IS901.

RESULTS

Patients and isolates. A total of 416 isolates of *M. avium* subsp. *hominissuis* from 121 patients and 80 household environmental biofilm sites, three reference strains of *M. avium* subsp. *avium* (one patient strain and two chicken strains), and two reference strains of *M. avium* subsp. *hominissuis* were characterized. Each different VNTR genotype was counted only once per patient, although multiple patients had more than one VNTR type during the follow-up period (and were counted more than once), and multiple isolates of the same VNTR types (range, 2 to 21 isolates) contributed to the total of 416 isolates. Patients had typical radiographic findings of nodular bronchiectasis and MAC disease. Additionally, most patients met the 2007 American Thoracic Society/ Infectious Disease Society of America guidelines for NTM lung disease (19). Two exceptions were reference strains MAC 104 and ATCC 700898, which were blood isolates recovered from patients with AIDS.

Species and subspecies identification. PCR for IS901 yielded positive results for the three reference strains of *M. avium* subsp. *avium* (ATCC 25291^T and ATCC 35712, both from chickens, and ATCC 35718^T, from a human cervical lymph node) (1, 2). The IS901 PCR also produced an amplicon of the same size as IS901 for four clinical VNTR types (type 8, which contained one isolate from one patient; type 40, which contained five isolates from two patients; type 37a, which contained two isolates from one patient; and type 51, which contained 36 isolates from three environmental sources and 11 patients) containing a total of 44 isolates from 15 patients (Fig. 1). PCR for IS901 yielded negative results (did not produce an amplicon) for all of the remaining clinical and environmental isolates and the two reference strains of *M. avium* subsp. *hominissuis* (strains MAC 104 and ATCC 700898) (data not shown.).

FIG 2 Examples of different amplicon sizes associated with different tandem-repeat copy numbers for isolates of *M. avium* subsp. *hominissuis* and MATR-7 primers. Gel electrophoresis was performed with a 2% agarose gel in 1× Tris-acetate-EDTA (TAE) buffer. Lane 1, 500-bp amplicon with 5 complete copies (MA-4214); lane 2, 450-bp amplicon with 4 complete copies (MA-4826); lane 3, 395-bp amplicon with 3 complete copies (ATCC 700898); lane 4, 320-bp amplicon with 2 complete copies (MA-4616); lane 5, 290-bp amplicon with 1 complete copy (MA-5029); lane 6, 100-bp ladder.

PFGE. Multiple MAC isolates from patients suspected of microbiological relapse underwent species or subspecies identification (including isolates obtained before and after the suspected relapse); the test isolates underwent PFGE, and *M. avium* isolates underwent VNTR typing. Single isolates of *M. avium* subsp. *hominissuis* from patients not suspected of relapse did not undergo PFGE analysis. A total of 81 isolates of *M. avium* subsp. *hominissuis* from 28 patients with suspected microbiological relapse underwent simultaneous VNTR typing and PFGE analysis. Ten patients had multiple different *M. avium* VNTR types at different times (mean, 2.4 types per patient [range, 2 to 6 types per patient]). All different VNTR types from the same patients had unrelated (different) PFGE patterns.

Seventeen patients had multiple sputum isolates of the same VNTR type at different times (mean, 3.4 isolates per patient [range, 2 to 6 isolates per patient]). A total of 14 of those patients had 42 compared isolates, with all of the isolates from each patient having indistinguishable PFGE patterns. A total of 9 patients had 10 isolates with related (clonal) PFGE patterns, compared to the predominant (most frequently occurring) PFGE clone, and 4 patients had 5 isolates with the same *M. avium* VNTR type as ≥1 isolate but unique (unrelated) PFGE patterns. Some patients had both indistinguishable and clonal patterns; therefore, the patient number was >17.

TABLE 1 VNTR tandem-repeat size, copy number, amplicon size, and assigned allele number for isolates of *M. avium* subsp. *hominissuis*, with seven tandem-repeat loci (8, 12)

Locus, amplicon size, and copy no. ^a	5 alleles	4 alleles	3 alleles	2 alleles	1 allele	0+ alleles	0 alleles
MIRU locus 3 (53 bp)							
Amplicon size (bp)	460 ^b	400	350	300	260	ND	ND
Copy no.	5.2 ^b	4.2	3.2	2.2	1.2	ND	ND
MATR-7 (57 bp)							
Amplicon size (bp)	500	450	395 ^b	330	280	ND	ND
Copy no.	5.1	4.1	3.1 ^b	2.1	1.1	ND	ND
MATR-1 (53 bp)							
Amplicon size (bp)	490	430	ND	330 ^b	285	ND	ND
Copy no.	5.4	4.4	ND	2.4 ^b	1.4	ND	ND
MATR-2 (53 bp)							
Amplicon size (bp)	ND	ND	350	300 ^b	250	195	ND
Copy no.	ND	ND	3.1	2.1 ^b	1.1	0.36	ND
MATR-3 (53 bp)							
Amplicon size (bp)	460 ^b	400	350	300	230	ND	ND
Copy no.	5.2 ^b	4.2	3.2	2.2	1.2	ND	ND
MATR-13 (56 bp)							
Amplicon size (bp)	ND	ND	ND	ND	340 ^b	295	240
Copy no.	ND	ND	ND	ND	1.9 ^b	0.9	0
MATR-14 (58 bp)							
Amplicon size (bp)	ND	450 ^b	450	395	335	280 ^c	ND
Copy no.	ND	3.7 ^b	3.7	2.7	1.7	0.7 ^c	ND

^a Copy numbers were rounded to the nearest tenth. The tandem-repeat allele numbers were determined by the numbers of complete repeats. The amplicon sizes include the flanking regions and the tandem repeats. The sizes of the complete tandem repeat are indicated in parentheses. ND, not detected.

^b Size of the tandem repeat of *M. avium* subsp. *hominissuis* reference strain MAC 104.

^c Seen only with the *M. avium* subsp. *avium* reference strain.

PFGE comparisons of *M. avium* with multiple other isolates of different MAC species (*M. intracellulare*, *M. chimaera*, or other MAC X species) from the same patient were also made for those 28 patients. All *M. avium* PFGE profiles were unique with respect to those isolates as well.

VNTR analysis. Multiple *M. avium* strains with different genotypes from the same patients that had undergone PFGE (range, 2 to 4 genotypes) and single isolates that had not undergone PFGE underwent VNTR typing, as did the five reference strains. Isolates from the same patients that were considered clonal on the basis of PFGE results had the same VNTR type. All genotypes of *M. avium* produced satisfactory VNTR amplicons for gel sizing and DNA sequencing except for three loci in single isolates, which resulted in no PCR product. Amplicons of indeterminate copy number were confirmed by sequencing. Examples of five amplicons of different sizes, with different numbers of complete tandem repeats for MATR-7, are shown in Fig. 2.

The different tandem-repeat copy numbers and amplicon sizes observed for each of the seven primers were given allele numbers that matched the numbers of complete tandem repeats (partial tandem repeats were excluded). Amplicons from each primer set were grouped together as a single allele if their size differences (generally <20 bp) did not allow separation on the sizing gel (Table 1). This resulted in 49 VNTR types of *M. avium* subsp. *hominissuis* (including the two reference strains) and three VNTR types of the three reference strains of *M. avium* subsp. *avium* (VNTR types 24, 29, and 43) (Table 2). Eight VNTR subtypes

(with a number followed by a letter) were isolates that had the same tandem-repeat copy numbers for all seven primer sets but different ITS sequences (i.e., 14 and 14a; 15 and 15a; 27 and 27a; 31, 31a, and 31b; 36 and 36a; 37 and 37a; and 38 and 38a). The 49 VNTR types differed by one or more tandem-repeat copy numbers. Most VNTR types of *M. avium* subsp. *hominissuis* included a single genotype from one patient (33/49 types [67.3%]). Only 13 VNTR types and subtypes included isolates from four or more different patients (range, 4 to 14 patients). Eight of these 13 groups (61.5%) had geographic clustering (same town), while five did not, based on home addresses (Table 3).

DNA fingerprinting of isolates with the same VNTR types, using PFGE and 3' *hsp65* gene sequencing. PFGE was performed for three VNTR types that included five or more patients or household water sites. PFGE was performed for VNTR type 51, which included three isolates from different patients from the same town and two isolates from other geographic sites (cities) (Fig. 3). Four of the isolates were closely related and one (MA-5511) (Fig. 3, lane 4) was possibly related. By 3' *hsp65* gene sequencing, all five isolates belonged to code 15 (2, 26).

PFGE was also performed on five isolates from four patients with VNTR type 10, including two reference strains (strains MAC 104 and ATCC 700898 from AIDS patients, collected in the same hospital) and two clinical isolates from other cities. The two reference strains were indistinguishable, as were two isolates obtained from the same patient, 6 months apart. Two of the patient isolates were only possibly related, which suggests that this cluster

TABLE 2 VNTR types among *M. avium* subsp. *hominissuis* isolates from environmental sources and patients with nodular bronchiectasis, determined using six MATR loci described by Inagaki et al. (9) and one MIRU VNTR locus described by Bull et al. (8)

Copy no.								No. of isolates			
VNTR type ^a	MIRU locus							From patients	From environmental sources	Total tested	ITS sequence
	3	MATR-7	MATR-1	MATR-2	MATR-3	MATR-13	MATR-14				
1	5	5	2	0	5	1	1	1	2	3	Mav-A
2	5	5	1	0	5	1	1	0	2	2	Mav-A
3	5	4	2	2	5	1	1	6		13	Mav-B
3a	5	4	2	2	5	1	1	1		3	Mav-B
4	5	4	2	2	5	0+	1	3		4	Mav-A
5	5	4	2	2	2	1	1	1		1	Mav-B
6	5	4	2	2	3	0+	2	1		1	Mav-B
7	5	4	2	0	2	1	1	1		1	Mav-B
8	5	4	2	1	5	1	1	1		1	Mav-B
9	5	3	2	3	5	1	1	1		1	NA
10	5	3	2	2	5	1	3	2 ^b (<i>M. avium</i> subsp. <i>hominissuis</i> blood isolates MAC 104 and ATCC 700898)		3	Mav-B
11	5	3	2	2	5	1	1	1		1	Mav-A
12	5	3	2	1	5	1	1	1			Mav-B
13	5	3	2	0	5	1	2	1	5	9	Mav-A
14	5	3	2	0	5	1	1	1		1	Mav-B
14a	5	3	2	0	5	1	1	9 ^b	22	30 ^b	Mav-A
15	5	2	2	0	5	1	1	1		3	Mav-B
15a	5	2	2	0	5	1	1	1		1	Mav-A
16	5	1	NP	0	2	1	1	1		1	Mav-A
17	5	1	1	0	5	1	1	0	1	1	Mav-B
18	4	3	2	2	5	1	3	2		2	Mav-B
19	4	3	2	2	4	1	3	5	1	13	Mav-B
20	4	3	2	2	4	0+	3	1		1	Mav-B
21	4	3	2	3	4	1	1	2		1	Mav-B
22	4	3	2	0	4	1	1	1	1	2	Mav-B
23	4	3	2	0	3	1	1	0	1	1	Mav-A
24	4	1	1	2	4	0+	2	ATCC 35718 (human)			Mav-A
25	3	5	2	3	3	1	1	1		1	Mav-B
26	3	5	1	0	3	1	1	1	2	8	Mav-A
27	3	4	2	2	3	1	1	1		3	Mav-B
28	3	3	2	3	3	2	2	1		2	Mav-A
29	3	3	2	3	3	1	2	ATCC 35712 (chicken)			Mav-B
30	3	3	2	3	3	1	2	4	6	29	Mav-B
31	3	3	2	3	3	1	1	2		2	Mav-A
31a	3	3	2	3	3	1	1	14 ^b	6	82	Mav-B
31b	3	3	2	3	3	1	1	1	2	1	Mav-G
32	3	3	2	2	3	1	1	1		1	Mav-B
33	3	3	2	2	3	0+	3	2		6	Mav-B
34	3	3	2	2	3	0+	1	1		1	Mav-B
35	3	3	2	0	4	1	1	0	2	2	Mav-B
36	3	3	2	0	3	1	2	6		6	Mav-A
36a	3	3	2	0	3	1	2	2 ^b	7	16	Mav-A
37	3	3	2	0	3	1	1	9 ^b	12	52	Mav-A
37a	3	3	2	0	3	1	1	6		9	Mav-B
38	3	3	1	3	3	1	1	2	1	4	Mav-A
38a	3	3	1	3	3	1	1	0	3	3	Mav-B
39	3	2	2	3	3	1	1	1		2	Mav-B
40	3	2	2	0	3	1	1	2		5	Mav-F
41	3	1	4	0	3	1	1	1		5	Mav-B
42	3	1	2	2	3	1	1	1		21	Mav-A
43	3	1	1	2	3	0+	0	ATCC 25291 ^T (chicken)			Mav-A
44	2	4	2	2	2	1	1	4		6	Mav-B
45	2	3	2	3	3	1	3	1		1	Mav-B
46	2	3	2	2	2	1	3	2		4	Mav-B
47	2	2	1	0	3	0	1	0	1	1	Mav-B

(Continued on following page)

TABLE 2 (Continued)

VNTR type ^a	Copy no.							No. of isolates			
	MIRU locus							From patients	From environmental sources	Total tested	ITS sequence
48	1	4	2	2	2	1	1	1		1	Mav-B
49	1	3	2	2	1	0	1	1		1	Mav-B
50	1	2	2	0	1	1	1	1		2	Mav-F
51	1	1	1	1	1	0	1	1 ^b	3	36 ^b	Mav-F
52	NP	4	2	2	2	1	1	6 ^b		6	Mav-B

^a Some patients had more than one isolate tested. Isolates with the same VNTR copy numbers but different ITS sequences or different 3' *hsp65* codes (sequevars) were given the same VNTR number plus a letter (e.g., 3a or 14a). Three reference strains of *M. avium* subsp. *avium* and two reference strains of *M. avium* subsp. *hominissuis* were controls. VNTR types were validated as *M. avium* by sequencing of the ITS1 region. The reference strains were as follows: ATCC 25291, type strain for *M. avium* subsp. *avium*; ATCC 35718, *M. avium* subsp. *avium*; ATCC 35712, *M. avium* subsp. *avium*; ATCC 700898, *M. avium* subsp. *hominissuis*; strain MAC 104, *M. avium* subsp. *hominissuis*. NA, not available; NP, no PCR product (amplicon).

^b Patients/isolates with geographic clustering.

may consist of more than one VNTR type (Fig. 4). By 3' *hsp65* gene sequencing, all five isolates belonged to code 1 (2). PFGE was performed on isolates of a third VNTR type, with all isolates appearing clonal, comparable to the results for VNTR type 51.

PCR and DNA sequencing of ITS, IS901 PCR amplicons, and 3' *hsp65*. (i) ITS. Among the 49 *M. avium* subsp. *hominissuis*

VNTR types, isolates from each patient underwent ITS sequencing. Isolates that had the same VNTR type but different ITS sequences were given the same VNTR number but one was given a letter designation (e.g., 15 and 15a). This analysis revealed that 33 types or subtypes were Mav-B (58.9%), 18 were Mav-A (32.1%), 3 were Mav-F (5.4%), 1 was Mav-G (1.8%), and 1 did not amplify. Other previously described ITS sequences in isolates of *M. avium* (Mav C, Mav-D, and Mav-E sequences) were not encountered (1, 24, 28). Of the three reference strains of *M. avium* subsp. *avium*, ATCC 35712 was surprisingly found to have the Mav-B type, while the remaining two strains, ATCC 35718 and ATCC 25291, had the expected Mav-A type (1).

The three VNTR types that contained Mav-F ITS sequences included 2 patients (VNTR type 40), 1 patient (type 50), and 10 patients (VNTR type 51). All three types had VNTR copy numbers for one or more loci seen infrequently among the other VNTR types (Table 2).

(ii) Insertion sequences. The three *M. avium* subsp. *hominissuis* VNTR types that contained the Mav-F ITS sequence (types 40, 50, and 51) and one that contained the Mav-B sequence (type 8) also produced an amplicon with IS901 PCR primers, along with VNTR type 8 (Mav-B) (a total of 44 isolates in the four VNTR types). Sequencing of the PCR product revealed that it had 100% identity with the recently described ISMav6 from Japan (GenBank accession no. AB447556) (29). Sequencing of the PCR product from the *M. avium* subsp. *avium* type strain ATCC 25291 and two other *M. avium* subsp. *avium* strains, ATCC 35718 and ATCC 35712, revealed 100% identity with IS901 (GenBank accession no. X59272).

(iii) 3' *hsp65* sequences. Twenty-two VNTR types, including all those that included two or more different patients, were sequenced (Table 3). The 3' *hsp65* codes (sequevars) identified included codes 1, 2, 3, 4, 9, and 15. All except three patient isolates with the same VNTR type belonged to the same 3' *hsp65* codes (sequevars) (Table 3); this included VNTR types 40 and 51, which were two of the three VNTR types with the Mav-F ITS sequence. The isolates containing the Mav-F ITS sequence were the only isolates studied that belonged to 3' *hsp65* sequence code 15.

Comparison of VNTR copy numbers with isolates from Japan. A comparison of the number of VNTR types with the same tandem-repeat copy numbers for each of the six MATR-VNTR loci used to separate the 52 VNTR types in the current U.S. study

TABLE 3 3' *hsp65* sequevars in 22 *M. avium* VNTR types or ITS subtypes, of which 20 were VNTR types with two or more patients (including outbreak isolates)

No. ^a	VNTR type or subtype ^b	No. of patients	Code no.	Associated with outbreak	Sequence type
1	3	6	1	?	
	3	1	2	—	
2	4	3	1	—	
3	10	4	1	—	
4	14a	10	1	+	
5	18	2	NA	—	
6	19	5	3	—	
7	21	2	NA	—	
8	30	4	9	—	
9	31	2	NA	—	
10	31a	14	2	+	
11	33	6	1	—	
12	36	5	1	+	
13	37	7	2	+	
14	37a	4	2	+	
	37a	1	9	—	
	37a	1	1	—	
15	38	2	NA	—	
16	40	2	2	—	Mav-F
17	42	1	2	—	
18	44	4	1	—	
19	46	2	1	—	
20	50	1	15	+	Mav-F
21	51	11	15	+	Mav-F
22	52	6	NA	+	

^a Arbitrary numerical identifier for type.

^b Only two VNTR types or subtypes consisted of more than one 3' *hsp65* sequevar (i.e., code numbers), and 6/8 types with five or more patients were part of a known cluster or outbreak. The total includes 20 VNTR types with two or more patient isolates and 2 with single isolates. NA, not available.

FIG 3 PFGE of XbaI digests of VNTR type 51 (*Mav-F* ITS sequence) isolates from five different patients. Lane 1, MA-5327; lane 2, MA-5308; lane 3, MA-4925; lane 4, MA-5295; lane 5, MA-5511; lane 6, ladder. The isolates in lanes 1 and 2 were from patients from different cities, and those in lanes 3 to 5 were from patients from the same city (different from the first two cities). The isolates in lanes 1 and 4 were indistinguishable, those in lanes 2 and 3 were closely related, and that in lane 5 was possibly related (4). The first four isolates and potentially all five isolates were considered clonal. All five isolates belonged to the relatively unusual 3' *hsp65* sequence code 15 (2, 26). XbaI and a 1% agarose gel were used.

and a prior study of 70 VNTR types among respiratory isolates from Japan (9) is shown in Table 4. *M. avium* subsp. *hominissuis* reference strain MAC 104 was included in both studies and showed the same tandem-repeat copy numbers for the same loci in both. These six loci showed the greatest diversity of all among the isolates from Japan, but only three of the loci (MATR-2, MATR-3, and MATR-7) had comparable diversity among the U.S. isolates (Table 4). Overall, the ranges of copy numbers for each locus were similar but the distributions were very different. These findings suggest many of the VNTR types differ between the two geographic areas.

DISCUSSION

We studied respiratory tract isolates and household water (bio-film) isolates from patients with nodular bronchiectatic MAC lung disease. Most isolates were recovered in the UTHSCT mycobacterial laboratory, and most patients lived within a 200-mile radius in northeast Texas. Isolates also included patient isolates and household water isolates from a Philadelphia suburb (Lankenau Medical Center) (20) and a small number of isolates from household water studies around the United States (5). We identi-

fied 49 VNTR types and eight subtypes (same VNTR number but identified with a or a b [e.g., 14a] to show a difference in the ITS sequence) of *M. avium* subsp. *hominissuis* based on the combination of the seven loci, including reference strains. Six VNTR types or subtypes were found only in an environmental source, 38 were identified only in a respiratory source, and 13 were identified in both sources. Eleven of 50 respiratory types (22%) contained isolates from three or more patients, while 33 types (66%) included only a single patient with that combination of seven VNTR patterns. The largest number of patients with a specific VNTR type, not associated with geographic clustering, was six patients.

The tandem-repeat loci in the current study were chosen from among 16 MATR-VNTR loci and four MIRU-VNTR loci (8, 9). We chose the MATR primers with the greatest allelic diversity (diversity values of ≥ 0.480) among respiratory isolates from Japan. It seems likely that some of the five larger current clusters (not clustered geographically) would have been separated into more VNTR types with the use of more loci (e.g., isolates from Japan are currently grouped using all 16 MATR loci) (9). For comparisons of isolates from the same patients or identification of

FIG 4 PFGE of XbaI digests of five VNTR type 10 isolates from four different patients, showing that some VNTR types did not consist of patient isolates with indistinguishable or closely related PFGE profiles. Lanes 1 and 9, linear standards; lane 2, strain MAC 104; lane 3, ATCC 700898 (MAC 102); lane 6, strain 07-6464; lanes 4 and 5, two isolates from the same patient, obtained 6 months apart; lane 7, ATCC 25291^T (control); lane 8, ATCC 33712 (control). The isolates in lanes 2 and 3 were indistinguishable, while the isolates in lanes 4 and 5 were considered indistinguishable. The isolates in lanes 2, 5, and 6 were considered possibly related. The control strains in lanes 7 and 8 were unrelated. The type 10 isolates all belonged to 3' *hsp65* sequevar code 1 (2).

geographic clusters, the current number of loci is likely adequate, less expensive, easier, and less time-consuming. For population studies, larger numbers of loci may be needed. Interestingly, all of the isolates in each of the larger clusters belonged to the same 3' *hsp65* code (sequevar). The six MATR loci chosen because of high allelic diversity in Japan were not as diverse among the U.S. isolates (especially MATR-1 and MATR-13) (Table 3), suggesting that an optimization study of different loci, similar to studies performed with *Mycobacterium tuberculosis*, would be needed for U.S. isolates of *M. avium* (17).

One of the VNTR types with multiple (four) patients (type 10) included reference strains ATCC 700898 (i.e., MAC 101) and MAC 104, both recovered in 1983 from the blood of AIDS patients (30). The isolates were indistinguishable by PFGE in the current study and were included in a study by Horan et al. that identified 10 patients, from five clinical sites in the western United States (30), with strains that were genotypically identical to MAC 104. Those authors utilized large sequence polymorphism typing as well as rep-PCR. That study supports the concept that some genotypes of *M. avium* are widespread geographically, as noted in the current study (30).

Tandem repeats rarely occur in exact multiples, and most VNTR loci contain complete as well as partial copies. Options for determining the numbers of tandem repeats include rounding up or down to a whole number or doing both, depending on the size of the partial copy (e.g., rounding down if the partial copy is <0.5 and rounding up if it is ≥0.5). We elected to report only the numbers of complete copies. This resulted in difficulties because with MATR-13 we encountered amplicons with 0.9 copies and 0

TABLE 4 Allelic diversity among 51 VNTR types for respiratory isolates from the United States and 70 previously published VNTR types for respiratory isolates from Japan (9)

VNTR locus ^a	No. of isolates with complete tandem-repeat no. of:									Allelic diversity
	0	1	2	3	4	5	6	7	NP	
MATR-1										
United States	0	3	45 ^b	0	1	1	0	0	1	0.217
Japan	1	30	38 ^b	0	0	1	0	0		0.514
MATR-2										
United States	17	3	20 ^b	11	0	0	0	0	0	0.685
Japan	29	34	5 ^b	2	0	0	0	0	0	0.581
MATR-3										
United States	0	2	7	24	5	13 ^b	0	0	0	0.684
Japan	0	42	10	1	3	14 ^b	0	0	0	0.581
MATR-7										
United States	0	4	4	30 ^b	10	2	0	0	1	0.601
Japan	0	20	27	3 ^b	0	5	14	1	0	0.718
MATR-13										
United States	0	7	44 ^b	0	0	0	0	0	0	0.237
Japan	38	3	29 ^b	0	0	0	0	0	0	0.525
MATR-14										
United States	0	0	39	6	6 ^b	0	0	0	0	0.388
Japan	0	1	45	22	2 ^b	0	0	0	0	0.480

^a Because the numbers of tandem repeats for MATR-13 and MATR-14 were rounded up in the study from Japan (e.g., 1.9 and 3.7 were reported as 2 and 4 copy numbers, respectively), the U.S. isolates were also rounded for those two loci, for comparison. These six loci had the greatest diversity among MATR loci studied among the isolates from Japan, but only three of the loci (MATR-2, MATR-3, and MATR-7) had comparable diversity among the U.S. isolates (9).

^b Copy number for *M. avium* subsp. *hominissuis* strain MAC 104.

copies, both technically meeting the definition of 0 copies but differing in amplicon size. We elected to term the 0.9 copy as 0+ and the 0 copy (i.e., the amplicon includes only the flanking regions) as 0. This dilemma was not seen with any of the other loci. No consensus definition of copy numbers has been agreed upon for nontuberculous mycobacteria.

The majority of VNTR types had the Mav-B and Mav-A ITS sequences (which differ by a single base pair), which has been noted in other studies (1, 24, 28). For multiple isolates of the same VNTR type from the same patient, only one was sequenced; the results for the rest were presumed.

Three of 49 VNTR types (6.1%) of *M. avium* subsp. *hominissuis*, which included 13 patients, had the Mav-F ITS sequence. This sequevar of ITS was first noted in 2002 in a study by Mijs et al. of the *M. avium* complex in the Netherlands and, to our knowledge, has appeared only rarely in subsequent MAC studies in Europe and the United States (1, 2, 24). All three VNTR types with the Mav-F sequence exhibited tandem-repeat copy numbers for one or more loci seen infrequently among the other VNTR types (e.g., only 6 of the 52 VNTR types exhibited two tandem-repeat copies with MATR-7, including all VNTR types with the Mav-F ITS sequence). This was similar to the difference noted in a recent VNTR study of *M. intracellulare* in a single VNTR type that was found to be a different but closely related species (*M. chimera*) (R. J. Wallace, Jr., unpublished observations) (12). The two species are also closely related in sequencing of the 16S rRNA gene (with a single base pair difference in the complete sequences) and the *hsp65* gene but differ in the ITS and the *rpoB* gene (12, 31).

The unique character of *M. avium* VNTR types containing a Mav-F ITS sequence was underscored by the recognition that the isolates of these three VNTR types were all PCR positive using IS901 primers. Only 4 of 49 VNTR types (8.2%) and 0 of 8 subtypes were IS901 PCR positive. Upon sequencing, the insertion element was found to have 100% identity to ISMav6, which was first reported in 2009 by Ichikawa et al. for Japanese isolates of *M. avium* subsp. *hominissuis* and which has 95% sequence identity to IS901 (29). ISMav6 was found in 61% of 146 respiratory isolates, 87% of bathroom water isolates, and 0.0% of porcine isolates from Japan. No ITS sequencing of the isolates was performed (32). The importance of sequencing of the IS901 PCR amplicon was emphasized by a 2014 study by Tran and Han of 257 clinical strains of *M. avium* (33). Those authors found that 19/257 U.S. isolates (7.4%) of *M. avium* produced amplicons with IS901 primers, and they presumed the isolates were *M. avium* subsp. *avium*, without sequencing. We suspect those isolates carried ISMav6 and not IS901 (33).

The dramatic difference in the distributions of 3' *hsp65* sequevars (codes) (2), the distributions of different VNTR copy numbers for the same allelic site, and the low prevalence of ISMav6 in the southwestern and northeastern United States, compared to Japan, suggests that *M. avium* subsp. *hominissuis* isolates from different geographic areas may vary widely in their genetic composition. It also points out the potential for misleading findings if IS901 PCR results, without sequencing confirmation, are used for subspecies differentiation.

An interesting finding in the current study was that 8/13 (61.5%) of the multiple patients (five or more) with the same *M. avium* VNTR type were geographically clustered. Preliminary studies of one geographic area showed the same *M. avium* VNTR

types in a local commercial water supplier and patient households (20).

There are limitations to this study. Single sputum samples with a unique VNTR type could not be validated by other methods. PFGE is so technically difficult, expensive, and time-consuming that its use for VNTR types with multiple isolates was limited (with the exception of support for the sequencing studies, this study was funded only by patient donations and institutional support). Thus, much of the testing resulted from clinical needs rather than testing of consecutive isolates. The use of PFGE for some isolates but not all might have resulted in a selection bias. Because VNTR analysis is so consistent, fast, and relatively inexpensive and does not require sequencing, it has replaced PFGE for comparisons of *M. avium* strains in our laboratory.

The creation of an international database is within reach for *M. avium* with the availability of VNTR analysis. The current VNTR method used the loci with the greatest allelic diversity and allowed for individual strain differentiation in the same patients and recognition of geographic clustering but was simple enough to be used clinically. The number of VNTR loci needed for optimal strain differentiation will need to be determined for larger population studies. The current technique does not require sequencing, with a few exceptions, but only PCR (six pairs or sets of isolates had the same VNTR copy numbers but differed in their ITS sequences). The finding that different patient isolates with the same VNTR type are mostly clonal by PFGE and 3' *hsp65* sequencing demonstrates that clonal groups do exist within *M. avium*. The presence of a typing system that identifies specific clones of *M. avium* should allow for comparisons with isolates from other diseases (e.g., disseminated disease or cavitary lung disease) and other geographic sources and thus the beginning of a risk analysis for *M. avium* in drinking water.

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ADDENDUM IN PROOF

Since acceptance of this article, three new studies have been published describing *M. avium* subsp. *hominissuis* isolates harboring the ISMav6 element. A study by K. Ichikawa et al. (Infect Genet Evol 36:250–255, 2015, <http://dx.doi.org/10.1016/j.meegid.2015.09.029>) showed the high prevalence of ISMav6 from South Korea and Japan, but low percentages in select isolates from the Netherlands, Germany, and the United States. In C. Vluggen et al. (Euro Surveill. 21(3):pii=30111, 2016, <http://dx.doi.org/10.2807/1560-7917.ES.2016.21.3.30111>), two clinical isolates from Belgium were described as IS1245 negative and false IS901 positive (confirmed as ISMav6 by sequencing). In South Korea, S.-Y. Kim et al. (PLoS One 11(2):e0148917, 2016, <http://dx.doi.org/10.1371/journal.pone.0148917>) found that 61% of isolates contained the ISMav6 element (consistent with findings from Japan) and that these correlated with a higher resistance to moxifloxacin. ISMav6-positive isolates from all three studies harbored *hsp65* codes 1, 2, 15, and 16.

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