Genetic relatedness of *Mycobacterium avium-intracellulare* complex isolates from patients with pulmonary MAC disease and their residential soils

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Abstract

Mycobacterium avium-intracellulare complex (MAC) strains were recovered from 48.9% of residential soil samples (agricultural farms (n = 7), residential yards (n = 79), and planting pots (n = 49)) of 100 pulmonary MAC patients and 35 non-infected control patients. The frequency of MAC recovery did not differ among soil types or among patients regardless of the presence of pulmonary MAC disease, infecting MAC species or period of soil exposure. Variable numbers of tandem repeats (VNTR) analysis for MAC clinical and soil isolates revealed 78 different patterns in 47 *M. avium* clinical isolates and 41 soil isolates, and 53 different patterns in 18 *M. intracellulare* clinical isolates and 37 soil isolates. Six clinical and corresponding soil isolate pairs with an identical VNTR genotype were from case patients with high soil exposure (≥2 h per week, 37.5% (6/16) with high exposure compared with 0.0% (0/19) with low or no exposure, $p < 0.01$), suggesting that residential soils are a likely source of pulmonary MAC infection.

Keywords: environmental exposure, genotype, *Mycobacterium avium-intracellulare* complex, soil, variable numbers of tandem repeats
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Introduction

Although *Mycobacterium avium-intracellulare* complex (MAC) is widely distributed in water and soil [1], the routes of transmission to humans are still unclear. MAC biofilms are common in drinking water systems and showerheads [2–8], and the genetic relatedness of clinical isolates from MAC patients with water isolates have been reported [4–8], supporting the hypothesis that engineered water systems are a source of MAC infections.

We have reported that patients with pulmonary MAC disease have significantly more soil exposure than non-infected control patients [9]. Furthermore, occupational exposure to agricultural soil is associated with MAC infection, as determined by *M. avium* sensitin skin test [10]. MAC is the most abundant mycobacterium in potting soils [11]. One previous study reported the identification of a pair of closely related *M. avium* clinical and potting soil aerosol isolates using pulsed-field gel electrophoresis [11]. Another study identified a pair of *M. avium* clinical and pot soil isolates with identical genotypes using restriction fragment length polymorphism [12]. However, those data are insufficient to establish soil as a source of MAC infections.

Therefore, we measured the prevalence of MAC strains in soils from the residences of patients with or without pulmonary MAC disease and compared genotypes of MAC clinical and soil isolates to investigate whether residential soils are a source of MAC infections.

Materials and Methods

Study population

We prospectively recruited 100 pulmonary MAC patients who met the American Thoracic Society guidelines for

diagnosis of MAC infection [13]. The controls were 35 patients with bronchiectatic findings on radiographic examination, but no evidence of MAC infection, between January 2007 and September 2011 at Kyoto University Hospital. All participants completed a standardized questionnaire that included questions about experience of farming and gardening or any activities involving soil exposure, and the frequency and period of time in which the participants performed these activities. The protocol was approved by the ethics review board of Kyoto University. All participants signed a written consent form.

Clinical MAC isolates

Forty-seven *M. avium* and 18 *M. intracellulare* clinical isolates from sputum were obtained from 100 pulmonary MAC patients. Identification of *M. avium* and *M. intracellular* strains was performed using the COBAS TaqMan MAI test (Roche Diagnostics, Basel, Switzerland). All isolates were subcultured on Middlebrook 7H11 agar plates (BD). For variable numbers of tandem repeats (VNTR) analysis, a single MAC colony was used for extraction of DNA using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA).

Soil sampling and isolation of MAC strains

The individual patients collected approximately 5 g of soil in a sterile plastic tube from their agricultural farms, residential yards and planting pots to which they had been exposed, and mailed the soils to Kyoto University Hospital. The choice of soils and depth of soil were left to the individual patients. Soil samples were processed as described by Parashar *et al.* [14].The final suspension was inoculated into an MGIT (BD) with 0.8 mL MGIT PANTA Antibiotic Mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin, BD) and MGIT OADC Enrichment (oleic acid, albumin, dextrose and catalase, BD). After cultivation using the BACTEC MGIT 960 system, positive cultures were subjected to PCR analysis for identification of *M. avium* or *M. intracellulare*, as reported previously [15]. PCR-positive cultures were subcultured on Middlebrook 7H11 agar plates to obtain single colonies. Two colonies were isolated for extraction of DNA to identify Mycobacterial species using PCR [15] and to perform VNTR analysis.

VNTR analysis

Primer sets for 15 *M. avium* VNTR loci (MATR-VNTR) and 16 *M. intracellulare* VNTR loci (MITR-VNTR) were used in VNTR analysis, as described previously [16,17]. When the VNTR profiles of two colonies from one soil sample were different, both VNTR genotypes were used for analysis. *M. avium* or *M. intracellulare* clinical isolates from case patients, *M. avium* or *M. intracellulare* soil isolates from the residences of case patients and *M. avium* or *M. intracellulare* soil isolates from the residences of controls were designated as MAnP or MInP, MAnSca or MInSca and MAnSco or MInSco, respectively. The Hunter–Gaston Discriminatory Index (HGDI) was calculated according to a previous report [18].

Statistical analysis

JMP version 9.0.0 (SAS Institute, Cary, NC, USA) was used for all statistical analysis. Group comparisons were made using the chi-square test and Fisher's exact test for categorical variables; p-values <0.05 were considered statistically significant.

Results

Mycobacterium strains and clinical features

Of 100 case patients, 67 patients (67.0%) had an *M. avium* infection and 26 patients (26.0%) had an *M. intracellulare* infection. Seven patients were infected with both *M. avium* and *M. intracellulare*. Patients with pulmonary MAC disease experienced significantly more soil exposure (≥2 h per week) than controls, as previously reported [9]. Thirty-four case patients had 10.3 years (3.0–50.0 year) of high soil exposure (≥2 h per week), of whom 32 patients (94.1%) had experienced soil exposure before diagnosis of pulmonary MAC disease. Twenty-two case patients had 9.8 years (2.0–40.0 year) of low soil exposure (<2 h per week), of whom 19 patients (86.4%) had experienced soil exposure before diagnosis of pulmonary MAC disease. Forty-four case patients (44%) had neither experience of farming or gardening.

Recovery of MAC strains from soil samples

In total, 135 soil samples were collected from seven agricultural farms, 79 residential yards and 49 planting pots. MAC, *M. avium* and *M. intracellulare* strains, respectively, were recovered from 66 (48.9%), 38 (28.1%) and 36 (26.6%) of 135 soil samples. *M. avium* and *M. intracellulare* were detected at similar frequencies in soil samples of both case patients and controls (Table 1). MAC strains were recovered from five of seven (71.4%) agricultural farm samples, 40 of 79 (50.6%) residential yard samples, and 21 of 49 (42.9%) planting pot samples, without significant differences in their frequencies in these samples (p 0.33). They were also detected at similar frequencies in the soil samples of case patients regardless of the infecting mycobacterial species (Table 2) and intensity of soil exposure (Table 3).

TABLE 1. Recovery of MAC strains from soil samples from the residences of patients with and without pulmonary MAC disease

Species isolated from soil	Case				Control				p value
	Farm (*n* = 6)	Yard (*n* = 58)	Pot (*n* = 36)	Total (*n* = 100)	Farm (*n* = 1)	Yard (*n* = 21)	Pot (*n* = 13)	Total (*n* = 35)	
MAC	5 (83.3)	29 (50.0)[a]	16 (44.4)[a]	50 (50.0)	0 (0.0)	11 (52.3)[b]	5 (38.5)[b]	16 (45.7)	0.66
M. avium	3 (50.0)[c]	16 (27.6)[c]	9 (24.3)	28 (28.0)	0 (0.0)	6 (28.6)	4 (30.8)	10 (28.6)	0.71
M. intracellulare	2 (33.3)[d]	17 (29.3)	8 (22.2)	27 (27.0)	0 (0.0)	7 (33.3)	2 (15.4)	9 (25.7)	0.54

Data show number (%) of samples positive for MAC strains. p values were calculated between total isolates from case and control patients.
[a]Both *M. avium* and *M. intracellulare* strains were detected in four residential yard soil samples and one planting pot soil sample from case patients.
[b]Both *M. avium* and *M. intracellulare* strains were detected in two residential yard soil samples and one pot soil sample from control patients.
[c]One identical pair of MATR-VNTR genotypes was detected in one agricultural farm soil sample and four identical pairs of MATR-VNTR genotypes were detected in residential yard soil samples.
[d]One identical pair of MITR-VNTR genotypes was detected in one agricultural farm soil sample.

TABLE 2. Recovery of MAC strains from soil samples from the residences of pulmonary MAC patients

Species isolated from soil	Mycobacterial species isolated from pulmonary MAC patients			p value
	M. avium (*n* = 67)	*M. intracellulare* (*n* = 26)	*M. avium* + *M. intracellulare* (*n* = 7)	
MAC	36 (53.7)[a]	10 (38.5)[a]	4 (57.1)	0.38
M. avium	23 (34.3)	4 (15.4)	1 (14.3)	0.19
M. intracellulare	17 (25.3)	7 (26.9)	3 (42.9)	0.36

Data show number (%) of samples positive for MAC strains.
[a]Both *M. avium* and *M. intracellulare* were detected in four soil samples from the residences of patients infected with *M. avium* and in one soil sample from the residence of a patient infected with *M. intracellulare*.

TABLE 3. Recovery of MAC strains from soil samples in pulmonary MAC patients stratified by intensity of soil exposure

Species isolated from soil	Intensity of soil exposure			p value
	High (*n* = 34)	Low (*n* = 22)	No (*n* = 44)	
MAC	20 (58.8)[a]	10 (45.5)	20 (45.5)[a]	0.45
M. avium	11 (32.4)[b]	7 (31.8)	10 (22.7)	0.29
M. intracellulare	11 (32.4)[b]	3 (13.6)	13 (29.5)	0.52

Data show number (%) of samples positive for MAC strains. High and low soil exposure were defined as ≥2 h per week and <2 h per week, respectively.
[a]Both *M. avium* and *M. intracellulare* strains were detected in two soil samples from the residences of patients with high exposure and three soil samples from the residences of patients without exposure.
[b]Five identical pairs of MATR-VNTR genotypes and one identical pair of MITR-VNTR genotypes were detected in patients with high exposure.

VNTR genotyping and phylogenetic analysis of MAC

Forty-four MATR-VNTR patterns were identified in the 47 *M. avium* clinical isolates examined. Three MATR-VNTR clusters were found in six case patients, (MA29P and MA84P, MA76P and MA92P, MA86P and MA107P). *M. avium* strains were recovered from soil samples for 19 case patients and eight controls. For eight case patients and six controls, two strains with different MATR-VNTR allele profiles were identified from the same soil sample. The *M. avium* soil isolates were diverse, with 26 MATR-VNTR patterns for 27 soil isolates from case patients, and each of 14 soil isolates from controls. Despite this diversity, five pairs of clinical isolates and corresponding soil isolates showed identical MATR-VNTR patterns (MA26P and MA26Sca, MA62P and MA62-2Sca, MA73P and MA73Sca, MA76P and MA76-2Sca, MA90P and MA90-2Sca; 5/45 (11.1%) of *M. avium* clinical isolates). Three clinical isolates had identical MATR-VNTR patterns to unrelated soil isolates (MA49P, MA76-1Sca and MA127-1Sca, MA92P and MA76-2Sca, MA123P and MA64-1Sco). In total, 78 different MATR-VNTR allele profiles were found in 88 *M. avium* isolates (20 clustered isolates and 68 unique isolates; HGDI, 0.997) and did not include distinct major clusters for clinical or soil isolates (Figure S1). This diversity makes it unlikely that a given genotype found in residential soil is more likely to be associated with disease.

All 18 *M. intracellulare* clinical isolates had different MITR-VNTR patterns. *M. intracellulare* soil isolates were recovered from the residences of 21 case patients and six controls. Two strains from the same soil sample with different MITR-VNTR profiles were identified in five case patients and five controls. Twenty-five MITR-VNTR patterns for 26 soil isolates from case patients and each of 11 soil isolates from controls were identified. One paired clinical isolate and soil isolate had an identical MITR-VNTR pattern (MI132P and MI132-1Sca; 1/18 (5.6%) of *M. intracellulare* clinical isolates). Two soil isolates from different residences had an identical MITR-VNTR pattern (MI132-2Sca and MI135Sca). In total, 53 different MITR-VNTR allele profiles were found in 55 *M. intracellulare* isolates (four clustered isolates and 51 unique isolates; HGDI, 0.999) and no distinct major clusters were observed for clinical or soil isolates (Figure S2). Both *M. avium* and *M. intracellulare* soil isolates (MA34Sca and MI34Sca)

were recovered from the residence of one case patient infected with *M. avium*.

Among the 35 case patients for whom we had both clinical and soil isolates, the 28 patients infected with *M. avium* had 23 soil isolates of *M. avium* and 13 of *M. intracellulare*, and the seven patients infected with *M. intracellulare* had two soil isolates of *M. avium* and 10 of *M. intracellulare*. In comparing VNTR genotypes of these 28 *M. avium* and seven *M. intracellulare* clinical isolates and 25 *M. avium* and 23 *M. intracellulare* soil isolates, we observed six clinical and soil isolate pairs with identical VNTR patterns in six of 16 patients with high soil exposure. These six soil isolates were from two agricultural farm samples and four residential yard samples. No identical VNTR patterns were detected in 19 patients with low or no soil exposure (37.5% vs. 0.0%, $p < 0.01$).

Discussion

In this study, we identified six MAC clinical and corresponding soil isolate pairs with identical VNTR patterns among patients with high soil exposure. The participants' soil exposures were due to various activities such as digging or carrying soils, mowing grass, planting flowers, and exposure to soil dusts when farming or gardening. De Groot *et al.* [11] reported that MAC was recovered from aerosols produced by pouring soils. Therefore, we considered that aerosols were likely to occur and MAC could be transmitted from the residential soils during these activities. However, we could not discriminate them on the quantitative basis of exposure to soil. Instead, we estimated the intensity of soil exposure on the basis of a period of time when participants dealt with any activities related to farming or gardening. Because the six identical pairs of clinical and soil isolates were found in patients with a longer duration of soil exposure (≥2 h per week) alone, period of time of exposure is probably a valid way to estimate the intensity of soil exposure.

Our study has some limitations. First, we did not save MAC clinical isolates from 35 case patients and only 65 isolates were available for the VNTR analysis. However, the results remained significant among these 65 patients (data not shown). Second, we attempted to culture MAC from only a single soil sample from each patient's home, and sampling area and depth of soil were non-uniform because the patients provided the samples. Third, pulmonary MAC disease is chronic, usually present for years before diagnosis, with prior infection that may have been present for many years before overt disease or diagnosis. Although case patients with soil exposure mostly had been exposed to soil before diagnosis of pulmonary MAC disease, the timing of MAC infection and collection of soil samples were not necessarily coincident. Lastly, patients probably had been exposed to many environments over the long time periods of interest, all of which would need to be identified and sampled in a comprehensive environmental study. Therefore, our study may be limited by presenting an incomplete picture of the sources of infection. However, our microbiological findings support the idea that high soil exposure in farming or gardening is likely to increase the risk of transmission of MAC from environmental soils.

In summary, we demonstrated that MAC strains can be isolated without significant difference from various types of residential soil samples and from patients regardless of the presence of pulmonary MAC disease, infecting MAC species or intensity of soil exposure. We found six pairs of clinical isolates and corresponding soil isolates with identical VNTR profiles among patients with high soil exposure. These results suggest that residential soils are likely to be a source of MAC infections.

Transparency Declaration

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree and allele profiles for *M. avium* constructed from MATR-VNTR genotypes.

Figure S2. Phylogenetic tree and allele profiles for *M. intracellulare* constructed from MITR-VNTR genotypes.

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