

Original Article

Mycobacterium avium Complex Organisms Predominantly Colonize in the Bathtub Inlets of Patients' Bathrooms

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SUMMARY: Medical treatment of pulmonary *Mycobacterium avium* complex (MAC) disease does not always provide curative effects and is frequently hampered by recurrence. This suggests the presence of a reservoir for MAC in the environment surrounding patients. We previously reported the recovery of MAC isolates from the residential bathrooms of outpatients. In the present study, to ascertain the colonizing sites and the possibility of an MAC reservoir in the bathrooms of patients, we tested the recovery and the genetic diversity of MAC isolates from 6 sites of specimens, including 2 additional sampling sites, inside the showerhead and the bathtub inlet, in the residential bathrooms of patients with pulmonary MAC disease. MAC isolates were recovered from 15 out of the 29 bathrooms (52%), including specimens from 14 bathtub inlets and 3 showerheads. Nearly half of these bathrooms (7/15) contained MAC strains that were identical or similar to their respective clinical isolates. Additionally, in 5 out of 15 bathrooms, polyclonal colonization was revealed by pulsed-field gel electrophoresis. The results imply that colonization of MAC organisms in the bathrooms of MAC patients occurs predominantly in the bathtub inlets, and there is thus a risk of infection and/or reinfection for patients via use of the bathtub and other sites in the bathroom.

INTRODUCTION

The incidence of pulmonary *Mycobacterium avium* complex (MAC) disease has increased over the past several decades (1-3). MAC disease occasionally leads to death even in patients without a history of lung diseases or immunodeficiency (2,4-6) and is characterized by multiple infection with genetically different strains (7,8). Although macrolide-based regimens are effective against MAC, the cure rate with these drugs is still low (56%) because patients drop out due to drug side effects, consecutive positive culture, and recurrence (2). Kobashi and Matsushima (9) reported that 41 of their 71 patients (58%) showed negative sputum cultures after successful completion of multidrug chemotherapy including a macrolide, and 16 of these 41 patients (39%) experienced recurrence. The genotyping research has demonstrated that patients with nodular bronchiectasis have multiple and/or repeated infections, and that frequent recurrence is due to reinfection with a genetically different strain or relapse with the original strain (7,8). To prevent recurrence of MAC disease, long-term treatment such as chemotherapy for 2 years (10) or 12-month treatment leading to culture-negative sputum (3) is recommended as a reasonable endpoint. The frequent recurrence and multiple infections suggest that polyclonal MAC colonization is likely to occur in the home environ-

ment surrounding patients with pulmonary MAC disease.

MAC is widely distributed both in the natural and living environment, and these environmental organisms are thought to be a source of infections (2,5). Drinking water systems are a possible source of disseminated MAC infection (11-16), which has been detected in biofilms of water distribution systems (13). In a recent study, we recovered MAC isolates from residential bathrooms but not from other sites within the residence (17). We also found that the appearance ratio in the bathrooms of patients with pulmonary MAC disease was significantly higher than that in the bathrooms of healthy volunteers ($P = 0.01$) (17). In the present study, therefore, we tried to determine the colonizing sites and to investigate the possibility of a reservoir of MAC organisms in the bathrooms of patients with MAC disease. We sampled specimens from 6 sites in the residential bathrooms of MAC-positive patients, including 2 sampling sites that were not included in our previous study: inside the showerhead and the bathtub inlet. A traditional Japanese bathtub has a water inlet below the water level, and we reasoned that biofilm produced by MAC organisms could develop inside this inlet, as well as within the showerheads. Finally, we examined the genetic diversity of the MAC isolates from specimens in the patients' bathrooms and sputa.

MATERIALS AND METHODS

Subjects and collection of samples: We collected 6 samples from the residential bathroom of each patient: 2 water samples (shower water and used bathtub water, 200 ml each), 3 scale samples (on the surface of the showerheads, inside

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the showerhead, and inside the bathtub inlet), and 1 sample from the slime on the bathroom drain. Participants were outpatients diagnosed with pulmonary MAC disease ($n = 29$). All patients lived with their family and shared their bathroom with at least one other family member. Patients were diagnosed with pulmonary MAC disease according to the American Thoracic Society 1997 diagnostic criteria (18). Informed consent was obtained from all participants before the collection of samples. This study was approved by the Toneyama National Hospital institutional review board and complies with international guidelines for studies involving human subjects. Information regarding the bathrooms was collected by a questionnaire survey.

Culture of residential samples: The collected residential samples were cultured as described previously (17). In brief, water samples were centrifuged at $11,800 \times g$ for 30 min at 4°C , and pellets from the shower water were suspended in 0.5 ml of phosphate buffer (PB) at pH 6.8, 200 μl of which was inoculated onto a Middlebrook 7H11-OADC agar plate containing the antibiotic mixture PANTA (7H11 PANTA plate). The pellet from the used bathtub water was treated with 3 ml of 2% sodium hydroxide solution for 10 min. After adding 6 ml of PB to this alkali-treated sample, it was centrifuged at $2,270 \times g$ for 15 min, and resuspended in 0.5 ml of PB. The collected samples on the swabs were preincubated for 3 h at 25°C in a tryptic soy broth followed by alkali treatment, and the pellets were suspended in 1 ml of PB solution. One hundred to 200 μl of these suspensions were inoculated onto 7H11 PANTA plates at 37°C for 3 weeks. Growing colonies were examined microscopically, followed by Ziehl-Neelsen staining. The isolated acid-fast bacterial species were identified by the PCR method (19).

Genotypic analysis: Genotypic analyses were carried out using pulsed-field gel electrophoresis (PFGE) as described previously (17). PFGE genotypic patterns were defined as follows: identical, when one case was not distinguishable from another; related, when the genotypic pattern differed by only 1-3 bands; and unrelated, when the genotypic pattern differed by 4 or more bands.

Statistical analysis: The chi-square and Mann-Whitney U-test were used for analysis. Findings of $P < 0.05$ were considered statistically significant, while those of $P < 0.1$ were considered as evidence of a statistical tendency.

RESULTS

Colonization of MAC in residential bathrooms: MAC isolates were frequently recovered from the residential bathrooms of outpatients with pulmonary MAC disease (Table 1). Twenty-nine bathrooms of patients were inspected in

the present study, of which 15 and 1 were found to harbor *Mycobacterium avium* (52%) and *M. intracellulare* (3.4%), respectively. MAC isolates were recovered from 4 shower specimens in 3 bathrooms (3/29, 10%); the shower specimens were taken from the inside and surface of the showerheads, and from the shower water. MAC isolates were most frequently recovered from the scale of the bathtub inlets (14/25, 56%), though they were distributed throughout the bathroom (Table 1). In 7 specimens from bathtub inlets, more than 100 colonies of MAC were recovered from each primary isolation plate. The additional sampling sites were responsible for the increase in the recovery rate from 18% in our previous study (17) to 52% in the present study. In order to ascertain whether MAC continuously inhabits the inside of the showerheads and bathtub inlets, we took additional samples from these sites after an interval of 3 months in 2 bathrooms. MAC isolates were recovered from both sites (data not shown), indicating a long-lasting colonization of MAC at these sites.

Polyclonal MAC colonization: MAC isolates were recovered from more than 2 sampling sites in the bathrooms of 10 participants. Additionally, in some cases we obtained multiple isolates of MAC possessing a colony morphology different from that of the primary isolation plate. In order to clarify the genetic diversity of multiple MAC isolates from individual bathrooms, we analyzed the polymorphism of MAC isolates using PFGE (Fig. 1). In each of 5 bathrooms, we found multiple isolates that possessed different genotypic profiles. This demonstrated that polyclonal MAC colonization can occur within a single bathroom. In the case of patient #29 (P29), 5 MAC isolates were recovered from 3 different sampling sites, and they had 3 different PFGE profiles (Fig. 1). Interestingly, 3 MAC isolates from the bathtub inlet, each possessing a different colony morphology, also showed 3 different PFGE profiles. Similarly, unrelated genotypes of plural isolates recovered from 1 sampling site were observed in the cases of P8 and P17. These results indicate that polyclonal MAC strains are capable of growing together in the same location. In the case of P27, MAC isolates were recovered from 4 different sampling sites, and all of them had related PFGE profiles with 1-3 band differences (Fig. 1). Moreover, in the case of P9, 3 of 4 isolates which were recovered from 2 different sampling sites showed related PFGE profiles (Fig. 1). These related PFGE profiles reflect the genomic variation which may have occurred due to mutation of one of the strains.

Identical/related polymorphism between environmental and clinical isolates: To assess the relationship between the patient-derived clinical MAC isolates and the respective environmental isolates, we compared their polymorphism in

Table 1. Recovery of *M. avium* and *M. intracellulare* from residential bathrooms of outpatients with pulmonary MAC disease

	Sampling site						Total Sample (Residence)
	Surface of the shower head	Inside the shower head	Shower water	Bathtub inlet	Bathtub water	Drain	
No. of test samples	29	24	29	25	26	29	162 (29)
<i>M. avium</i>	1	2	1	14 ¹⁾	7	7 ²⁾	32 (15)
<i>M. intracellulare</i>	0	0	0	0	0	1	1 (1)

Data are represented as the number of samples from which *M. avium* or *M. intracellulare* were recovered. Numbers in parenthesis represent the number of residences.

¹⁾: Over 100 colonies of *M. avium* in a primary isolation plate were recovered from 7 of 14 culture-positive samples.

²⁾: Over 100 colonies of *M. avium* in a primary isolation plate were recovered from 2 of 7 culture-positive samples.

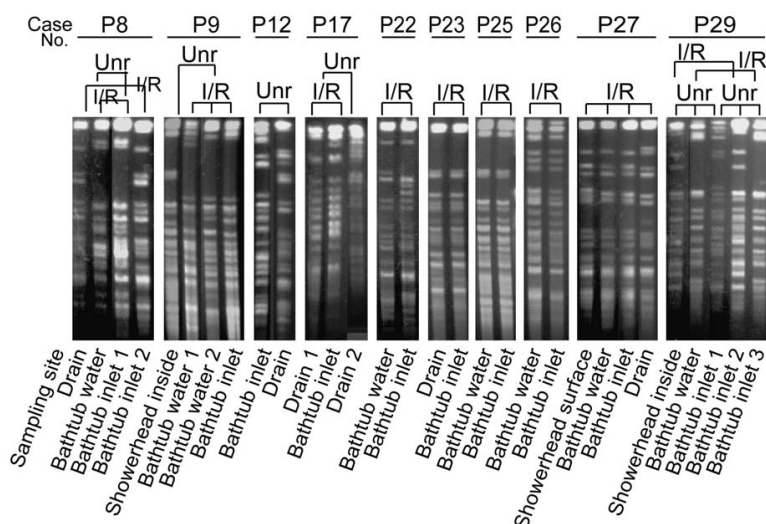


Fig. 1. Polyclonal colonization of *M. avium* complex (MAC) in residential bathrooms. Pulsed-field gel electrophoresis (PFGE) profiles of chromosomal *Xba*I digests of MAC isolates. Unr, unrelated profiles; I/R, identical or related profiles.

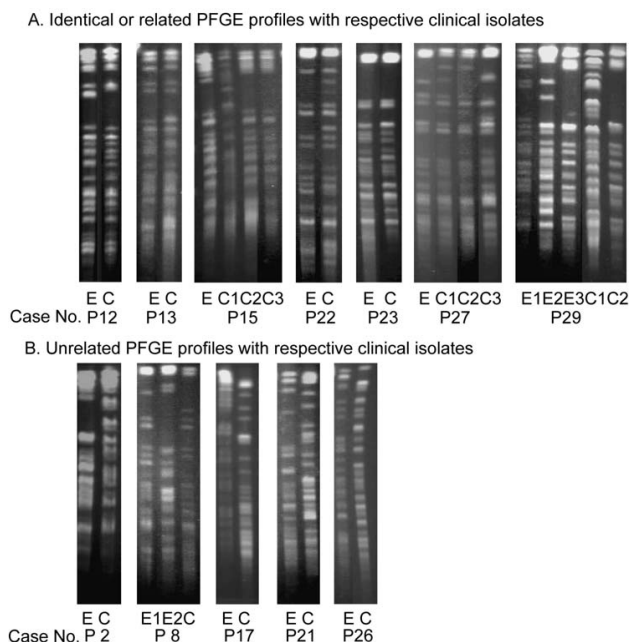


Fig. 2. Molecular typing of environmental and clinical *M. avium* complex (MAC) isolates. (A) Identical or related PFGE profiles of isolates from bathrooms with respective clinical isolates. E, environmental isolates; C, clinical isolates. C1 of P15, isolate from sputum 1 in December 2001; C2 of P15, isolate from sputum 2 in May 2003; C3 of P15, isolate from sputum 3 in January 2004; E of P27, isolate from bathtub inlet; C1 of P27, isolate from sputum 1 in December 2006; C2 of P27, isolate from sputum 2 in February 2007; C3 of P27, isolate from sputum 3 in July 2007; E1 of P29, isolates from bathtub inlet 1; E2 of P29, isolate from bathtub inlet 2; E3 of P29, isolate from bathtub inlet 3; C1 of P29, isolate from sputum 1 in June 2004; C2 of P29, isolate from sputum 2 in March 2006. (B) Unrelated PFGE profiles of isolates from bathrooms with respective clinical isolates. E1 of P8, isolate from drain; E2 of P8, isolate from bathtub inlet 1; E of P17, isolate from drain 2; E of P26, isolate from bathtub inlet.

individual cases. We recovered MAC in 15 cases, of which 11 and 1 cases were determined to contain *M. avium* isolates and *M. intracellulare* isolates, respectively, in both their environmental and clinical specimens. In the remaining 3 cases, *M. avium* isolates were recovered from the environment and *M. intracellulare* isolates were recovered from clinical specimens. Therefore, we compared the MAC genotypes in the

former 12 cases. In 7 of 15 cases (47%), MAC isolates from the patients' bathrooms and their respective sputa had identical or related molecular profiles by PFGE analysis (Fig. 2). In the other 5 cases, MAC isolates from the bathrooms possessed profiles different from those of their respective sputa (Fig. 2). These results are in agreement with our previous findings (17) that environmental isolates exhibit the identical polymorphism with their respective clinical isolates. The rate of identity obtained in the present study (47%, 7/15) is higher than that observed in the previous study (22%, 2/9) (17). We could compare the genetic diversity of retrospective sputum cultures in 3 cases. Genotypic analyses showed unrelated polymorphisms in 2 of these cases (Fig. 2, P15 and P29). The genotypes of the latent clinical strains were related to those of isolates from their respective bathrooms.

Bathtub types and bathroom maintenance: MAC bacilli appear to colonize in the bathroom preferentially over other household locations. However, no MAC isolates were recovered from half of the bathrooms examined in the present study. Therefore, to identify the risk factors for MAC colonization, we collected information regarding the type of bathtub and the method of maintaining the bathroom by a questionnaire survey. The characteristics of culture-negative versus culture-positive bathrooms are presented in Table 2. In the traditional Japanese bath, a bathtub inlet is installed inside the bathtub and below the water level. Some of the bathtub inlets are attached to the hot-water supply, while others are attached to the bath-boiler. MAC organisms are more likely to colonize in the bathtub inlet attached to the bath-boiler than in the inlet attached to the hot-water supply ($P < 0.01$, chi-square test). The bath-boiler can be classified into two types: a natural circulation type having two holes, an inlet and outlet, and a forced circulation type having one hole. In the present study, 8 participants used the natural circulation type of bath-boiler and all the bathtub inlets were found to retain MAC bacilli. In Japan, used bathtub water is sometimes reserved in the bathtub until the next day for washing clothes or to provide for a disaster such as an earthquake. MAC isolates were more frequently recovered from the samples taken from participants who were in a habit of reserving bathtub water; this relation reached the level of a tendency but was not statistically significant ($P = 0.09$, chi-square test). There was also a nonsignificant tendency for

Table 2. Participants' residential bathtub type and ventilation method of bathrooms

	Recovery of <i>M. avium</i> and <i>M. intracellulare</i>		
	Culture positive	Culture negative	Total
Type of bathtub			
Bathtub supplied with hot-water	3	10	13*
Bathtub attached to a bath-boiler			
Natural circulation type ¹⁾	8	0	8
Forced circulation type ²⁾	4	4	8
The time of draining off water from bathtub after bathing			
Shortly after bathing	2	6	8**
Next day	12	8	20
Unknown	1	0	1
Ventilation of a bathroom			
Bathroom dryer	0	3	3**
Ventilating fan			
Regular use	0	1	1
1 - 8 h after bathing	2	2	4
0,5 - 1 h after bathing	3	2	5
No bathroom dryer or no ventilating fan	10	6	16

Data are represented as the number of residences.

¹⁾: Natural circulation type of bath-boiler which has two holes in a bathtub.

²⁾: Forced circulation type of bath-boiler which has one hole in a bathtub.

*, $P < 0.01$. **, $P < 0.1$.

the use of a bathroom dryer or a ventilating fan to decrease the recovery rate of MAC ($P = 0.08$, Mann-Whitney's U test). Our results showed that the reservation of bathtub water and/or the continuance of high humidity in the bathroom appear to be conducive to MAC colonization.

DISCUSSION

The characteristics of pulmonary MAC disease, frequent recurrence and multiple infections, suggest that polyclonal MAC colonization is likely to occur in the home or hospital environment surrounding patients. Here, we demonstrated that polyclonal MAC organisms colonize predominantly inside the showerhead and the bathtub inlet of patients' bathrooms. In our previous study, MAC isolates were recovered from residential bathrooms but not from other sites within the residence (17). This uneven distribution and polyclonal colonization of MAC in the residential bathrooms suggested that MAC concentrates and colonizes in the bathroom preferentially. Furthermore, we found that MAC organisms colonized in the bathrooms for a long period of at least 3 months. These findings were in accord with a previous report that nontuberculous mycobacteria (including MAC) formed persistent colonies in a drinking water system (20). Thus MAC may also persistently colonize in bathtub inlets and the inside of the showerhead, where biofilm can be formed.

For 7 cases (47%, 7/15), the genotypes of the environmental isolates showed PFGE patterns related to their respective clinical isolates. Such a high rate of related polymorphism suggests that the bathroom is one of source of infection, although there is still a possibility that MAC bacilli might be transmitted from patients to their bathrooms. Therefore, the source of primary infection with MAC is a controversial issue. However, the fact that patients' bathrooms colonize MAC supports the idea that patients were reinfected by inhaling the MAC organisms each time they bathed. When patients repeatedly inhale pathogens, the efficacy of chemotherapy is reduced, and reinfection may be caused by the same patho-

gen or a genetically different strain. In fact, the cure rate with macrolide-based regimens is still low, and MAC infection frequently recurs after the sputum culture is converted to negative upon successful completion of therapy (2,3,9). Most recurrences of MAC disease after discontinuation of therapy are interpreted as reinfections with new MAC strains rather than relapse of the initial MAC strains (3,7,8). In the present study, we also demonstrated that the genotypes of reinfected strains were related to those of isolates from their respective bathrooms. This fact supports a risk of reinfection in patients. Furthermore, polyclonal infection may be involved in transferring organisms from a polyclonal environmental colonization in the bathrooms to patients. A recent report also provided evidence that showers may serve as a source of pulmonary infection caused by waterborne *M. avium* (21). The results of our questionnaire survey suggest that, in order to prevent the transference of MAC from bathroom to patient, it is important to keep the bathroom free from MAC colonization by desiccating. Indeed, Archuleta et al. (22) reported that desiccated *M. avium* loses its viability at a constant rate. It is difficult to prove that the bathroom is the source of primary infection for pulmonary MAC disease. In part, this is because pulmonary MAC disease is often asymptomatic during the early stage of infection and progresses slowly, so that many patients are uncertain of the number of months or years that have passed since the initial infection. If MAC bacilli in the bathroom are transferred to a susceptible host, they might be capable of moving between the bathroom and host during the asymptomatic time periods. Therefore, a prospective cohort study would be required to clarify that the bathroom is a source of infection.

In conclusion, we found that polyclonal MAC organisms were distributed throughout the bathrooms of our patients, but predominantly colonized in the bathtub inlets. Nearly half of the 15 bathrooms that harbored MAC strains (47%, 7/15) had strains with a genetically close relationship to their respective clinical isolates. Thus, it is considered that there is a risk of infection in bathrooms colonized by MAC.

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