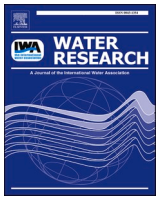
Water Research 275 (2025) 123188

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Unveiling significant regrowth and potential risk of nontuberculous mycobacteria in hospital water supply system

Yalan Gan [a], Futoshi Kurisu [a,b], Dai Simazaki [c], Mitsunori Yoshida [d], Hanako Fukano [d], Takeshi Komine [d], Hiromi Nagashima [e], Yoshihiko Hoshino [d], Ikuro Kasuga [a,f,*]

[a] Department of Urban Engineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan
[b] Research Center for Water Environment Technology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8656, Japan
[c] Department of Environmental Health, National Institute of Public Health, 2-3-6 Minami, Wako City, Saitama 351-0197, Japan
[d] Department of Mycobacteriology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aoba, Higashimurayama City, Tokyo 189-0002, Japan
[e] Iwate Medical University, 2-1-1, Idai-dori, Yahaba, Shiwa-gun, Iwate 028-3695, Japan
[f] Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1, Komaba, Meguro, Tokyo 153-8904, Japan

ARTICLE INFO

Keywords:
Building plumbing
Hospital
Microbial regrowth
Mycobacterium abscessus
Nontuberculous mycobacteria

ABSTRACT

The health burden of waterborne nontuberculous mycobacteria (NTM) is a rising concern. While the water supply systems can serve as a potential reservoir for NTM, their abundance, diversity, and transmission pathways remain unknown. This study aimed to characterize the prevalence and regrowth of NTM in building water supply system in a hospital where many *M. abscessus* were isolated from patients. The depletion of residual chlorine after stagnation and supply of warm water at the point of use promoted significant microbial regrowth, including NTM, in the hospital. The absolute abundance of *Mycobacterium* spp. 16S rRNA genes in tap water and shower water samples increased to approximately 10^4 copies/mL, while it was below the quantification limit in the finished water from a drinking water treatment plant. Amplicon sequencing of NTM-specific *hsp*65 genes revealed that *M. abscessus* was prevalent in all samples, while the dominant NTM species varied depending on locations even in the same building. The presence of *M. abscessus* in water suggested the possibility of waterborne transmission in the hospital. *M. abscessus* was frequently isolated from tap water, shower water, and shower biofilms. These isolates demonstrated high clonality and were closely affiliated with the ABS-GL4 cluster of *M. abscessus* subsp. *abscessus*. Even though the automatic mixing equipment at the point of use was replaced with new one, the settlement and growth of NTM were reproducibly observed, suggesting mixing equipment as a hotspot for NTM proliferation. Additional interventions including water quality control are required as the hospital water supply system is a hot spot for NTM regrowth.

1. Introduction

Nontuberculous mycobacteria (NTM) are atypical mycobacteria other than *Mycobacterium tuberculosis* complex as well as *M. leprae/lepromatosis*. NTM, such as *M. avium* complex (MAC) and *M. abscessus*, typically act as opportunistic pathogens with the potential to cause pulmonary infections, disseminated infections, cervical lymphadenitis, and skin/soft tissue diseases to individuals, especially the ones with compromised immune systems. The incidence rate of NTM pulmonary disease in Japan has been increasing since 2007, with an estimated rate of 14.7 cases per 100,000 person-years in 2014 (Namkoong et al., 2016), which is one of the highest rates globally. Therefore, NTM has been listed on the Japanese Priority Pathogen List (Priority 1), posing a serious concern for human health (AMED Public and Private Partnerships for Infectious Diseases R&D, 2021).

NTM exposure is mainly suspected to be involved with aerosols of NTM-colonized water from plumbing systems in residential buildings and healthcare facilities (Gan et al., 2022). Waterborne NTM infection accounted for 57% of domestic waterborne deaths and 44% of domestic waterborne hospitalizations in the USA (Collier et al., 2021), representing the largest health burden among various waterborne infections. A correlation has been observed between the abundances of pathogenic NTM (MAC, *M. abscessus*, and *M. fortuitum*) in showerhead biofilm and the prevalence of NTM disease (Gebert et al., 2018). Some studies have

* Corresponding author at: Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1, Komaba, Meguro, Tokyo 153-8904, Japan.
E-mail address: kasuga@env.t.u-tokyo.ac.jp (I. Kasuga).

https://doi.org/10.1016/j.watres.2025.123188
Received 6 May 2024; Received in revised form 18 October 2024; Accepted 21 January 2025
Available online 22 January 2025
0043-1354/© 2025 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

demonstrated that NTM isolates from patients were genetically identical to those obtained from the plumbing system and point of use (POU) (Thomson et al., 2013). Building plumbing and POU may provide favorable habitats for the colonization and regrowth of NTM (Donohue et al., 2015; Gebert et al., 2018) as they can form biofilms, associate with amoebae (Delafont et al., 2014), and resist to disinfectants due to their acid-rich cell walls (Taylor et al., 2000). *Mycobacterium* was the most dominant taxon in the biofilms collected from showerheads across the United States (Webster et al., 2021). Surprisingly, 25% and 35% of the potable water samples in the United States were positive for *M. avium* and *M. intracellulare*, respectively (Pfaller et al., 2022), indicating the contamination of NTM in water supply systems is a rising concern. Notably, *M. avium* and *M. abscessus* have been listed as waterborne pathogens on Contaminant Candidate List 5 for water safety regulation by U.S. EPA.

The waterborne NTM has been the major control target in healthcare-associated infection via water use. Several factors in hospital water supply system further promote the regrowth of pathogenic bacteria including NTM: warm water temperature applied in healthcare activities is ideal for bacterial growth; the presence of many POU and complicated lines cause frequent stagnation and slow flow, resulting in the decay of residual chlorine. A previous study investigated the outbreak of *M. abscessus* linked to hospital tap water (Baker et al., 2017), demonstrating the attention to waterborne infection of NTM in healthcare-associated water system. However, the fate and transmission of NTM community developed in hospital water supply system remains uncharacterized well.

This study aims to reveal the occurrence and diversity of NTM in building plumbing and POU in a large hospital where many *M. abscessus* had been isolated from patients. Although the waterborne transmission was suspected, the fate of NTM in the complex hospital water supply system remains unknown. Confirmation of NTM contamination in the hospital water supply system is indispensable for implementing control measures against pathogenic NTM.

2. Materials and methods

2.1. Hospital water supply system

A large hospital with 1000 beds in the northern part of Japan was surveyed in this study, which relocated to its current location in 2019. After starting operation in the new building, *M. abscessus* was isolated from approximately 50 patients until 2022. The hospital receives only municipal drinking water sourced from groundwater and treated by inline coagulation by poly aluminum chloride, followed by rapid sand filtration and disinfection with sodium hypochlorite. The finished water meets the drinking water quality standards in Japan, with the free residual chlorine level maintained at approximately 0.5 mg/L. After receiving municipal drinking water in a water-receiving tank (RS) in the hospital, the water is pumped up to a roof-top storage tank (RT) and hot water generation and storage tank (HS). In HS, hot water at 65°C is generated. Then, cold and hot water are separately supplied to each floor via stainless and cross-linked polyethylene pipes. Cold and hot water are further supplied to 1565 faucets and 106 showers. Automatic mixing equipment at each POU, including faucets and showers, mixes the water to maintain a constant temperature of approximately 30–40°C.

2.2. Intensive sampling from drinking water treatment plant to hospital

Three source groundwater samples (GW1, GW2, and GW3) and finished water after treatment (FW) were collected from the drinking water treatment plant (DWTP) supplying water to the hospital in February 2023. In the same month, intensive sampling of water and biofilm was conducted at the hospital. Three water samples, including RS, RT, and HS, were collected from the point of entry (POE) in the hospital. POU samples (faucets and showers) were collected from different floors (4^{th}, 6^{th}, 7^{th}, 8^{th}, 9^{th}, and 10^{th} floor) of the building. Tap water samples (TW, mixture of cold water and hot water) were collected from faucets in different rooms (n=8). They had been stagnant overnight (approximately 12 h) before sampling. In addition, shower water samples (SW, mixture of cold water and hot water) which had been stagnant overnight (approximately 12 h) were collected (n=6). Twenty biofilm samples (SH) were collected from showerheads and junction tubes in showering rooms using sterilized swabs.

2.3. Monitoring before and after the replacement of an automatic mixing equipment

Automatic mixing equipment for a faucet in room A was replaced with a new one in May 2023, and subsequent microbial growth after replacement was monitored. The mixing condition of cold water and hot water was maintained before and after replacement. Before replacement, overnight stagnant tap water was first collected, and then the used equipment was removed. Biofilm samples were collected from different parts of the equipment and the internal surface of the faucet (n=8). In addition, fresh cold water and fresh hot water were collected separately from the lines before entering the equipment. After replacing the equipment with a new one, fresh tap water was collected after flushing for 5 min. Tap water samples before and after overnight stagnation were collected on days 2, 14, 28, 42, 70, 105, 154, and 182 after the replacement.

2.4. DNA extraction from water and biofilm samples

One liter each of the water samples was filtered through 0.2 μm polycarbonate filters (0.20 μm GTTP, Millipore Isopore^{TM}, Billerica, MA, USA). The filters were put inside the Lysing Matrix E tube and stored at -20°C until further processing. The swabs used for collecting biofilms were immersed in 10 mL of sterile phosphate-buffered saline (PBS) solution. They were subjected to vortex for 2 min to detach biomass. Nine milliliters of biomass solution were treated in the same way as the water samples before DNA extraction. DNA extraction was performed according to the previous study (Rahmatika et al., 2022). Briefly, total DNA was extracted from the filters using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) with phenol–chloroform–isoamyl alcohol (25:24:1) (Nippon Gene, Japan). The DNA concentration and purity (absorbance ratio at 260/280) were measured by NanoDrop spectrophotometer (NanoDrop Technology, Delaware, USA).

2.5. Quantitative PCR

The abundance of prokaryotic 16S rRNA genes (total bacteria and archaea) was quantified by SYBR green-based qPCR with a QuantStudio^{TM} 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The primers and the thermal condition used in this study are shown in **Table S1** (Caporaso et al., 2011). LightCycler 480 SYBR Green I Master Solution (Roche, Mannheim, Germany) was used according to the instructions. All reactions were analyzed in triplicate. Artificially synthesized plasmid containing the target sequence was serially diluted to prepare for the standard curve (5.0×10^3–5.0×10^7 copies/μL). Amplification efficiency was in the range of 84.0–86.0%. The limit of quantification (LOQ) was set as the lowest concentration of the standards (5.0×10^3 copies/μL).

The abundance of *Mycobacterium* spp. 16S rRNA gene was quantified by TaqMan-based qPCR after DNA extraction. The primers and probes as well as the thermal condition are shown in **Table S1** (van der Wielen, 2013). LightCycler 480 Probes Master Solution (Roche, Mannheim, Germany) was used according to the instructions. All reactions were analyzed in triplicate. Artificially synthesized plasmid containing the target sequence was serially diluted to prepare for the standard curve (5.0×10^1–5.0×10^8 copies/μL). Amplification efficiency was in the

range of 96.2–97.1%. LOQ was set as the lowest concentration of the standards (5.0×10^1 copies/μL).

2.6. Amplicon sequencing of procaryotic 16S rRNA genes and Mycobacterium hsp65 genes

Microbial community structures were determined by amplicon sequencing targeting the V4 region of prokaryotic 16S rRNA genes (Caporaso et al., 2011). On the other hand, NTM community structures were determined by targeting *hsp*65 genes, which were commonly possessed by *Mycobacterium* spp. (Telenti et al., 1993). Amplicon sequencing was performed on an Illumina MiSeq platform with 300-bp paired-end reads at the Bioengineering Lab (Japan). Read sequences that completely matched the primer sequence used were filtered using the fastx_barcode_splitter tool of FASTX-Toolkit (ver. 0.0.14). Primer sequences were removed from the extracted reads using fastx_trimer of the FASTX-Toolkit. Sequences with a quality value of less than 20 and discarded sequences with a length of 130 bases (for 16S rRNA gene amplicon sequencing)/40 bases (for *hsp*65 gene amplicon sequencing) or less, and their paired sequences were removed using sickle (ver. 1.33). Reads were joined using the paired-end read combination script FLASH (ver. 1.2.11). Chimera sequences and noise sequences were removed using the DADA2 plugin of Quantitative Insights into Microbial Ecology 2 (QIIME2) (ver. 2023.2). As for 16S rRNA gene amplicon sequencing, representative sequences were clustered with 97% similarity using feature-classifier plugin of QIIME2. As for *hsp*65 gene, BLASTN (ver. 2.13.0) was performed on the obtained representative sequence against an in-house database of 151 different *hsp*65 genes of *Mycobacterium* spp. to infer the phylogenetic lineage. All sequences obtained from 16S rRNA gene and *hsp*65 gene amplicon sequencing were submitted to the DNA Data Bank Japan (DDBJ) Sequence Read Archive with accession numbers DRR541395–DRR541432 and DRR541433–DRR541469, respectively.

2.7. Cultivation of NTM isolates

Water samples (1 L) and suspended solution of biofilm samples (1 mL) were treated with 0.04% cetylpyridinium chloride (CPC) for 30 min at room temperature to decontaminate other bacteria (Gebert et al., 2018). Half of the decontaminated water samples were filtered through an EZ-Fit filter (0.45 μm, Millipore SAS, Molsheim, France) and inoculated on Middlebrook 7H10 agar (with 10% OADC and 10% PANTA). Another half of the decontaminated water samples were suspended in 5 mL of PBS after being filtered through EZ-Fit filters (0.45 μm, Millipore SAS, Molsheim, France). Then, the solution was vortexed well and centrifuged at 8000 rpm for 5 min. 500 μL of concentrated pellet were inoculated in 5 mL of Middlebrook 7H9 broth (with 10% OADC enrichment and 10% PANTA). For the suspended biofilm solution, 100 μL of decontaminated solution was directly inoculated on Middlebrook 7H10 agar (with 10% OADC and 10% PANTA), while 500 μL of decontaminated solution was inoculated in 5 mL of Middlebrook 7H9 broth (with 10% OADC and 10% PANTA).

Samples on Middlebrook 7H10 agar were incubated at 37°C until colonies were formed. The representative colonies were re-streaked and subcultured onto Middlebrook 7H10 agar to obtain a single isolated colony. Middlebrook 7H9 broth cultures were cultivated at 37°C with constant agitation until visible turbidity was observed. After that, they were grown on Middlebrook 7H10 agar in the same manner.

Colony direct PCR targeting NTM-specific *hsp*65 genes was applied to the isolated colonies. The same primers used for amplicon sequencing of *hsp*65 genes were used (**Table S1**). The PCR products were checked by electrophoresis on 1.5% agarose gel. PCR products were purified by QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions and submitted to FASMAC (Japan) for bidirectional sequencing. Sequence homology search was conducted using BLAST. The sequences of *hsp*65 genes of the isolates have been deposited on DNA Data Bank Japan (DDBJ)/EMBL/GenBank databases under accession numbers LC780736–LC780764, LC791672–LC791686, LC791704–LC791711, LC791714–LC791718, LC791721–LC791722, LC791725–LC791728, and LC791731–LC791732.

2.8. Whole genomic sequencing of M. abscessus isolated from water and biofilm samples

All *M. abscessus* environmental isolates (n=50) were sub-cultured on 7H10 agar plates supplemented with 10% OADC (BD BBL, MD, USA). The genomic DNA of each isolate was extracted as described previously (Yoshida et al., 2022). The DNA concentration and purity (absorbance ratio at 260/280) were measured by the Qubit Fluorometer (ThermoFisher, Waltham, USA). Genomic sequencing was performed as described elsewhere (Yoshida et al., 2021). All raw read data of newly sequenced isolates in this study (50 isolates) were deposited into the DNA Data Bank of Japan (DDBJ) and the National Centre for Biotechnology Information (NCBI) under BioProject accession number PRJDB17850. The raw read data of each isolate were *de novo* assembled in the Shovill pipeline (https://github.com/tseemann/shovill) with the "–trim" option. After assembly, basic statistics and the percentage of contaminating reads were estimated using the CheckM2 software and the GTDB-Tk v2 software (Chaumeil et al., 2022; Chklovski et al., 2024). Phylogenetic analysis was performed as described previously with some modifications (Yoshida et al., 2022). In brief, we combined our data set with publicly available WGS data of 452 *M. abscessus* clinical isolates from CF and non-CF patients (Bryant et al., 2016; Yoshida et al., 2022). All additional raw read data were obtained from the Sequence Read Archive (SRA) and assembled as previously mentioned. The Gubbins software with the "–tree-builder iqtree –best-model" options (Croucher et al., 2015) and the TreeGubbins (https://github.com/simonrharris/tree_gubbins) software were used to estimate phylogenetic trees and to identify significant phylogenetic clusters. The recombination-free whole-genome alignment generated by the Gubbins was used to calculate the SNP distances among *M. abscessus* isolates using snp-dists (https://github.com/tseemann/snp-dists). The phylogenetic tree and associated data were visualized using ggtree R package. A minimum spanning network of *M. abscessus* isolates was assessed using the igraph R package (Csárdi and Nepusz, 2006).

2.9. Water quality analysis

Free chlorine was measured on site by HACH chlorine pocket colorimetric according to N, N-diethyl-p-phenylemediamine (DPD) colorimetric method (Hach, Germany). The LOQ of the free chlorine measurement was 0.02 mg/L. The temperature was measured by a digital thermometer (SK-250WP II-R; Sato Keiryoki, Japan). Total cell counts (TCC) were analyzed using flow cytometry (Accuri C6, BD, NJ, USA) according to the previous study (Rahmatika et al., 2022). LOQ of TCC was set as 10^2 cells/mL.

2.10. Statistical analysis

All statistical analyses were conducted by using R statistical software (ver. 4.2.1). Logarithmic transformation was applied to TCC and qPCR data prior to statistical analysis. The significance of difference was analyzed using the Mann-Whitney U test or Kruskal-Wallis test. Correlation analysis regarding TCC, water temperature, free residual chlorine, and absolute abundance of *Mycobacterium* spp. was performed based on Spearman's rank order correlation using Hmisc package in R. $P < 0.05$ was considered as significant.

3. Results and discussion

An intensive sampling of water and biofilm was conducted in a hospital water supply system where *M. abscessus* had been previously

isolated from patients. First of all, water quality and microbial community structures were analyzed. Then, NTM abundances were determined by qPCR. The diversity of NTM communities was revealed by amplicon sequencing targeting NTM-specific *hsp*65 genes. In addition to culture-independent approach, NTM isolates were collected from the water and biofilm samples, and whole genome sequencing was applied to characterize *M. abscessus* strains. Finally, the repetitive settlement and proliferation of NTM were revealed after the replacement of a used automatic mixing equipment at POU.

3.1. Changes in water quality from DWTP to POU in the hospital

Fig. 1 shows the changes in the water temperature and free chlorine from the DWTP to the POU in the hospital. As shown in Fig. 1 **(a)**, water temperature was maintained at approximately 14°C from DWTP to POE (RS and RT). The hot water temperature in HS was 61.8°C. The water temperatures in tap water and shower water regulated by automatic mixing equipment were 32.7 ± 11.0°C and 40.6 ± 11.1°C (average ± standard deviation), respectively.

The level of free residual chlorine from FW in DWTP to RS and RT in POE was maintained at 0.32–0.38 mg/L, which was above the required level (>0.1 mg/L) of drinking water in Japan (Fig. 1 **(b)**). However, the average free residual chlorine in the tap water and shower water after overnight stagnation decreased to 0.16 ± 0.12 mg/L and 0.05 ± 0.02 mg/L, respectively. The decrease in free residual chlorine in the building plumbing and POU could be accelerated by the mixing of hot water with low free residual chlorine (0.08 mg/L) with cold water.

Overall microbial abundance was evaluated by TCC (**Fig. S1**). TCC level was maintained at 1.0×10^2–2.0×10^2 cells/mL at the POE in the hospital, which was equivalent to the level in finished water in the DWTP. However, a significant increase in TCC was observed in tap water (6.5×10^3–2.3×10^5 cells/mL) and shower water (1.3×10^4–4.7×10^5 cells/mL) in the hospital, indicating that microbial regrowth occurred in building plumbing and POU. According to the correlation analysis (**Table S2**), the TCC showed significant positive and negative correlations with water temperature and free residual chlorine, respectively, which were known as key factors for microbial regrowth in plumbing system (Prest et al., 2016).

3.2. Microbial community in hospital water supply system

Fig. 2 shows the microbial community analyzed by 16S rRNA gene amplicon sequencing, revealing a noticeable shift in the microbial community structures from DWTP to POU. The dominant microbial groups in the raw groundwater were *Acinetobacter* (GW1), *Crenothrix* (GW2), and Comamonadaceae (GW3). After treatment, *Crenothrix* and Comamonadaceae emerged as the major groups in FW. At the POE, RT demonstrated an increased proportion of *Mycobacterium* (9.5%).

In tap water samples, the predominance of *Mycobacterium* (11.9%–53.7%) with Rhizobiales (7.5%–36.6%) and *Methylotenera* (0.04%–40.0%) was observed at different sites. The microbial community structures in shower water differed depending on the sampling site even within the same building. *Mycobacterium* was predominant in SW5 (36.6%) and SW6 (17.2%), while Methylobacteriaceae (64.3%) and *Blastomonas* (29.8%) were dominant in SW3 and SW4, respectively. Showerhead biofilms were featured with the predominance of *Enhydrobacter* (1.1%–66.9%) with an average abundance of 34.5%. *Mycobacterium* was also present in some showerhead biofilms, with the highest relative abundance of 15.2%.

In the raw groundwater, *Mycobacterium* was not dominant, and the microbial communities varied among the different wells despite their proximity within the same geographical area. At the POE, *Mycobacterium* was relatively enriched in RT, while TCC levels were almost stable from FW to POE (**Fig. S1**). In most tap water samples, the microbial community structures differed significantly from those in POE. The regrowth and prevalence of distinct bacterial groups, particularly *Mycobacterium*, were observed, suggesting the conditions of tap water in the hospital could be suitable for their regrowth. *Mycobacterium* was also the predominant taxon in some shower waters. The microbial community in stagnant water appeared to be site-specific, owing to several environmental factors, such as water usage frequency and residual disinfectant level (Rahmatika et al., 2022; Webster et al., 2021). The proportion of *Mycobacterium* in showerhead biofilms appeared relatively lower, while *Mycobacterium* has been found to constitute 32% of the total bacterial taxa in showerhead biofilms in the United States (Feazel et al., 2009). *Enhydrobacter* was the primary population in the microbial communities of showerhead biofilm. In the complex hospital water supply system, microbial interactions could be one key factor affecting the occurrence and proliferation of NTM, warranting further research.

3.3. NTM abundance in hospital water supply system

Fig. 3 **(a)** shows the absolute abundance of *Mycobacterium*-specific 16S rRNA genes in water samples. The copy numbers of *Mycobacterium*

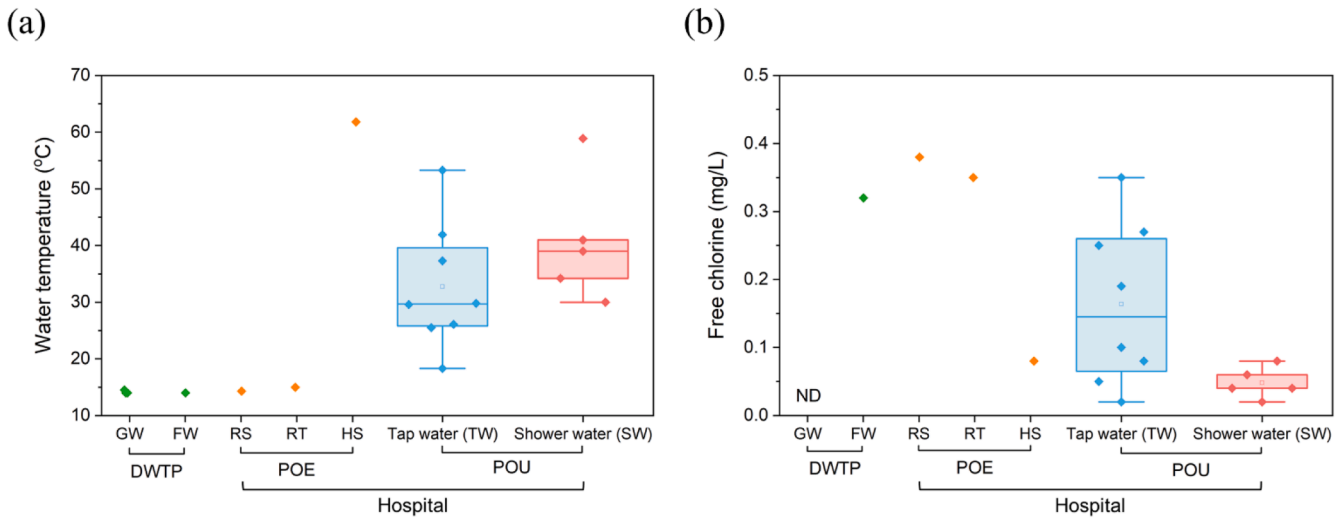


Fig. 1. (a) Water temperature and (b) free chlorine of water samples. GW (raw groundwater) and FW (finished water) were collected from DWTP (drinking water treatment plant). POE samples include RS (receiving tank water), RT (roof-top storage tank water), and HS (hot water generation and storage tank water). POU samples include TW (tap water) and SW (shower water).

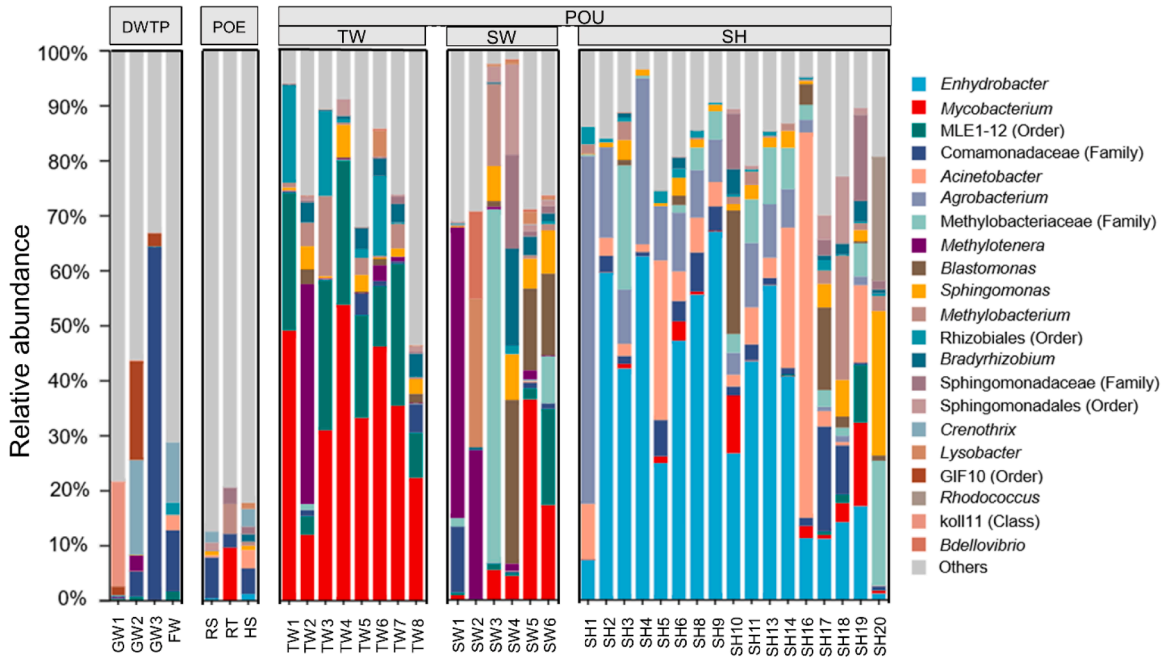


Fig. 2. Microbial community structures identified by amplicon sequencing targeting prokaryotic 16S rRNA gene. GW: raw groundwater; FW: finished water; RS: receiving tank water; RT: roof-top storage tank water; HS: hot water generation and storage tank water; TW: tap water; SW: shower water; SH: showerhead biofilm.

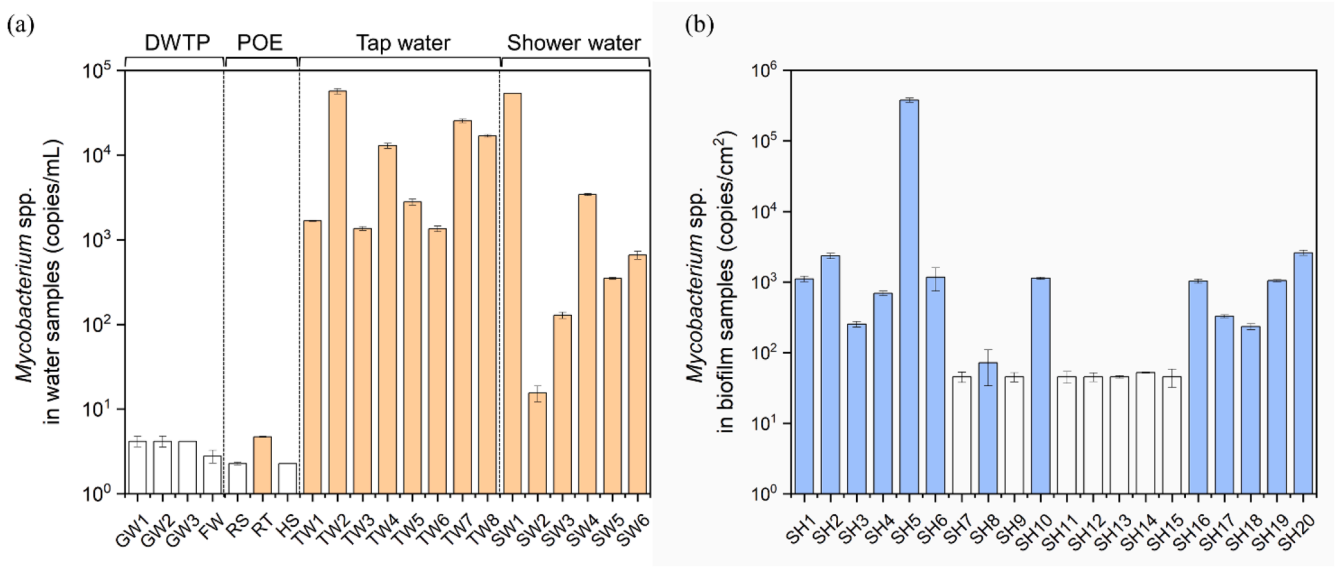


Fig. 3. The absolute abundance of *Mycobacterium* spp. (16S rRNA gene) in (a) water samples and (b) biofilm samples. GW (raw groundwater) and FW (finished water) were collected from DWTP. POE samples include RS (receiving tank water), RT (roof-top storage tank water), and HS (hot water generation and storage tank water). POU samples include TW (tap water), SW (shower water), and SH (showerhead biofilm). Error bars indicate the standard deviation of triplicate measurement of quantitative PCR. Symbols with blank filling mean *Mycobacterium* spp. was under the LOQ in those samples.

16S rRNA genes were below the LOQ in the source groundwater (GW1–GW3), finished water (FW), water samples from the water-receiving tank (RS) and the hot water storage tank (HS), while the concentration of *Mycobacterium* 16S rRNA genes was just above the LOQ (4.7 copies/mL) in the roof-top tank water (RT). 100% (14/14) of tap water and shower water samples and 65% (13/20) of showerhead biofilm samples from the hospital were positive for *Mycobacterium* spp. The absolute abundance of *Mycobacterium* spp. in tap water was in the range of 1.4×10^3–5.7×10^4 copies/mL, while that in shower water was in the range of 1.6×10^1–5.4×10^4 copies/mL. In showerhead biofilms (Fig. 3 **(b)**), the average absolute abundance of *Mycobacterium* spp. was 7.2×10^1–3.8×10^5 copies/cm^2. The abundance of *Mycobacterium* spp. on pipe wall was 6.0×10^2–4.8×10^6 copies/cm^2 in a chloraminated distribution water (Waak et al., 2019).

The relative abundances of *Mycobacterium* spp. 16S rRNA genes normalized by those of prokaryotic 16S rRNA genes in the water and showerhead biofilm samples were compared (**Fig. S2**). The relative abundances of *Mycobacterium* 16S rRNA genes increased from 2.8% in RT to 11.0% ± 6.0% in tap water in the hospital. The relative abundances in tap water were significantly higher than those in shower water (1.3% ± 2.0%) and showerhead biofilm (0.8% ± 1.4%) (Kruskal-Wallis test, $P < 0.05$). This result suggests that NTM was enriched in hospital

tap water, which is consistent with the results of amplicon sequencing of 16S rRNA genes (Fig. 2).

These results indicated that NTM regrowth occurred in building plumbing and POU in the hospital. The same primer set was applied to investigate *Mycobacterium* spp. in unchlorinated cold tap water in the Netherlands (2.0×10^1 to 1.3×10^4 copies/mL) (van der Wielen and van der Kooij, 2013) and stagnant building cold tap water in Japan (1.7×10^0 to 7.0×10^2 copies/mL) (Rahmatika et al., 2022). Compared to these studies, the abundance of *Mycobacterium* spp. in water samples in the hospital was significantly higher, suggesting that warm water temperature and less free residual chlorine conditions in building plumbing and POU in the hospital were preferable for NTM regrowth. As NTM are more resistant to disinfectants than other bacteria (Taylor et al., 2000), they can selectively survive during water distribution with residual chlorine. Once the residual chlorine decays and the water temperature increases in building plumbing and POU, NTM can occupy such a niche among the microbial community. In the hospital studied in this study, *M. abscessus* was frequently isolated from patients after relocation to the new building. It should be noted that NTM regrowth was observed in the new building plumbing system, suggesting that the age of the pipes may not be a primary factor contributing to NTM colonization and proliferation. Careful attention is required to suppress the colonization and regrowth of NTM even though the plumbing system is new.

3.4. NTM diversity in hospital water supply system

Amplicon sequencing targeting *Mycobacterium*-specific *hsp*65 genes was applied to reveal NTM diversity (Fig. 4). There was a noticeable variation in the NTM community composition among different sample groups. All identified NTM species, other than *M. sediminis* and *M. pyrenivorans*, can be pathogenic NTM (Matsumoto et al., 2019), posing a potential health risk to immunocompromised patients.

Although NTM abundance was low in the roof-top storage tank (RT) (Fig. 3), the major NTM in RT was *M. abscessus* (50.2%), indicating that slight amounts of *M. abscessus* were prevalent in POE. Interestingly, tap water samples from the hospital building, where significant regrowth of NTM was observed, demonstrated that NTM diversity varied across samples. Major NTM groups in tap water included *M. abscessus* (2.2%–93.3%), *M. gordonae* (0.5%–90.6%), *M. phocaicum* (0.0%–72.7%), and *M. paragordonae* (0.1%–78.8%). For shower water samples, *M. abscessus* (6.5%–96.2%), *M. gordonae* (0.0%–58.1%), *M. llatzerense* (0.0%–40.3%), and *M. phocaicum* (0.0%–68.4%) were dominant, while their relative abundances were different in different samples. In addition, the major NTM in showerhead biofilms included *M. abscessus* (0.0%–97.0%), *M. fortuitum* (0.0%–97.8%), *M. phocaicum* (0.0%–90.2%), *M. wolinskyi* (0.0%–93.8%), and *M. sediminis* (0.0%–45.5%). Although the relative abundance of NTM in the showerhead biofilm community was lower than that in tap water (Fig. 2), *M. abscessus* was predominant in many showerheads.

Previous studies applying amplicon sequencing for NTM community analysis reported *M. avium* and *M. chelonae* were mainly observed from chloraminated household tap water in the USA (Haig et al., 2018), while *M. gordonae, M. paraterrae*, and *M. lentiflavum* were dominant in chlorinated tap water in building in Japan (Rahmatika et al., 2023). Compared with these studies, the predominance of *M. abscessus* observed in the hospital water supply systems is unique. The type of disinfectants, water age, temperature, and organic carbons could play an important role in shaping drinking water-associated mycobacterial communities (Donohue et al., 2015). Thus, it is likely that *M. abscessus* has some advantages in growth competition among NTM communities at POU in the hospital.

The water supply system in healthcare facilities also serves as a suitable reservoir for *M. abscessus* (Baker et al., 2017; Davidson et al., 2022). An outbreak of *M. abscessus* infection related to tap water use and cardiac surgery through the heater-cooler unit use in a hospital demonstrated that *M. abscessus* subsp. *abscessus* isolated from hospital water were genetically identical to those from patients (Davidson et al., 2022). Therefore, it is necessary to prevent the proliferation and transmission of pathogenic NTM in hospital water.

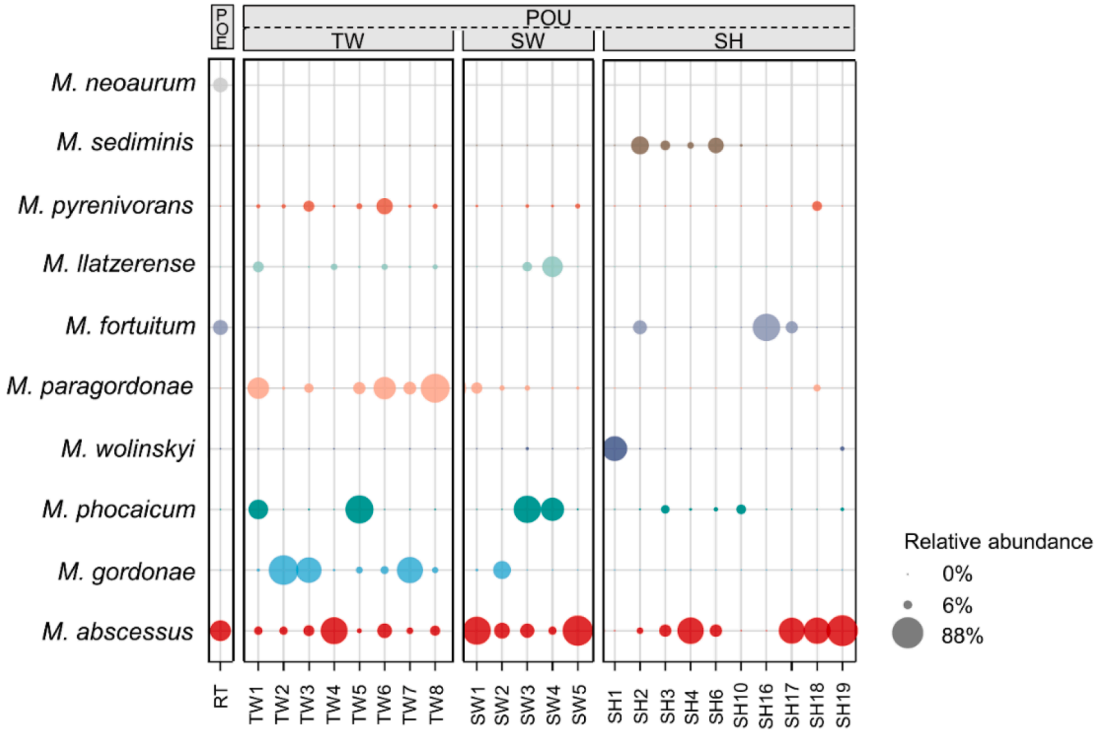


Fig. 4. NTM community identified by amplicon sequencing targeting *hsp*65 gene. POE samples include RT (roof-top storage tank water). POU samples include TW (tap water), SW (shower water), and SH (showerhead biofilm).

3.5. Characterization of NTM isolates

The NTM isolates obtained from tap water, shower water, and showerhead biofilm samples were identified (**Fig. S3, Table S3**). No NTM isolates were obtained from DWTP and POE samples. In total, 29 isolates were obtained, including *M. abscessus* (n=21), *M. phocaicum* (n=3), *M. mucogenicum* (n=2), *M. fortuitum* (n=1), *M. wolinskyi* (n=1), and *M. neoaurum* (n=1). These NTM isolates are reported to have the pathogenicity to humans (Matsumoto et al., 2019). Notably, the percentages of *M. abscessus* isolates were 89%, 89%, and 57% in the isolates cultivated from tap water, shower water, and showerhead biofilm, respectively. The high prevalence of viable *M. abscessus* in POU indicates the potential risk of infection via water use. *M. gordonae* and *M. paragordonae*, which were detected by amplicon sequencing analysis, were not detected by cultivation, probably because they are slow-growing NTM species that cannot compete with other fast growers in the medium (Rahmatika et al., 2023).

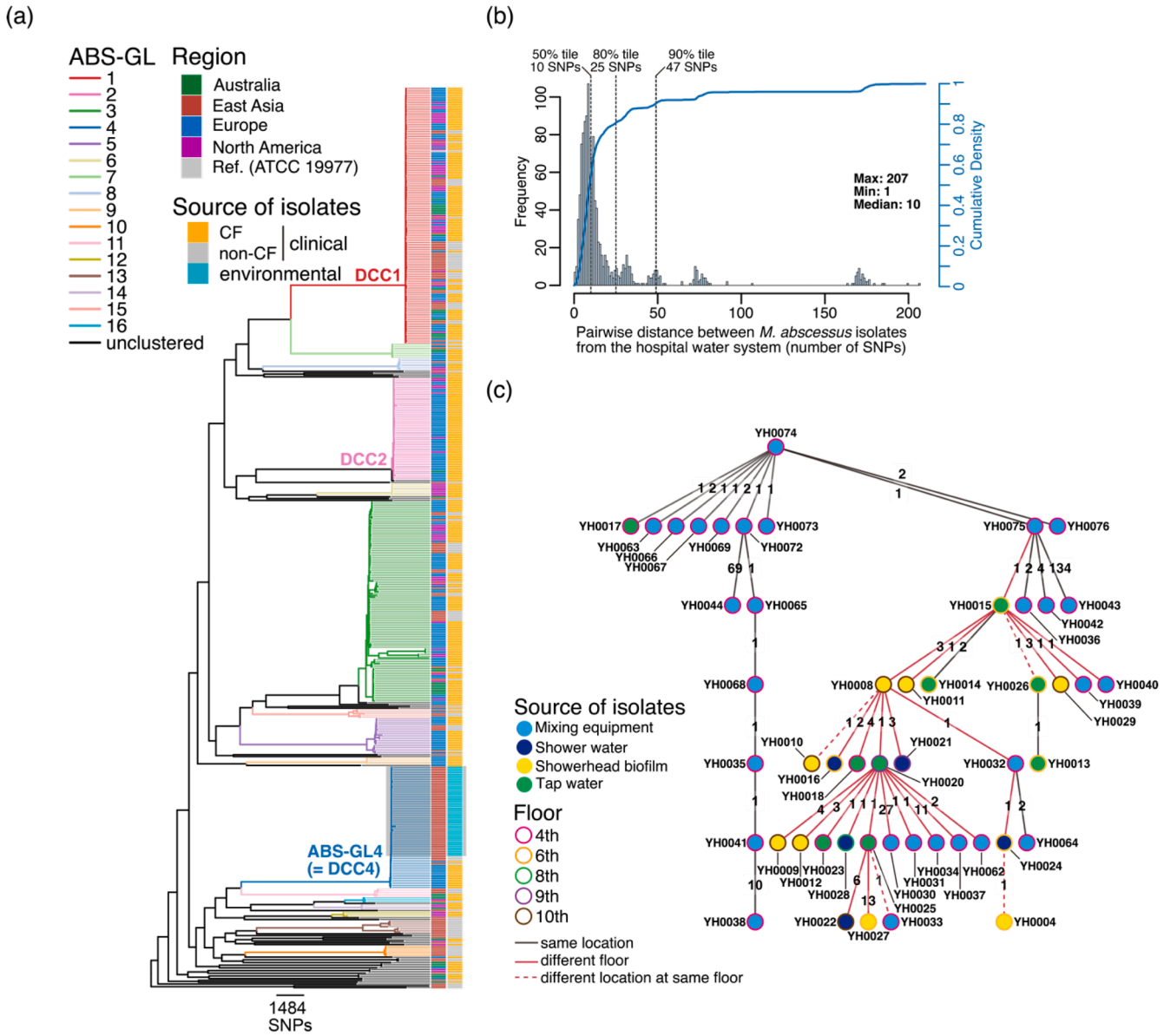


Fig. 5. Phylogenetic analysis of *M. abscessus* isolates derived from the hospital water system. (a) A core genome alignment of 502 *M. abscessus* isolates was used to determine the phylogeny of isolates from the hospital water system. The complete genome sequence of *M. abscessus* ATCC 19977 (Accession number = CU458896) was used as a reference. The alignment was used with Gubbins to construct a maximum likelihood tree with 1000 bootstrap replicates. The 16 monophyletic clusters (ABS-GL1 to ABS-GL16) identified using TreeGubbins are presented. Each color box corresponds to the region where the clinical or environmental isolate was isolated. The corresponding source of isolates (cystic fibrosis [CF] = yellow; non-CF = grey; environmental = cyan) are presented. The scale bar indicates the mean number of nucleotide substitutions on the respective branch. (b) Histogram of pairwise single nucleotide polymorphism (SNP) distances among *M. abscessus* isolates obtained from the hospital water system. The 90, 80, and 50 percentiles of the SNP distances among isolates are presented. The blue line indicates the cumulative density of these SNP distances. (c) A minimum spanning network of *M. abscessus* isolates from the hospital water system (n = 50) was constructed using the core genome alignment same as (a). Each circle's inner or outer color represents the source or the floor from which the corresponding environmental isolate was isolated. The number on each edge represents the SNP distance between each node, representing the corresponding environmental isolate. Red lines indicate that the two corresponding isolates were separated at different locations, black lines indicate that the two corresponding isolates were separated at the same location, and red dashed lines indicate that the two corresponding isolates were separated at different locations on the same floor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.6. Clonal expansions of *M. abscessus subsp. abscessus* in the hospital water system

Several genomic epidemiological studies have reported that specific strains of *M. abscessus* are transmitted globally in patients with cystic fibrosis (CF) (Bryant et al., 2016; Ruis et al., 2021). In addition, these strains were also distributed in patients without CF in East Asian countries (Bronson et al., 2021; Yoshida et al., 2022). These findings prompt us to investigate the association of our environmental isolates with clinical isolates derived from CF and non-CF patients. Using a whole genome sequence-based method (Yoshida et al., 2021), all of our isolates were identified as *M. abscessus* subsp. *abscessus*. All the isolates did not have T28C substitution or truncation for *erm*(41) gene, suggesting that they could induce macrolide resistance. Subsequently, we performed a phylogenetic analysis of the environmental isolates in combination with clinical isolates from patients with or without CF. We found that all isolates from the hospital water system exclusively belonged to a monophyletic cluster ABS-GL4 (Yoshida et al., 2022). ABS-GL4 is phylogenetically associated with one of the dominant circulating clones, DCC4, predominantly distributed in patients with CF worldwide (Fig. 5 **(a)**). To estimate the clonality of these isolates, we assessed pairwise genetic distances among them. Of the genetic distances for all combinations of the environmental isolates, half were less than 10 SNPs, and 80% were less than 24 SNPs (Fig. 5 **(b)**). A minimum spanning network illustrated that there were many instances in which isolates from different locations in the hospital were most closely related (Fig. 5 **(c)**). For example, YH0015 was isolated from tap water on the 6th floor of the hospital, while YH0008 and YH0011 were isolated from biofilms in shower heads on the 10th floor. At the same time, YH0015 was also connected to YH0014 isolated from the exact location (Fig. 5 **(c)**).

Given that the maximum genetic distance between *M. abscessus* strains consecutively isolated from the same patient was up to 24 SNPs (Fujiwara et al., 2022; Komiya et al., 2023), we infer that the majority of our environmental isolates were cloned from a single source. Moreover, the fact that closely related *M. abscessus* strains were isolated from different hospital locations implies that this strain has colonized in the water pipes before they branched off to their respective floors. *M. abscessus* ABS-GL4 strains have been clinically isolated from CF patients in Europe and non-CF patients in Japan and Taiwan but less frequently (Yoshida et al., 2022). The reason that *M. abscessus* DCC4 strain was predominantly isolated from the hospital water system is currently unknown. Further studies are needed to elucidate the mechanism of transmission of *M. abscessus* from the environment to humans.

3.7 Repetitive growth of NTM after replacement of automatic mixing equipment at POUWe

suspected that automatic mixing equipment could be a hot spot for NTM proliferation. Many NTM strains related to *M. abscessus* were isolated from the biofilms on the inner surface of the mixing equipment (**Table S4**). Before installing the new equipment, we collected cold and hot water from the inlet tubes to the equipment. The abundance of *Mycobacterium* 16S rRNA genes in cold water was $3.6 \times 10^1 \pm 1.5 \times 10^1$ copies/mL, while it was below LOQ in the hot water. *M. abscessus* was isolated from fresh cold water but not from fresh hot water (**Table S4**).

The used equipment was replaced with a new one. After replacement, tap water was used as usual under the same mixing condition. Fig. 6 **(a)** shows the changes in water temperature and free residual chlorine after replacement. The temperature of tap water after the replacement was maintained at 32–40°C. The concentration of free residual chlorine before overnight stagnation was around 0.2 mg/L, while it was often below LOQ (0.02 mg/L) in overnight stagnant water. Fig. 6 **(b)** shows the changes in the absolute abundances of *Mycobacterium* 16S rRNA genes after replacement. The abundance of *Mycobacterium* 16S rRNA genes in stagnant water decreased from $5.3 \times 10^3 \pm 4.3 \times 10^2$ copies/mL to lower than 5.0×10^1 copies/mL after replacement, which was consistent with 2-log reduction of TCC (**Fig. S4**). *Mycobacterium* 16S

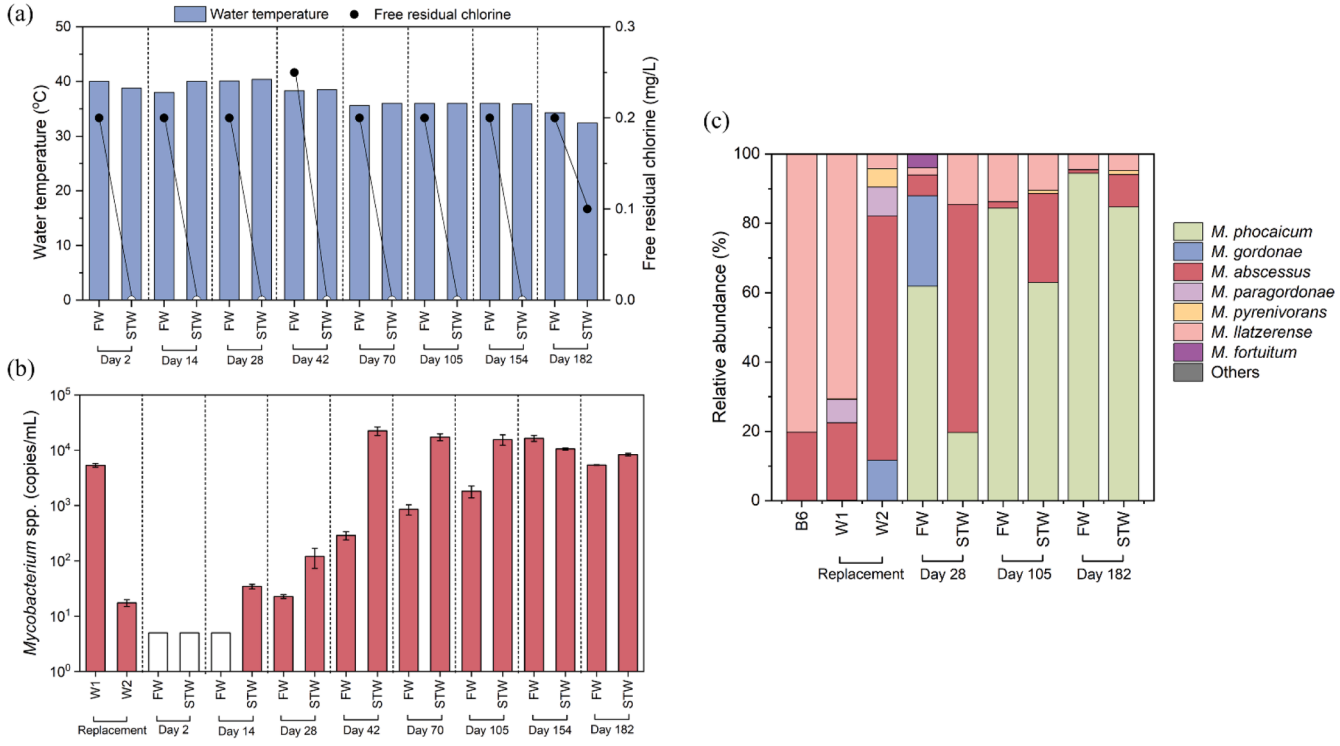


Fig. 6. (a) The changes in water temperature and free residual chlorine after replacement of mixing equipment. (b) *Mycobacterium* spp. 16S rRNA genes in water samples before and after replacement of mixing equipment. Error bars indicate the standard deviation of triplicate measurement of quantitative PCR. (c) NTM community identified by amplicon sequencing targeting *hsp*65 gene. FW: fresh tap water before overnight stagnation; STW: tap water after overnight stagnation; W1: Stagnant tap water before replacement; W2: Fresh tap water after replacement; B6: Biofilm from the internal motor in the used equipment.

rRNA genes were below the LOQ in fresh and stagnant tap water after one day of replacement. However, from day 14 to day 42, the gradual increase of *Mycobacterium* 16S rRNA genes was observed in both fresh and stagnant tap water. Finally, the level of *Mycobacterium* 16S rRNA genes was comparable to that before replacement. Additionally, *M. abscessus* strains can be continuously obtained from the fresh and stagnant tap water even after the replacement of mixing equipment (**Table S4**).

Amplicon sequencing targeting *hsp*65 genes was applied to the samples (Fig. 6 **(c)**). Before replacement of the equipment, *M. abscessus* accounted for 19.7% of NTM biofilm on the surface of the internal motor in the used equipment. The percentages of *M. abscessus* were 22.4% in stagnant tap water before replacement (W1) and 70.3% in fresh tap water immediately after replacement (W2). After 28, 105, and 182 days of replacement, the percentages of *M. abscessus* in tap water before stagnation decreased to 1.0%–6.0%, while the contribution of *M. phocaicum* increased. In the stagnant water samples, *M. abscessus* accounted for 65.7%, 25.6%, and 9.2%, respectively. Although the percentage of *M. abscessus* decreased, it could still settle and regrow at POU.

The occurrence of NTM in cold water and the absence of NTM in hot water suggests that NTM proliferation in hot water was suppressed due to high water temperature (65°C) at HS. After the replacement of mixing equipment, the reduction of NTM in tap water and the increase of NTM with the operation of new equipment indicates that the used automatic mixing equipment was the primary source of NTM in tap water, and mixing equipment was a hotspot for NTM repetitive growth at POU. Additional interventions on the management of water quality, such as an increase in free residual chlorine level and a decrease in mixed water temperature, should be considered to suppress *M. abscessus* regrowth in the whole hospital water supply system.

4. Conclusion

In a large hospital where many *M. abscessus* strains were isolated from patients, NTM communities including *M. abscessus* were prevalent and regrew in the building plumbing and POU. Complex plumbing lines and many POUs with frequent stagnation in the hospital could cause decay of residual chlorine and microbial regrowth including pathogenic NTM. Continuous supply of warm water at POU also contributed to the regrowth of *M. abscessus*. Although direct linkage between waterborne *M. abscessus* and *M. abscessus* isolated from patients remains to be studied, transmission of NTM via water use and dynamic circulation of NTM in the hospital was suspected. Currently, NTM are not regulated in drinking water quality standards in Japan. However, the risk of NTM infection has been increasing in the aging society. In particular, more attention should be paid to healthcare-associated water as it could serve as a reservoir for waterborne NTM. Effective prevention and control practices in water supply system from DWTP to POU are required to mitigate the regrowth of pathogenic NTM associated with water use in the hospital.

Funding

This study was supported by Japan Society for the Promotion of Science KAKENHI (Grant Number 20H02282 and 24H00331: Ikuro Kasuga) and the Japan Agency for Medical Research and Development (AMED) (23wm0225022: Yoshihiko Hoshino).

CRediT authorship contribution statement

Yalan Gan: Writing – original draft, Visualization, Investigation, Conceptualization, Data curation. **Futoshi Kurisu:** Writing – review & editing, Data curation. **Dai Simazaki:** Writing – review & editing, Project administration, Investigation, Data curation. **Mitsunori Yoshida:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Hanako Fukano:** Writing – review & editing, Investigation, Data curation. **Takeshi Komine:** Writing – review & editing, Investigation, Data curation. **Hiromi Nagashima:** Writing – review & editing, Project administration, Investigation, Data curation. **Yoshihiko Hoshino:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Data curation. **Ikuro Kasuga:** Writing – original draft, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Ms. Ginko Kaneda, Maki Okuda, Akiko Yamashita, and Yukari Nogi for their technical support. Yalan Gan acknowledges support from the Chinese Scholarship Council.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123188.

Data availability

Data will be made available on request.

References

AMED Public and Private Partnerships for Infectious Diseases R&D, 2021. List of pathogen [WWW Document]. URL https://id3catalyst.jp/apid/en/list.html (accessed 10.12.23).

Baker, A.W., Lewis, S.S., Alexander, B.D., Chen, L.F., Wallace, R.J., Brown-Elliott, B.A., Isaacs, P.J., Pickett, L.C., Patel, C.B., Smith, P.K., Reynolds, J.M., Engel, J., Wolfe, C. R., Milano, C.A., Schroder, J.N., Davis, R.D., Hartwig, M.G., Stout, J.E., Strittholt, N., Maziarz, E.K., Saullo, J.H., Hazen, K.C., Walczak, R.J., Vasireddy, R., Vasireddy, S., McKnight, C.M., Anderson, D.J., Sexton, D.J., 2017. Two-phase hospital-associated outbreak of Mycobacterium abscessus: investigation and mitigation. Clin. Infect. Dis. 64, 902–911. https://doi.org/10.1093/cid/ciw877.

Bronson, R.A., Gupta, C., Manson, A.L., Nguyen, J.A., Bahadirli-Talbott, A., Parrish, N. M., Earl, A.M., Cohen, K.A., 2021. Global phylogenomic analyses of Mycobacterium abscessus provide context for non cystic fibrosis infections and the evolution of antibiotic resistance. Nat. Commun. 12, 5145. https://doi.org/10.1038/s41467-021-25484-9.

Bryant, J.M., Grogono, D.M., Rodriguez-Rincon, D., Everall, I., Brown, K.P., Moreno, P., Verma, D., Hill, E., Drijkoningen, J., Gilligan, P., Esther, C.R., Noone, P.G., Giddings, O., Bell, S.C., Thomson, R., Wainwright, C.E., Coulter, C., Pandey, S., Wood, M.E., Stockwell, R.E., Ramsay, K.A., Sherrard, L.J., Kidd, T.J., Jabbour, N., Johnson, G.R., Knibbs, L.D., Morawska, L., Sly, P.D., Jones, A., Bilton, D., Laurenson, I., Ruddy, M., Bourke, S., Bowler, I.C., Chapman, S.J., Clayton, A., Cullen, M., Daniels, T., Dempsey, O., Denton, M., Desai, M., Drew, R.J., Edenborough, F., Evans, J., Folb, J., Humphrey, H., Isalska, B., Jensen-Fangel, S., Jönsson, B., Jones, A.M., Katzenstein, T.L., Lillebaek, T., MacGregor, G., Mayell, S., Millar, M., Modha, D., Nash, E.F., O'Brien, C., O'Brien, D., Ohri, C., Pao, C.S., Peckham, D., Perrin, F., Perry, A., Pressler, T., Prtak, L., Qvist, T., Robb, A., Rodgers, H., Schaffer, K., Shafi, N., van Ingen, J., Walshaw, M., Watson, D., West, N., Whitehouse, J., Haworth, C.S., Harris, S.R., Ordway, D., Parkhill, J., Floto, R.A., 2016. Emergence and spread of a human-transmissible multidrug-resistant nontuberculous mycobacterium. Science 354, 751–757. https://doi.org/10.1126/science.aaf8156.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522. https://doi.org/10.1073/pnas.1000080107.

Chaumeil, P.-A., Mussig, A.J., Hugenholtz, P., Parks, D.H., 2022. GTDB-Tk v2: memory friendly classification with the genome taxonomy database. Bioinformatics 38, 5315–5316. https://doi.org/10.1093/bioinformatics/btac672.

Chklovski, A., Parks, D.H., Woodcroft, B.J., Tyson, G.W., 2024. Author correction: checkM2: a rapid, scalable and accurate tool for assessing microbial genome quality using machine learning. Nat. Methods 21. https://doi.org/10.1038/s41592-024-02248-z, 735–735.

Collier, S.A., Deng, L., Adam, E.A., Benedict, K.M., Beshearse, E.M., Blackstock, A.J., Bruce, B.B., Derado, G., Edens, C., Fullerton, K.E., Gargano, J.W., Geissler, A.L.,

Hall, A.J., Havelaar, A.H., Hill, V.R., Hoekstra, R.M., Reddy, S.C., Scallan, E., Stokes, E.K., Toder, J.S., Beach, M.J., 2021. Estimate of Burden and Direct Healthcare Cost of Infectious Waterborne Disease in the United States. Emerging Infectious Diseases.

Croucher, N.J., Page, A.J., Connor, T.R., Delaney, A.J., Keane, J.A., Bentley, S.D., Parkhill, J., Harris, S.R., 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucl. Acids Res. 43. https://doi.org/10.1093/nar/gku1196 e15–e15.

Csárdi, G., Nepusz, T., 2006. The igraph software package for complex network research.

Davidson, R.M., Nick, S.E., Kammlade, S.M., Vasireddy, S., Weakly, N., Hasan, N.A., Epperson, L.E., Strong, M., Nick, J.A., Brown-Elliott, B.A., Stout, J.E., Lewis, S.S., Wallace, R.J., Baker, A.W., 2022. Genomic analysis of a hospital-associated outbreak of Mycobacterium abscessus: implications on transmission. J. Clin. Microbiol. 60. https://doi.org/10.1128/JCM.01547-21 e01547-21.

Delafont, V., Mougari, F., Cambau, E., Joyeux, M., Bouchon, D., Héchard, Y., Moulin, L., 2014. First evidence of amoebae-mycobacteria association in drinking water network. Environ. Sci. Technol. 48, 11872–11882. https://doi.org/10.1021/es5036255.

Donohue, M.J., Mistry, J.H., Donohue, J.M., O'Connell, K., King, D., Byran, J., Covert, T., Pfaller, S., 2015. Increased frequency of nontuberculous mycobacteria detection at potable water taps within the United States. Environ. Sci. Technol. 49, 6127–6133. https://doi.org/10.1021/acs.est.5b00496.

Feazel, L.M., Baumgartner, L.K., Peterson, K.L., Frank, D.N., Harris, J.K., Pace, N.R., 2009. Opportunistic pathogens enriched in showerhead biofilms. Proc. Natl. Acad. Sci. U. S. A. 106, 16393–16399. https://doi.org/10.1073/pnas.0908446106.

Fujiwara, K., Yoshida, M., Murase, Y., Aono, A., Furuuchi, K., Tanaka, Y., Ohta, K., Ato, M., Mitarai, S., Morimoto, K., 2022. Potential cross-transmission of Mycobacterium abscessus among non-cystic fibrosis patients at a tertiary hospital in Japan. Microbiol. Spect. 10. https://doi.org/10.1128/spectrum.00097-22 e00097-22.

Gan, Y., Rahmatika, I., Kurisu, F., Furumai, H., Simazaki, D., Fukano, H., Hoshino, Y., Kasuga, I., 2022. The fate and risk of nontuberculous mycobacteria in the water supply system: a review. H2Open J. 5, 180–197. https://doi.org/10.2166/h2oj.2022.144.

Gebert, M.J., Delgado-Baquerizo, M., Oliverio, A.M., Webster, T.M., Nichols, L.M., Honda, J.R., Chan, E.D., Adjemian, J., Dunn, R.R., Fierer, N., 2018. Ecological analyses of mycobacteria in showerhead biofilms and their relevance to human health. Mbio 9, e01614–e01618. https://doi.org/10.1128/mBio.01614-18.

Haig, S.-J., Kotlarz, N., LiPuma, J.J., Raskin, L., 2018. A high-throughput approach for identification of nontuberculous mycobacteria in drinking water reveals relationship between water age and Mycobacterium avium. Mbio 9. https://doi.org/10.1128/mBio.02354-17 e02354-17.

Komiya, K., Yoshida, M., Uchida, S., Takikawa, S., Yamasue, M., Matsumoto, T., Morishige, Y., Aono, A., Hiramatsu, K., Yamaoka, Y., Nishizono, A., Ato, M., Kadota, J., Mitarai, S., 2023. Massive and lengthy clonal nosocomial expansion of Mycobacterium abscessus subsp. *massiliense* among patients who are ventilator dependent without cystic fibrosis. Microbiol. Spectrum 11, e04908–e04922. https://doi.org/10.1128/spectrum.04908-22.

Matsumoto, Y., Kinjo, T., Motooka, D., Nabeya, D., Jung, N., Uechi, K., Horii, T., Iida, T., Fujita, J., Nakamura, S., 2019. Comprehensive subspecies identification of 175 nontuberculous mycobacteria species based on 7547 genomic profiles. Emerg. Microbes Infect. 8, 1043–1053. https://doi.org/10.1080/22221751.2019.1637702.

Namkoong, H., Kurashima, A., Morimoto, K., Hoshino, Y., Hasegawa, N., Ato, M., Mitarai, S., 2016. Epidemiology of pulmonary nontuberculous mycobacterial disease, Japan. Emerg. Infect. Dis. 22, 1116–1117. https://doi.org/10.3201/eid2206.151086.

Pfaller, S., King, D., Mistry, J.H., Donohue, M., 2022. Occurrence revisited: Mycobacterium avium and Mycobacterium intracellulare in potable water in the USA. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-022-11849-7.

Prest, E.I., Frederik, H., Van, L., Vrouwenvelder, J.S., 2016. Biological stability of drinking water: controlling factors, methods, and challenges. Front. Microbiol. 7, 1–24. https://doi.org/10.3389/fmicb.2016.00045.

Rahmatika, I., Kurisu, F., Furumai, H., Kasuga, I., 2022. Dynamics of the microbial community and opportunistic pathogens after water stagnation in the premise plumbing of a building. Microbes Environ. 37, ME21065. https://doi.org/10.1264/jsme2.ME21065.

Rahmatika, I., Simazaki, D., Kurisu, F., Furumai, H., Kasuga, I., 2023. Occurrence and diversity of nontuberculous mycobacteria affected by water stagnation in building plumbing. Water Supply 23, 5017–5028. https://doi.org/10.2166/ws.2023.318.

Ruis, C., Bryant, J.M., Bell, S.C., Thomson, R., Davidson, R.M., Hasan, N.A., van Ingen, J., Strong, M., Floto, R.A., Parkhill, J., 2021. Dissemination of Mycobacterium abscessus via global transmission networks. Nat. Microbiol. 6, 1279–1288. https://doi.org/10.1038/s41564-021-00963-3.

Taylor, R.H., Falkinham, J.O., Norton, C.D., LeChevallier, M.W., 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. Appl. Environ. Microbiol. 66, 1702–1705. https://doi.org/10.1128/aem.66.4.1702-1705.2000.

Telenti, A., Marchesi, F., Balz, M., Bally, F., Böttger, E.C., Bodmer, T., 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 31, 175–178. https://doi.org/10.1128/jcm.31.2.175-178.1993.

Thomson, R., Tolson, C., Carter, R., Coulter, C., Huygens, F., Hargreaves, M., 2013. Isolation of nontuberculous mycobacteria (NTM) from household water and shower aerosols in patients with pulmonary disease caused by NTM. J. Clin. Microbiol. 51, 3006–3011. https://doi.org/10.1128/jcm.00899-13.

van der Wielen, P.W.J.J., 2013. Opportunistic pathogens in drinking water in the Netherlands. In: van der Kooij, D. (Ed.), Microbial Growth in Drinking Water Supplies. IWA Publishing, London, pp. 177–203.

van der Wielen, P.W.J.J., van der Kooij, D., 2013. Nontuberculous mycobacteria, fungi, and opportunistic pathogens in unchlorinated drinking water in the Netherlands. Appl. Environ. Microbiol. 79, 825–834. https://doi.org/10.1128/AEM.02748-12.

Waak, M.B., Lapara, T.M., Halle, C., Hozalski, R.M., 2019. Nontuberculous mycobacteria in two drinking water distribution systems and the role of residual disinfection. Environ. Sci. Technol. 53, 8563–8573. https://doi.org/10.1021/acs.est.9b01945.

Webster, T.M., McFarland, A., Gebert, M.J., Oliverio, A.M., Nichols, L.M., Dunn, R.R., Hartmann, E.M., Fierer, N., 2021. Structure and functional attributes of bacterial communities in premise plumbing across the United States. Environ. Sci. Technol. 55, 14105–14114. https://doi.org/10.1021/acs.est.1c03309.

Yoshida, M., Chien, J.-Y., Morimoto, K., Kinjo, T., Aono, A., Murase, Y., Fujiwara, K., Morishige, Y., Nagano, H., Jou, R., Hasegawa, N., Ato, M., Hoshino, Y., Hsueh, P.-R., Mitarai, S., 2022. Molecular epidemiological characteristics of Mycobacterium abscessus complex derived from non-cystic fibrosis patients in Japan and Taiwan. Microbiol. Spectrum 10. https://doi.org/10.1128/spectrum.00571-22 e00571-22.

Yoshida, M., Sano, S., Chien, J.-Y., Fukano, H., Suzuki, M., Asakura, T., Morimoto, K., Murase, Y., Miyamoto, S., Kurashima, A., Hasegawa, N., Hsueh, P.-R., Mitarai, S., Ato, M., Hoshino, Y., 2021. A novel DNA chromatography method to discriminate Mycobacterium abscessus subspecies and macrolide susceptibility. EBioMedicine 64, 103187. https://doi.org/10.1016/j.ebiom.2020.103187.