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Heterogeneity of clinical and environmental isolates of *Mycobacterium fortuitum* using repetitive element sequence-based PCR: municipal water an unlikely source of community-acquired infections

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SUMMARY

M. fortuitum is a rapidly growing mycobacterium associated with community-acquired and nosocomial wound, soft tissue, and pulmonary infections. It has been postulated that water has been the source of infection especially in the hospital setting. The aim of this study was to determine if municipal water may be the source of community-acquired or nosocomial infections in the Brisbane area. Between 2007 and 2009, 20 strains of *M. fortuitum* were recovered from municipal water and 53 patients' isolates were submitted to the reference laboratory. A wide variation in strain types was identified using repetitive element sequence-based PCR, with 13 clusters of $\geqslant 2$ indistinguishable isolates, and 28 patterns consisting of individual isolates. The clusters could be grouped into seven similar groups (>95% similarity). Municipal water and clinical isolates collected during the same time period and from the same geographical area consisted of different strain types, making municipal water an unlikely source of sporadic human infection.

Key words: Clinical microbiology, infectious disease, mycobacteria, water-borne infections.

INTRODUCTION

Mycobacterium fortuitum is a non-pigmented rapidly growing mycobacterium, often associated with skin and soft tissue infections, although pulmonary disease can occur [1]. Wound infections [2], post-injection abscesses, and post-pedicure infections [3] are well documented. *M. fortuitum* is also a recognized cause of nosocomial infections [4] and pseudo-outbreaks [5]. It is usually considered that municipal and hospital water are the source of these outbreaks, although *M. fortuitum* is also found in soil and house dust [6]. *M. fortuitum* has been found in municipal water [7–9] and is relatively resistant to chlorine and other disinfectants [10, 11]. While there have been many outbreaks reported where water has been the suspected vehicle of transmission, very few have been realized in a timely fashion that would allow full environmental sampling to adequately document the source of the infection [2, 5, 12–14].

In order to determine if strains of *M. fortuitum* resident in municipal drinking water in Brisbane, Australia were a likely source of infections with this

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organism in the community, we undertook a prospective study (2007–2009) to compare isolates from municipal water and infected patients resident in the area supplied by that water.

METHODS

During 2007–2008 citywide water sampling of about 220 sites (trunk main, reservoir and distribution water samples) was performed and 19 isolates were identified as *M. fortuitum* using 16S rDNA gene fragment sequencing [15]. A home sampling study of water, swabs and aerosols from the homes of non-tuberculous mycobacteria (NTM) patients was also conducted during 2009–2010 and one isolate of *M. fortuitum* was recovered from a shower aerosol [16]. Water isolates were stored in Dubos broth at −20 °C and were thawed and subcultured onto 7H11 plates as well as Lowenstein–Jensen slopes and incubated at 35 °C until sufficient growth was available.

Human samples from patients with a residential address within the water distribution catchment, were submitted to the Mycobacterium Reference Laboratory during the same time period (2007–2009) and were digested and decontaminated using 4% NaOH, neutralized with phosphoric acid and centrifuged at 3000 ***g*** to concentrate the acid-fast bacilli (AFB). Smears were prepared from the sediment and stained by the Ziehl–Neelsen (ZN) method. One Lowenstein–Jensen slope (±pyruvate) and 7 ml Mycobacterial Growth Indicator Tube (MGIT) were inoculated and incubated at 35°C until growth was detected. ZN staining of colonies confirmed AFB. Multiplex polymerase chain reaction (PCR) [17] was performed to discriminate between *M. tuberculosis, M. avium, M. intracellulare, M. abscessus* and other *Mycobacterium* spp. Isolates identified as other *Mycobacterium* spp. were further speciated using Hain Lifescience's GenoType® Mycobacterium AS (additional species) kit (2004–7 only) (Hain Lifescience, Germany) and/or 16S rRNA sequencing in conjunction with phenotypic characteristics. Patients' clinical information was obtained from the NTM database at the Queensland Tuberculosis Control unit. Additional isolates from patients living outside the water distribution network were included for comparison.

The clonality of clinical and water *M. fortuitum* isolates was determined using a repetitive sequence-based PCR (rep-PCR) method (Diversilab® system, bioMérieux, Australia). DNA was extracted from clinical and water isolates using the Ultraclean Microbial DNA Isolation kit (MoBio Laboratories, USA). The PCR mixture was prepared using AmpliTaq polymerase and PCR buffer (Applied Biosystems, USA) and *Mycobacterium* Diversilab primer mix according to the manufacturer's instructions. Separation and detection of rep-PCR products was performed by micro-fluidic chips of the Diversilab system. Fingerprints were analysed with Diversilab software v. 3.4.38 using the Pearson correlation coefficient and unweighted pair-group method with arithmetic means to compare isolates and determine clonal relationship. Pulsed-field gel electrophoresis (PFGE) was performed on 10 clinical isolates and a control strain and the results compared to the patterns generated by automated rep-PCR.

PFGE was performed using the method outlined in the BioRad Genpath® Group 6 kit (BioRad, France) with modifications outlined by Mazurek *et al.* [18] and Burki *et al.* [19]. Organisms were inoculated into 10 ml Middlebrook 7H9 broth (Difco, Becton Dickinson and Company, USA, in-house media) supplemented with 0·2% OADC (Difco, Becton Dickinson and Company), 0·1% Tween-80 (MP Biochemicals LLC, USA), 1 mg/ml cycloserine (Sigma-Aldrich, USA) and 0·1 mg/ml ampicillin (Sigma-Aldrich) and incubated for 3 days. One millilitre of broth was centrifuged and the supernatant discarded.

Gel plugs were prepared and incubated in 500 μl lysis buffer 1 and 20 μl lysozyme (25 mg/ml) at 36°C. Following a wash step, 500 μl Proteinase K buffer and 20 μl Proteinase K (>600 U/ml) were added to each sample. Plugs were incubated for 48 h at 50°C. The plugs were then washed four times in 1x wash buffer. After the final wash, the plugs were stored in 1x wash buffer. Digestion was performed using *Xba*I enzyme (10 U/ml) and the samples were incubated for 18 h at 36°C.

The plugs were loaded into wells of a 1% PFGE agarose (BioRad) electrophoresis gel ensuring there were no air bubbles. Sufficient 0·5x TBE was added to the electrophoresis cell and cooled to 14°C and electrophoresis performed using the following parameters: initial A time (i.e. switch time) 1 s, final A time 40 s, voltage 200 V and time 22 h. The gel was stained using ethidium bromide (BioRad), de-stained in running distilled water for 30 min and then photographed.

Based on the Tenover classification of isolates using PFGE, the Diversilab rep-PCR similarity cut-offs were determined as >97% (indistinguishable), >95% similar, and <95% different.

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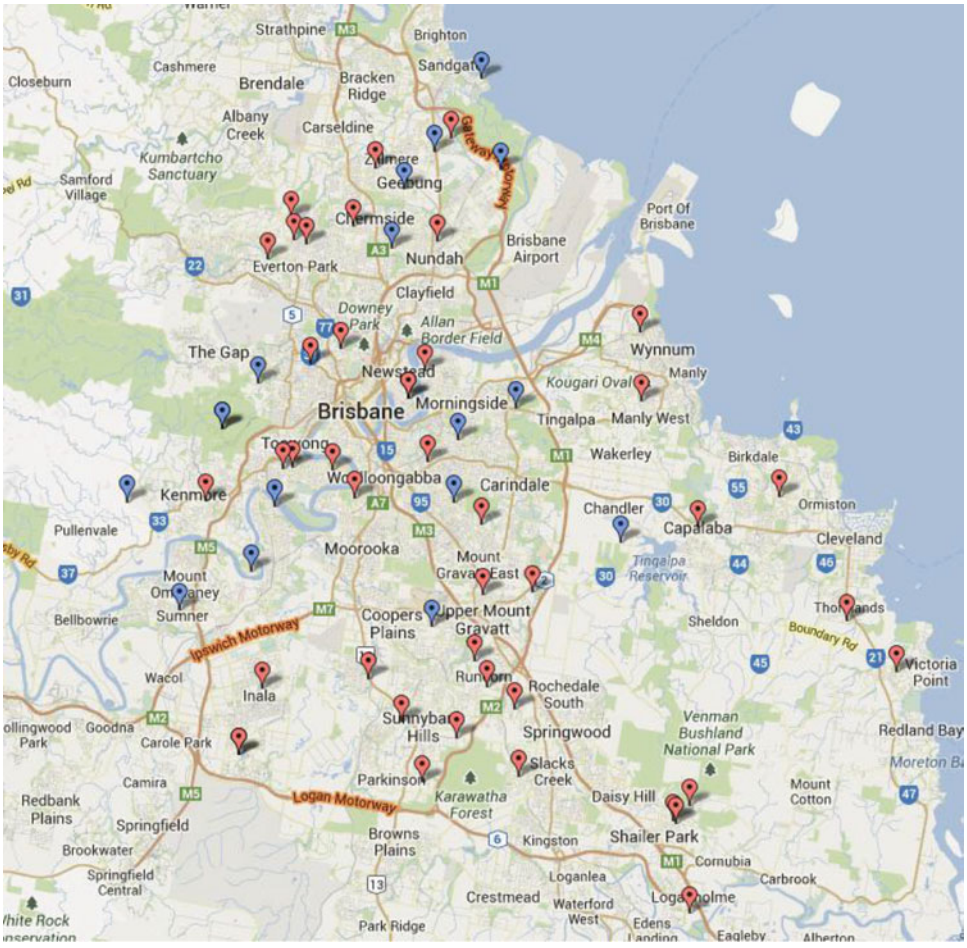

Fig. 1 [*colour online*]. Map of Brisbane area with water sites indicated by blue symbols and patient locations indicated by red symbols. (Map data © 2013 GBRMPA, Google.)

RESULTS

The geographical distribution of water sites and patients' residential addresses is demonstrated in Figure 1. The clinical isolates came from 53 patients, 27 (50·9%) of whom were male. There were 27 pulmonary isolates (50·9%, 24 considered not to be causing invasive disease, and three associated with significant disease according to ATS/IDSA criteria [20]). Twelve patients had underlying bronchiectasis and four had cavities by chest radiograph. There were 26 soft tissue isolates (five insulin injection site infections, three laparoscopic band infections, two isolates from blood associated with line infections, two surgical wound infections, and 14 other community-acquired soft tissue infections).

PFGE and rep-PCR were performed on 10 isolates of *M. fortuitum* associated with laparoscopic gastric band infections. Isolates from the same patient that were indistinguishable by PFGE, were associated with a >97% similarity cut off by rep-PCR. (See Figures in supplementary online material.)

Three of the water isolates had low-intensity band patterns generated by rep-PCR and were removed from the analysis. There was a wide variation in strain types evident with 41 different patterns generated by 70 isolates (Fig. 2). These formed 13 clusters (P1–13) of two or more isolates; the remaining patterns consisted of single isolates only (P14–41). These clusters were further grouped according to similarity of ~95% into seven groups. Of the larger clusters, P4 consisted of nine clinical isolates, eight of which were associated with soft tissue infections. P5 (a cluster similar to P4), however, consisted of six isolates, five of which were pulmonary isolates and the other associated with an injection site infection.

There was no evidence of geographical clustering of strains apart from the two isolates making up P6 that came from patients living in adjacent suburbs. A husband and wife both presented with soft tissue

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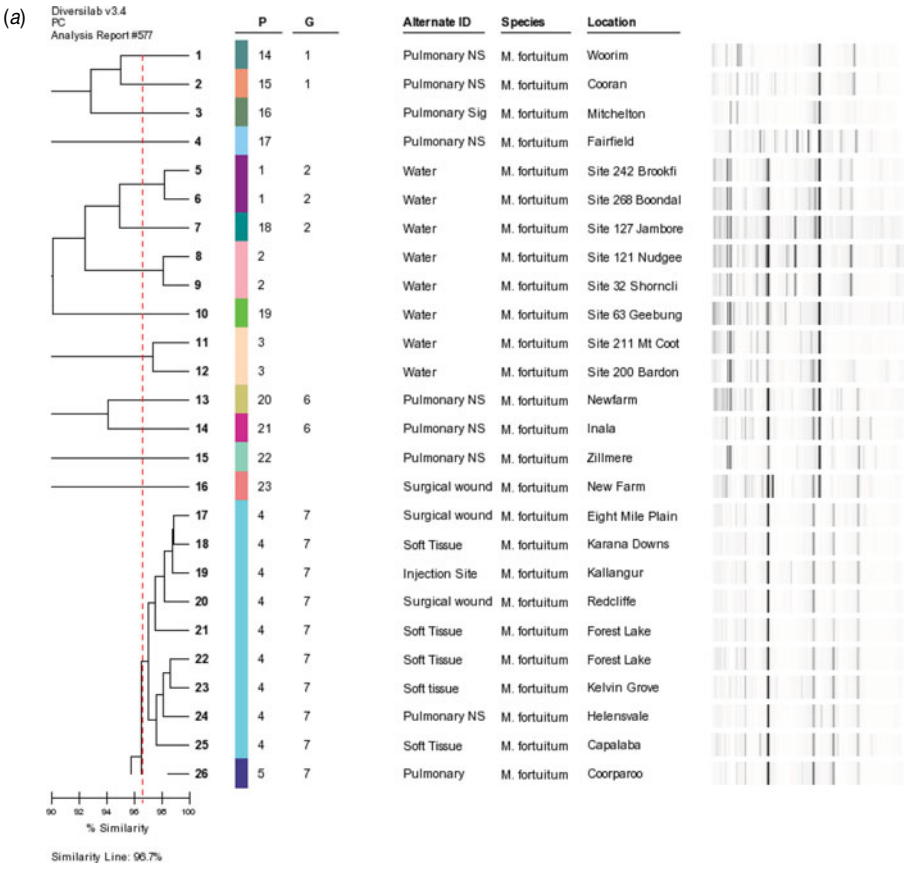

Fig. 2 [*colour online*]. (*a–c*) Rep-PCR dendrograms of *M. fortuitum* water and clinical isolates. Red indicates interrupted line of similarity at 96·7%. Coloured bars under P represent indistinguishable patterns (⩾97% similarity), grouped under G into similar groups (⩾95% similarity). NS, Non-clinically significant isolates; Sig., clinically significant isolates. (Figure continues on next page.)

infections of the lower limbs and had indistinguishable isolates (P4, nos. 21 and 22). The water isolate groups were distinctly separate from the clinical isolates, best appreciated by viewing the scatterplot in Figure 3.

DISCUSSION

The aim of this study was to determine if strains of *M. fortuitum* resident in the municipal water were likely to be the source of sporadic infections with *M. fortuitum* in the community. The correlation with PFGE and the wide variety of strain types identified confirms the utility of rep-PCR as a discriminatory tool for this species. No patient isolates were indistinguishable from water isolates. There did appear to be a dominant group of clinical strains, with a degree of non-significant clustering according to infection type (P4 predominantly soft tissue and P5 predominantly pulmonary) but this finding may have occurred by chance as the clusters P4 and P5 were similar. The water strains in contrast formed three separate groups that differed from all clinical isolates.

Other studies have demonstrated heterogeneity in strains of *M. fortuitum* using a variety of molecular techniques, including PFGE [13, 21–24], 16 s-23 s rRNA ITS genotyping, RAPD and ERIC-PCR [21] mainly in the investigation of nosocomial outbreaks. Legrand *et al.* [22] examined 51 isolates of *M. fortuitum* from 47 patients in Guadeloupe, Martinique and French Guiana between 1996 and 1999, suspecting a microepidemic. Only five isolates were found to cluster, confirming significant genomic heterogeneity in clinical strains and suggesting a 'diversity of ecological niches in which this organism may develop'.

One of the first reports of nosocomial transmission of *M. fortuitum* through water was published by Burns *et al.* [4] who investigated a cluster of cases of positive sputum cultures in patients in an alcoholic rehabilitation ward. The common factor in cases was usage of two ward showers, and *M. fortuitum* was isolated

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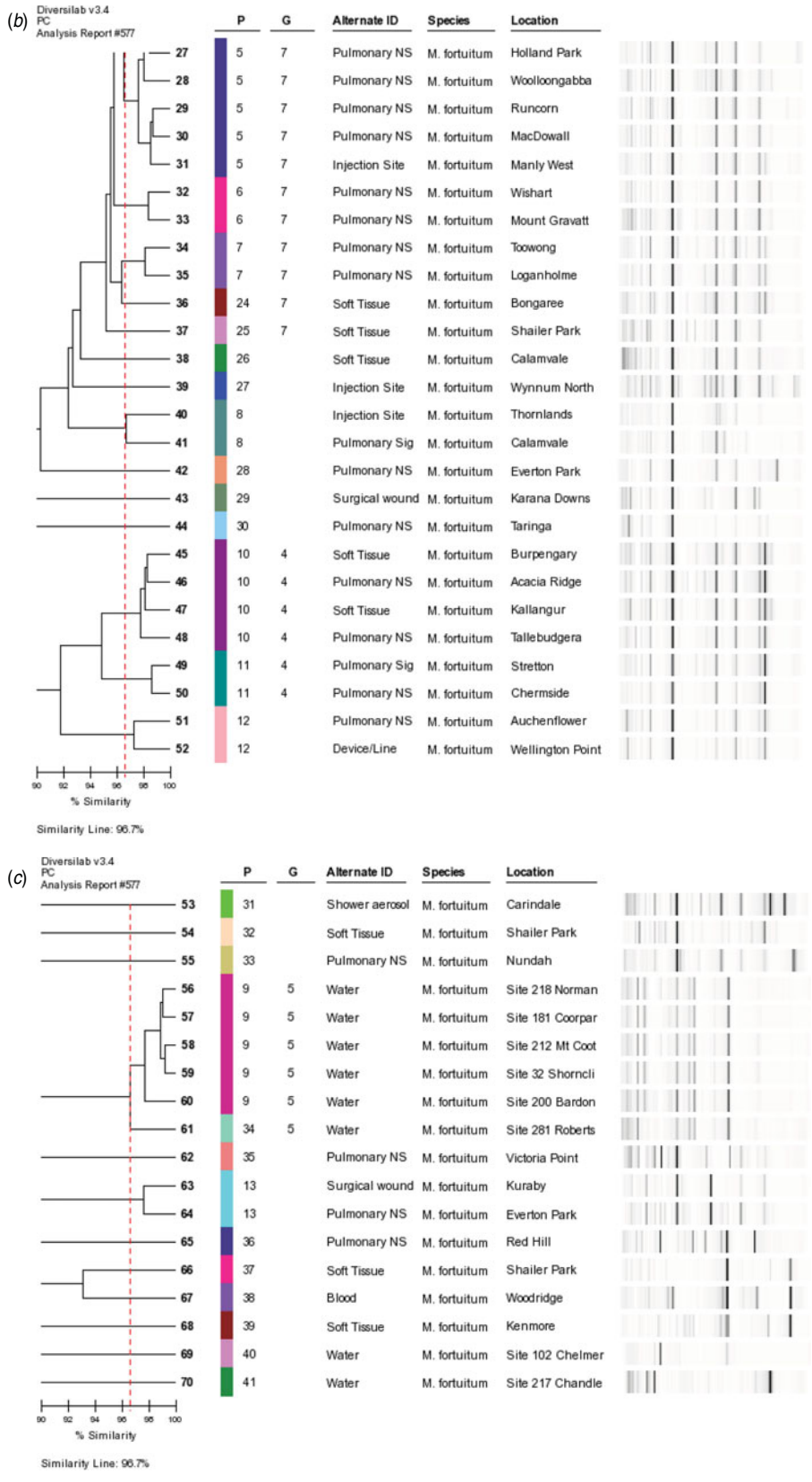

Fig. 2 (*cont.*). For legend see previous page.

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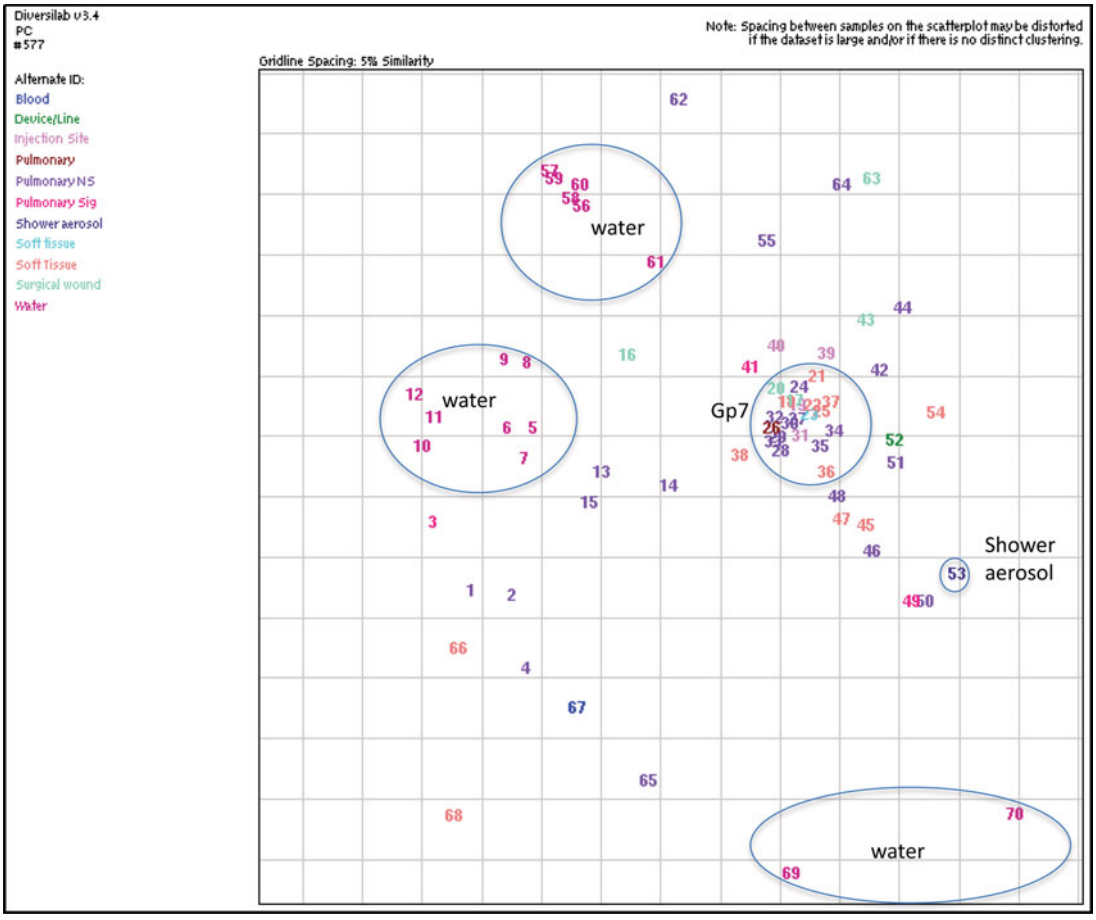

Fig. 3 [*colour online*]. Scatterplot of numbered *M. fortuitum* isolates, colour coded according to site of infection, with water isolates in pink (legend at top left). Gridline spacing correlates with 5% similarity. NS, Non-clinically significant isolates; Sig., clinically significant isolates.

from the tap water connecting to the showers but not from the showers themselves. Using PFGE, the 16 case isolates were found to be identical to the water isolate. However, there were no other parts of the hospital where such cases were identified and no further cases were reported after the showers had been disconnected and decontaminated.

Wallace *et al*. investigated the sources of *M. fortuitum* associated with infections following cardiac bypass surgery [13] across 12 states in the USA. In the analysis of a particular outbreak in Texas in 1981, one isolate of *M. fortuitum* was recovered from an operating room waterbath and had a similar phenotype and enzyme electrophoretic pattern as a cardiac associated isolate. A similar isolate was also recovered from the municipal water coming into the hospital and from an ice machine on one of the hospital floors. Two patients with other surgical wound infections (neck and abdominal) were infected with the same strain. In the 5 years following that outbreak an additional 21 clinical isolates of *M. fortuitum* were recovered from patients in the same hospital. Six were studied. All differed in phenotypic markers or plasmid profile from the outbreak isolates.

In a separate outbreak (Colorado 1976), an isolate of *M. fortuitum* recovered from a settle plate in an operating room had the same phenotype and electrophoretic pattern as four of the epidemic strains.

Winthrop *et al.* [23] reported 110 cases of furunculosis acquired following pedicures at a single nail salon in California (*M. fortuitum* cultured from 32 cases). All patients had their feet and lower legs soaked in a whirlpool footbath. Large amounts of hair and skin debris were found behind the inlet suction screens of the whirlpool footbath and *M. fortuitum* was cultured from these areas from all 10 footbaths. The authors concluded that *M. fortuitum* had entered via the salon tap water, seeded the accumulated organic debris behind the footbath inlet screens then multiplied and circulated within the footbath basin. They felt

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that because all of the footbaths yielded rapid growers including multiple strains of *M. fortuitum* that it was unlikely the baths were contaminated by a client. Based on our results and the fact that NTM are so prevalent in soil, it could be argued that in the absence of identical strains of *M. fortuitum* being discovered in the salon tap water (only *M. chelonae/abscessus* were isolated), it is highly likely that the footbaths were contaminated by soil or organic matter from the feet of clients. The salon owner reported that these inlet suction areas were never cleaned.

The heterogeneity of strains we have demonstrated, suggest that if water is the vehicle for transmission of infection, then point-source contamination probably occurs from another environmental reservoir such as soil or dust. Such soil organisms can readily enter water distribution systems via cracks in pipes caused by tree roots or other trauma, or may contaminate surface source water and survive disinfection.

Many nosocomial outbreaks in the literature have been reported in the midst of long periods without NTM surgical infections. If municipal water was the source of organisms, then it could be postulated that a transient drop in disinfection or an increase in contamination must have occurred. It seems more likely that episodic point-source contamination may be responsible.

The susceptibility of *M. fortuitum* to quaternary ammonium disinfection was studied by Cortesia *et al.* [10] and there were phenotypic and presumably genotypic changes in those strains that persisted after exposure to disinfection. It is possible that the differences we noted between clinical and water strains occurred because we only detected strains that survived exposure to disinfection in municipal water and that strains responsible for some clinical infections are protected by survival in amoebae or biofilm inside pipes and hence were not detected by our sampling approach. The changes that occur in strains upon exposure to the chlorine/chloramine disinfection used in drinking water have not been studied adequately to permit comment on whether this theory is plausible [10].

In summary, we have compared isolates of *M. fortuitum* resident in a municipal drinking-water distribution system, to those found to cause community and medically acquired infections. We have confirmed significant heterogeneity in strains of *M. fortuitum* from patients and municipal water. These results and appraisal of previous studies, would suggest that most community infections probably originate in soil/dust, and nosocomial outbreaks occur as a result of point contamination of water sources. However, further comparison of strain types found in soil and other environmental sources (such as amoebae and biofilm) are needed to prove this is the case.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268813003257.

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DECLARATION OF INTEREST

None.

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