Short report

Investigation of two cases of *Mycobacterium chelonae* infection in haemato-oncology patients using whole genome sequencing and a potential link to the hospital water supply.

Inkster T [1], Peters C [1], Seagar AL [2], Holden MTG [3], Laurenson IF [2]

[1] Queen Elizabeth University Hospital, Glasgow

[2] Scottish Mycobacteria Reference Laboratory, Royal Infirmary of Edinburgh

[3] School of Medicine, University of St Andrews

Corresponding author; Dr Teresa Inkster, Department of Microbiology, Queen Elizabeth University Hospital, 1345 Govan Road, Glasgow, G51 4TF.

Email; teresaink@hotmail.com

Telephone; 0141 9544192

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Summary

Haemato-oncology patients are at increased risk of infection from atypical mycobacteria such as *M. chelonae.* Atypical mycobacteria are commonly found in both domestic and hospital water systems. We describe the utility of whole genome sequencing in the investigation of two patient cases of *M. chelonae* , and positive water samples result from a hospital water supply. We discuss relevant control measures and the potential for chemical dosing of water systems to enhance proliferation of atypical mycobacteria.

Introduction

Mycobacterium chelonae is an environmental opportunistic mycobacterium found in hospital water systems and household plumbing.[1] It belongs to the non-tuberculous (atypical) group of mycobacteria and is classified as a rapid grower. [2] Clinically it can cause cutaneous lesions, bacteraemia and invasive infections of bone and lung.

We describe WGS analysis of *M. chelonae* isolates from patients and hospital water samples following investigation of *M. chelonae* infection in two paediatric haemato-oncology patients during a 12-month period. One patient presented with a bacteraemia and the other with skin lesions in proximity to a Hickman line site. Both children are now in remission from original disease. Analysis of data in the preceding ten years for all Glasgow hospitals revealed four other cases of *M. chelonae* bacteraemias in haemato-oncology patients (one in each of the following years: 2011, 2013, 2016 and 2018). Three of these were in a different city hospital campus.

Methods

Water testing

In response to a second case of *M. chelonae* infection in a 12-month period water testing of outlets (pre- and post-flush) in wards and departments where patients had been nursed was undertaken. Water was also taken from basement storage tanks and the incoming mains supply .100ml of water was filtered on to Middlebrooks agar and incubated aerobically at 35°C for 42 days. Plates were examined weekly for evidence of bacterial growth. If bacteria were present smear microscopy using pre- and post-flush staining was performed. AFB smear-positive samples were sent to the Scottish Mycobacteria Reference Laboratory for identification. Clinical and water isolates were identified using GenoType Mycobacteria CM v2.0 (Hain Lifescience, Nehren, Germany) after Genolyse extraction, according to the manufacturer`s instructions.

Whole genome sequencing (WGS) of patient and environmental isolates

There is no recognised molecular typing scheme available for *M. chelonae* to assess genetic relatedness. The mutation rate for *M. chelonae* is unknown, and published literature on the utility of WGS for investigation of *M. chelonae* is difficult to find, so this investigation was performed on a research basis only.

Briefly, genomic DNA was extracted from heat-inactivated mycobacteria using a modified QIAamp DNA Mini kit (Qiagen, Hilden, Germany) protocol using silica-glass beads (adapted from Köser *et al*., 2013).[3] Following Nextera XT DNA library preparation (Illumina, Cambridge, UK) paired-end sequencing was performed using 500 v2 Illumina MiSeq kits producing 2 x 250bp reads.

Reference-based mapping to was utilised to determine the genetic relationship between different strains based on single nucleotide polymorphism (SNP) analysis. To identify SNPs, sequence reads were aligned to a reference genome of *M. chelonae* CCUG47445 (accession number CP007220) [4] using SMALT (http://www.sanger.ac.uk/science/tools/smalt-0). The default mapping parameters and SNP filtering have as previously described. [5] Recombination was detected in the genomes by using Gubbins (http://sanger-pathogens.github.io/gubbins/). [6] The results of Gubbins were used to filter out SNPs predicted to be associated with recombination and identify core genome SNPs.

Phylogenetic reconstruction using core SNPs was performed with RAxML (version 8.2.8) [7], using a GTR model with a gamma correction for among site rate variation.

To look more closely at the genetic relationship between each of the clades, reads were then mapped to a *de novo* genomic assembly of a representative of each clade i.e., a more similar sequence than *M. chelonae* CCUG47445. Fastq files from MiSeq sequencing were assembled *de novo* with Velvet (Zerbino and Birney, 2008). [8] This has been done in WGS investigations of other mycobacteria to increase the number of successfully mapped reads thus improving the accuracy of the mapping. [9]

Results

147 unfiltered water samples from outlets (taps and showers) in two paediatric haemato-oncology inpatient wards and an operating theatre complex were tested between 16/4/19 and 24/6/19. Atypical mycobacteria, subsequently identified as *M. chelonae*, were detected from all three areas. 68 of 147 (46%) water samples from outlets tested positive with 34/68 (50%) having counts > 100 cfu/ml. An additional five samples were taken from water storage tanks (post filtration) and all were negative. Three mains samples were taken by Scottish water and one of these tested positive for *M. chelonae.* Multiple other Gram negative isolates (predominantly *Cupriavidus pauculus*) and fungi were also isolated from water as previously described[10]. Whole genome sequencing was undertaken on 31 isolates.

The WGS results showed that there were two clonal populations within the group (Clades 1 and 2; Figure 1). The pairwise core genome SNP distance distinguishing the two clades is ~20,000 SNPs illustrating that they do not share a recent common ancestor and are genetically distinct. Isolates within each Clade appeared to be closely related (<100 SNPs SNP differences distinguish the isolates in each clade), which is suggestive that each population had separate recent common ancestors. The isolate from Patient 1 was genetically diverse from the isolates in Clade 1 and Clade 2 and therefore considered unrelated; the SNPs distance distinguishing patient 1 from Clade 2, the genetically closest Clade, was approximately 2,000 SNPs. In Clade 1, the isolate from Patient 2 was firmly placed amongst the isolates sampled from the environment although on a longer branch. The environmental isolates appear to be closely related (in terms of SNP differences), with some shown to be indistinguishable from each other. The maximum pairwise SNP distance distinguishing isolates in this Clade was 19 SNPs. In addition, a number of environmental isolates (3854, 3123, 3856, 3800, 3846, 3862 and 3808) are closely related to Patient 2 as shown by SNP distances of 6 to 8 SNP on the tree. (Figure 2). The positive water sample from the mains supply was in Clade 1 and unrelated to patient isolates.

Discussion

M. chelonae was first discovered from the lung tissue of sea turtles by Friedmann in 1903.[11] The organism is ubiquitous in the environment but is a rare cause of infection. Most commonly it is associated with skin lesions but immunosuppressed patients such as haemato-oncology patients may present with bacteraemias and disseminated infections. *M. chelonae* has been found in rivers, lakes, sea water, wastewater from hospitals and drinking water samples. [12] Atypical mycobacteria in water systems are more resistant to chlorine, chlorine dioxide and chloramine than other organisms.

[13] Warm water in premise plumbing is ideal for their proliferation and there is extensive surface available for biofilm formation. [13]

In some studies water samples obtained from homes had lower rates of cultures yielding NTM than hospitals. Rates of atypical mycobacterial colonisation of potable water systems ranged from 60-100% in hospitals, haemodialysis units and dental offices. [14] In one study in Berlin which tested two hospitals and four homes, mycobacteria were isolated predominantly from hospitals. Over 50% of samples from both hospitals contained mycobacteria compared to only 9% from a home environment.[15] *Mycobacterium gordonae* was the most common atypical mycobacterium isolated, *M. chelonae* was found also.

With regards to virulence factors *M. chelonae* and other NTM have a lipid rich outer membrane which aids survival in the environment. [16] This membrane also protects from the effects of high temperature and disinfectants. NTM demonstrate surface hydrophobicity enabling attachment to surfaces where they will grow in biofilm. The surface hydrophobicity also means that they are readily aerosolised which is one of the routes of transmission to patients.[16] Direct contact with contaminated water can also lead to infection.

Few nosocomial outbreaks of *M. chelonae* have been reported. The first reported outbreak of nosocomial infection with an identified source was in patients undergoing liposuction in the United States.[17] Twelve patients were infected and the organism was traced to plumbing in the procedure room. Multiple sources of contamination were identified involving rinsing of instrumentation in tap water and inadequate disinfection/sterilisation processes.[17] In another large outbreak involving 35 patients with laparoscopy port site infections the source of *M. chelonae* was traced to water stored used for rinsing instruments. [18] The organism was found in biofilm in the base of disinfectant trays and in the outer sleeves of reusable instruments. No further cases were detected following dismantling and manual cleaning of laparoscopic equipment followed by ethylene oxide gas sterilisation.[18] A contaminated humidifier was implicated in four cases of *M. chelonae* eye infections in patients who had undergone a LASIK (laser eye) procedure. Three of the four patient isolates were indistinguishable on PFGE from the humidifier isolate with the 4th being closely related. No further cases were detected after disposal of the humidifier and upgrading of the air handling system. [19]

In oncology patients' atypical mycobacteria can cause exit site infections, tunnel infections or catheter related bacteraemias, some of which can lead to disseminated infection. [20] Outbreaks and isolated cases of atypical mycobacteria have been described in haemato-oncology patients with links to the hospital water. Five patients in one adult haemato-oncology ward developed bloodstream infection secondary to atypical mycobacteria, four cases with *Mycobacterium mucogeniucm* and one with *Mycobacterium canariasense*. Both of the organisms were identified in the water supply to the ward and were identical to patient isolates on 16S-rRNA sequencing. [21]

In another paediatric setting a 20-year review of oncology patient in a US Children's hospital combined with a literature review of other reported cases, described 85 atypical mycobacteria infections in this patient group. Eighteen of these which were disseminated infection and 42 cases were central line associated blood stream infections (CLABSI). 31% of the CLABIS infections were associated with hospital outbreaks. [22]

In a paediatric haemato-oncology ward five patients developed *M. mucogenicum* blood stream infection over a six-month period. Samples of water from taps indicated they were the likely source and this was confirmed by typing. [2] In this incident levels of chlorine in the water were intermittently low and may have contributed to bacterial growth. A review of exit sites of central lines revealed

these were not properly covered during bathing which may have facilitated colonisation. [2] Similarly, following a cluster of atypical mycobacterial infections in haemato-oncology patients no more cases were identified following the introduction of central venous catheter control measures despite the bacteria continuing to be identified in the water system. [22] These control measures included new connectors and a line dressing which could remain in- situ, covering the line site whilst patients showered. [23] A single case of disseminated *Mycobacterium fortuitum* infection in a leukaemia patient was investigated and linked to shower head water in one hospital. [24]

Control measures for atypical mycobacteria in water systems include; increasing the water temperature to 55°C, application of point of use filters (POUF), replacement of showerheads to those with large holes and monthly disinfection of showerheads. Aerators should also be removed from taps. [16] In our hospital POUFs were in situ on our haemato-oncology wards following a previous water incident and these were subsequently extended to all areas that patients might visit in the hospital. A programme of regular disinfection of showerheads was also undertaken. Quarterly cleaning and disinfection of the showerhead and hose was undertaken via a service exchange procedure. Following removal, parts were dismantled and physically cleaned. They were then submerged in a solution of Showerhead Plus *Legionella* specific descaler and biocide for a minimum contact time of two minutes. Showerheads in particular offer a moist, warm, and nutrient rich environment for atypical mycobacteria to proliferate and form biofilms. [25] In one study biofilms from showerheads were highly contaminated with atypical mycobacteria, 100-fold above background water contents. [20]

Our hospital was a new build hospital, which opened in 2015. It is not clear whether atypical mycobacteria were present prior to 2019 testing or whether their presence and detection has been enhanced by the introduction of a Chlorine dioxide dosing system in late 2018 to control a widespread water contamination issue .[10] . On completion of each stage of the installation chemical treatment of the domestic cold water system commenced at a level of 1.0mg/l for 24-48 hours. Once the residual level of chlorine dioxide reached 0.1mg/l at each part of the system microbiological monitoring commenced. Once the residual reached 0.2mg/l at each part of the system the associated plant output was reduced in stages until the residual achieved 0.4mg/l and the plant output was between 0.5-0.7mg/l as a final continual water treatment baseline value. The domestic hot water system presented an additional challenge as chlorine dioxide when exposed to high temperatures gasses off. Therefore, the treatment was set at 2-4mg/l to achieve a residual of 0.5-1.0mg/l at hot water outlets.

The WGS results show that there are two main populations of *M. chelonae* within the group of sampled isolates. The limited number of SNP differences observed within each population suggests that the diversification may have happened fairly recently, but it is not possible to confirm the time scale over which this has occurred without understanding the mutation rate of the organism and/or the time of sampling. Our WGS results suggest that the isolate from one patient (Patient 2) is closely related to environmental isolates from water outlets. Epidemiologically this fitted, with the patient linked in time and place to these outlets. Whilst there was no link established with patient one, there were no contemporaneous water results available from the time this patient developed infection so a water source in the hospital cannot be completely excluded. Whilst there were POUF on outlets in wards there were none in theatre anaesthetic rooms where patients underwent procedures such as line insertions. There was therefore a splash risk from proximity of sinks. This highlights the importance of applying POUF throughout the full patient pathway.

In conclusion haemato-oncology patients are at increased risk of infection from atypical mycobacteria such as *M. chelonae*. Atypical mycobacteria are a common finding in hospital water

systems and a single case of atypical mycobacteria infection should prompt consideration of water as a source. Water systems which are being treated with disinfectants may be of particular risk as they remove competing organisms and enable atypical mycobacteria to proliferate. Whole genome sequencing is a promising adjunct for the investigation of outbreaks due to atypical mycobacteria.

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Figure 1 – **Diversity of the sampled *M. chelonae* population.** The Maximum likelihood phylogenetic tree was built using core SNPs with SNPs in regions of recombination removed. Sequence data was mapped to the *M. chelonae* CCUG47445 reference. The tree is midpoint rooted and 2 clades identified that are distinguished by less than 100 SNPs. The approximate SNP distance that distinguishes the two clades is approximately 20,000 SNPs.

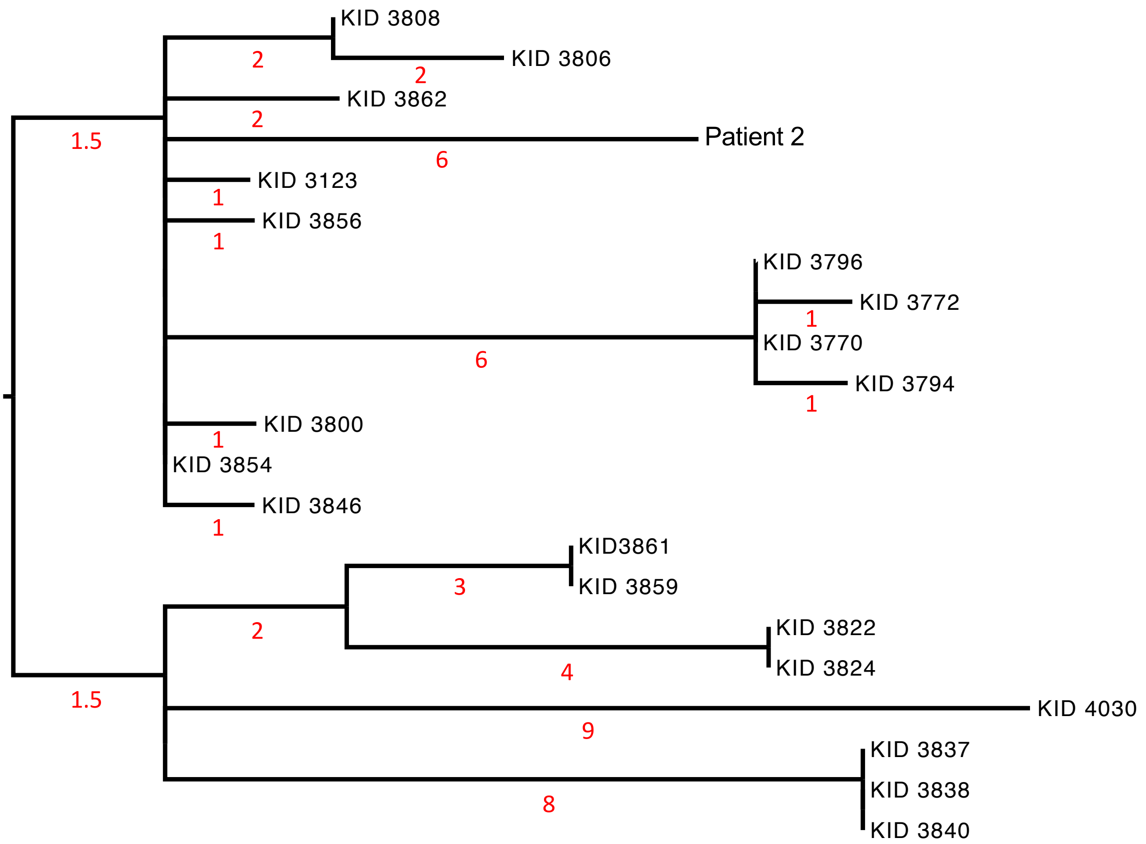

Figure 2 - **Diversity of the Patient 2 and environmental *M. chelonae* population.** The Maximum likelihood phylogenetic tree was built using core SNPs. Sequence data was mapped to a *de novo* assembly of Patient 2 isolate reference. The tree is midpoint rooted and the SNP distances associated with the branch lengths are indicated in red text.