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Effect of monochloramine treatment on the microbial ecology of *Legionella* and associated bacterial populations in a hospital hot water system

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ABSTRACT

Opportunistic pathogens, including *Legionella* spp. and non-tuberculous mycobacteria, can thrive in building hot water systems despite municipal and traditional on-site chlorine disinfection. Monochloramine is a relatively new approach to on-site disinfection, but the microbiological impact of on-site chloramine use has not been well studied. We hypothesized that comparison of the microbial ecology associated with monochloramine treatment versus no on-site treatment would yield highly dissimilar bacterial communities. Hot water samples were collected monthly from 7 locations for three months from two buildings in a Pennsylvania hospital complex supplied with common municipal water: (1) a hospital administrative building (no on-site treatment) and (2) an adjacent acute-care hospital treated on-site with monochloramine to control *Legionella* spp. Water samples were subjected to DNA extraction, rRNA PCR, and 454 pyrosequencing. Stark differences in the microbiome of the chloraminated water and the control were observed. Bacteria in the treated samples were primarily Sphingomonadales and *Limnohabitans*, whereas *Flexibacter* and Planctomycetaceae predominated in untreated control samples. Serendipitously, one sampling month coincided with dysfunction of the on-site disinfection system that resulted in a *Legionella* bloom detected by sequencing and culture. This study also demonstrates the potential utility of high-throughput DNA sequencing to monitor microbial ecology in water systems.

Introduction

Contamination of a hospital's water supply with waterborne pathogens such as *Legionella* has been shown to be a source of infection for hospitalized patients [32]. The case fatality rate of healthcare associated Legionnaires' disease is quite high, ranging from 38% to 53% [33]. Supplemental disinfection of the water distribution system in a healthcare facility is an effective approach to prevention of this mode of transmission [21,33]. Many options for disinfection exist, including copper–silver ionization, chlorine dioxide, point-of-use-filtration, hyperchlorination, and UV light; however, each of these methods has benefits and shortfalls [21,33].

Water treatment with monochloramine has been used at the municipal level but is a new strategy for supplemental disinfection at the building level and has not been extensively evaluated in long-term studies [21,33]. A recent study in Italy evaluated the use of monochloramine in one hot water network of a hospital's hot water distribution system [23]. They found that monochloramine significantly reduced the levels of *L. pneumophila* without a major change in nitrite and nitrate concentrations, but had no effect on *P. aeruginosa* [23]. However, the total microbial composition in hospital hot water systems treated with monochloramine, in contrast to those with no secondary disinfection, remains largely unknown. The on-site monochloramine generation system used in this study reduced the overall percentage of sites that were culture positive for *Legionella* spp. (distal site positivity) and total bacterial

Abbreviations: PCR, polymerase chain reaction; BCYE, buffered charcoal yeast extract; DGVP, buffered charcoal yeast extract with dyes, glycine, vancomycin, and polymyxin B.

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concentrations but did not significantly alter mycobacterial concentrations [7].

Culture-based protocols for assessing microbial populations require organism-specific conditions and make population studies complicated and expensive. High throughput sequencing technologies provide an approach to identify many types of bacteria in parallel. Sequence-based approaches can characterize entire microbial populations in biofilms, water, and aerosols of water distribution systems and hospitals [2,9,10,16,22,25,38]. These methods identify the presence of bacterial taxa by sequencing segments of their DNA in a culture-independent manner. We previously examined the effects of monochloramine treatment over time within this treated building and saw a significant shift in the microbial ecology of the community before and during treatment [3]. In this previous study, we were unable to compare the effects of treatment on community composition in samples taken simultaneously with the same source water or the effects of seasonality, especially that of the summer months, which can affect initial community structure [3].

In this study, we seek to further investigate the effects of monochloramine on the microbial ecology of a hospital's hot water system compared to an untreated control building using high throughput sequencing. Our study is the first to examine the shift in bacterial assemblages between common source water and monochloramine treated water due to on-site chloramination in a hospital's hot water system using high throughput sequencing. Characterization of the selective pressures of monochloramine on bacterial populations may yield new information to assess the risks and benefits of this disinfection strategy based upon changes in bacterial ecology, including the populations of waterborne pathogens.

Materials and methods

Hospital setting and monochloramine system

This study was conducted in a hospital complex in Pittsburgh, PA. The complex consists of a 12-story, 495-bed tertiary care facility and an 11-story administrative building. Both facilities are supplied by the same chlorinated municipal water source but have independent circulating hot water systems (Fig. 1). The hospital's hot water system had been treated using a monochloramine generation system since September 2011 (Sanipur, Lombardo, Flero, Italy) [7]. The administrative building received no supplemental water treatment and served as an appropriate physically adjacent control to determine the shift between the microbial ecology of the source water and the monochloramine treated building.

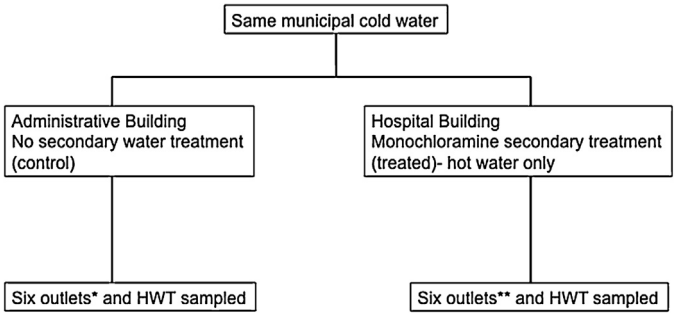

Fig. 1. Diagram of the sampling plan. * Immediate and Post-flush 1 L hot water samples taken from outlets on floors 3, 7, 8, 9, 10, and 11 in May, June and July 2012. ** Immediate and Post-flush 2 L hot water samples taken from outlets on floors 2, 8, 9, 10, 11, and 12 in May, June, and July 2012.

Sample collection and water processing

Immediate-draw (or "first-catch") hot water samples were collected in sterile Nalgene high density polyethylene (HDPE) bottles (Thermo Scientific; VWR) from seven sites (six faucets and the hot water tank) from each of the two buildings monthly in May, June, and July 2012 (Fig. 1). Due to low microbial biomass in the monochloramine treated building, two liters of water were collected, whereas one-liter samples were collected from the untreated control building (Fig. 1). In addition, a second sample was collected from each outlet after a one-minute water flush to assess the differences in microbial populations at the site versus upstream in the pipe (Fig. 1). The temperature of each sample was taken using an infrared thermometer (MiniiiIR Traceable; Control Company; Fisher Scientific). The monochloramine and free chlorine concentrations of the treated and control samples were tested using a Hach DR/890 Colorimeter using Monochlor F Reagent (Hach) and DPD Free Chlorine Reagent (Hach), respectively. Collected water was filtered through 0.2 μm, 47 mm, Supor® 200 Polyethersulfone membrane disc filters (Pall Corporation) housed in sterile, single-use Nalgene filter funnels (Thermo Scientific; Fisher). Filter membranes were folded and stored at −80 °C until DNA extraction.

Samples of the adjacent sink or hot water tank were collected in sterile HDPE bottles with enough sodium thiosulfate to neutralize 20 ppm of chlorine (Microtech Scientific) for enumeration of *Legionella* and total heterotrophic bacteria. Culturing for *Legionella* spp. and total heterotrophic bacteria was performed according to standard methods using BCYE and DGVP agar plates for *Legionella* spp. [17] and R2A media for total bacteria [4].

DNA extraction and PCR

Genomic DNA was extracted from filter membranes using a bead-beating, phenol-chloroform extraction as described previously [6,30]. Test PCR was performed for confirmation of successful extraction using universal 16S ribosomal RNA primers 515F and 1391R [20,30]. A 1% agarose gel was used to confirm the presence of an appropriate PCR product.

Barcoding, pooling, and sequencing

DNA was amplified in triplicate with barcoded bacterial PCR primers 8F and 534R that included adaptors for the Roche 454 sequencing platform [19]. A negative PCR control was performed for each barcode, and PCR was repeated for any sample where the control was positive. Amplicons were pooled after normalization of DNA concentration using the Invitrogen SequalPrep Kit [13] and sequenced using the Roche 454 FLX Titanium platform per the manufacturer's instructions (University of Pittsburgh Genomics and Proteomics Core Laboratories). Sequence data were submitted to NCBI and are available under accession number SRP035587.

Data analysis

Sequence reads were assigned to sample of origin using the bar code sequence added during PCR and screened for basic quality defects (short sequences <200 nucleotides [nt] in length; >1 nt ambiguity, best read with quality ≥20 over a 10 nt moving window) by the software program BARTAB [11]. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32) [8] using the Schloss Silva reference sequences [31] were removed from subsequent analysis. Filtered sequences (308,799 sequences; average 4173 sequences/sample) were aligned and classified with SINA (1.2.11) [26] using the 244,077 bacterial sequences in Silva 111NR [28] as reference configured to yield the Silva taxonomy (tax_slv). Sequences with identical taxonomic assignments were clustered to