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Examination of microbial contaminants of emergency showers and eyewash stations

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To evaluate the effects of regular flushing, water from fifty emergency eyewash and shower stations was cultured for the presence of potentially pathogenic protozoa, heterotrophic bacteria, and *Legionella* species. This study also provided the opportunity to evaluate a commercially available molecular assay for the direct detection of *Legionella* sp in environmental samples. The Perkin Elmer Legionella EnviroAmp polymerase chain reaction (PCR) kit and culture on buffered charcoal yeast extract agar were used to detect *Legionella* species in water samples. Chemical and physical parameters of station water measured included: pH, hardness, alkalinity, turbidity, conductivity, total chlorine and assimilable organic carbon. Protozoal isolates were identified by classical identification methods, and isolates from the stations were identified as *Hartmannella* sp, *Vexillifera* sp, *Vahlkampfia* sp, *Acanthamoeba* sp, and *Vanella* sp. Heterotrophic plate counts ranged from 10^2 to 10^6 CFU ml^{-1} and acridine orange total counts ranged from 10^3 to 10^6 cells ml^{-1} after regular flushing. PCR and gene probe analysis showed that 89% of the stations (eyewash and shower) were positive for *Legionella* species by PCR, while 6% of the samples were culture positive. These results indicate that routine flushing alone is not sufficient to control microbial contamination and disinfection must also be included in a routine maintenance program. In addition, regular maintenance, disinfection, and monitoring of emergency eyewash and shower stations is important in preventing potential secondary microbial infections by either direct inoculation or aerosol transmission.

Keywords: emergency shower; eyewash stations; PCR; *Legionella*; protozoa

Introduction

Emergency eyewash and shower stations are relied upon to provide a cleansing stream of water in personal injuries resulting from chemical spills, burns or other accidents. Since many of these emergency showers and eyewash stations go unused for months to years, a significant microbial load can accumulate in the pipes and hoses in the form of biofilms [19]. Primary organisms of concern include protozoa and a number of opportunistic bacterial pathogens including *Legionella* and *Pseudomonas* sp. The primary route of transmission for *Legionella* is through the inhalation of contaminated aerosols, although wound infections have also been reported [6,8,14]. One study found that 90% of the *L. pneumophila* cells which were recovered from showerhead and faucet aerosols were in the 1–5 mm range, in the size range to penetrate the human lower respiratory tract and initiate infection [4]. Similarly, emergency showers can produce aerosolized water droplets in a similar particle size that may represent not only a route of transmission but also a potential health risk to the end user.

Certain species of free-living amoebae belonging to the genera *Acanthamoeba* and *Naegleria* are well established 'opportunistic pathogens' capable of causing disease and death in humans [17]. Another species of protozoa, *Hartmannella* is involved in a complex interaction with *Legionella*, by providing an ideal intracellular multiplication and amplification niche. In addition, these protoza provide growth-supporting factors and shield the internalized *Legionella* from unfavorable environmental conditions (ie disinfection) [13,20,25].

One month prior to the initiation of this study, regular flushing of the stations, as suggested by the American National Standards Institute (Z358.1–1981) and [23] was implemented to evaluate the effects of regular flushing on microbiological water quality. This study was undertaken to determine if immediate implementation of a maintenance program might preclude the need for costly repairs or disinfectant applications. Prior to the initiation of this study, all 50 stations were subjected to regular flushing (bi-monthly for several minutes). Flushing consisted of turning the units on for a period of 3–5 min to flush the stagnant water from the supply line.

Materials and methods

Sample collection

Sample volumes of 100–250 ml were collected in sterile polypropylene sample bottles directly from the opening of the eyewash station or emergency shower. Samples for Assimilable Organic Carbon (AOC) were collected in 1-L acid-washed and baked glass bottles, and AOC calculations were determined as previously described [10]. A total of 50 emergency station water samples were collected, 25 eyewash stations and 25 emergency showers. In addition, a single floor drain sample was also collected to serve as a control. Parallel tap water samples were also collected as controls. Samples were collected from two states and from eight different buildings (which all contained copper plumbing systems). The eight buildings represented three

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different municipal water supplies, all three similar in terms of water quality. The plumbing serving the emergency showers and eyewash stations in the eight buildings sampled could be broken down into three age groups, <2 years, <12 years, and >40 years. Only stationary eyewash and shower stations were examined. The eyewash units included in the study were all twin eyewash head units and the shower units were overhead (singlehead) drench type showers supplied by a vertical water supply line (in house copper plumbing). Both the eyewash and shower stations were constructed of stainless steel.

Measurement of chemical and physical parameters

Sample pH was recorded using a Corning pH meter (model 125) (Corning, NY, USA). Chlorine concentrations were determined by using the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) titration method (American Public Health Association, Standard Methods for the Examination of Water and Wastewater 1985). Hardness, alkalinity, turbidity, and conductivity were all measured according to the Standard Methods for the Examination of Water and Wastewater [1].

Protozoal identification

Samples were concentrated by centrifugation ($1500 \times g$) for 5 min to a final volume of 2 ml of which 100 μl was analyzed. Minimal agar plates (15 g agar (Difco Laboratories, Detroit, MI, USA), 0.1 g each of malt extract and yeast extract) streaked with *Klebsiella pneumoniae* (ATCC 27889) were inoculated with 100 μl of the concentrate in duplicate and incubated at room temperature (21–22°C). In addition, a liquid medium, ATCC Medium 802 (rye grass cerophyll in distilled water) supplemented with 10^6 cells ml^{-1} *K. pneumoniae* was also inoculated with 100 μl of the sample concentrate. Samples were examined with a Leitz inverted microscope over a 2-week period. Amoebae were identified to the genus level based on morphological features, measurement of living trophozoites and cysts. Ciliates, flagellates, yeasts, fungi, and nematode eggs were also isolated. One fungal isolate was identified to the species level.

Bacterial enumeration

The heterotrophic plate count was determined by membrane filtration on R2A agar (Difco Laboratories) with incubation at 28°C for 7 days and by the pour plate method at 35°C for 48 h. Acridine Orange Direct Counts (AODC) were performed according to the method of Hobbie *et al* [11]. *Pseudomonas* species were identified by biochemical tests as previously described [19]. Assimilable Organic Carbon (AOC) measurements were made according to the method of van der Kooij *et al* [24].

PCR analysis for Legionella species

The EnviroAmp Legionella PCR kit obtained from Perkin-Elmer (Foster City, CA, USA) was used for the molecular detection of *Legionella* species as per manufacturer's instructions. Emergency shower and eyewash samples were analyzed directly, however a few samples contained inhibitors of PCR and required dilution (1 : 10 or 1 : 100) or treatment with bovine serum albumin (0.2% BSA). The BSA served to stabilize the AmpliTaq polymerase and may also act to bind inhibitors. Water samples were analyzed for *Legionella* species by filtering a minimum of 10 ml of each water sample. PCR was used to amplify specific sequences from a conserved region of *Legionella's* 5S rRNA [16] and from the mip (macrophage infectivity potentiator) gene specific for the *L. pneumophila* species [7]. Biotinylated amplification products 5S rRNA and/or mip, are detected following hybridization on nylon membranes to complementary immobilized probes with detection by streptavidin-horseradish peroxidase conjugate (via a reverse dot blot technique). The assay is able to detect approximately 10 *Legionella* cells ml^{-1} in the original sample, however, sensitivity can be enhanced by increasing the volume that is filtered. The sensitivity of the PCR assay was confirmed through seeding studies using *Legionella* ATCC type strains. Genus and species identification is achieved by interpreting the nylon membranes; two blue dots of equal intensity next to the 'L' and the 'P' symbols indicates the presence of *L. pneumophila*. Each strip has a positive and negative control, the intensity of the positive control equals 10^3 cells ml^{-1}. In these experiments, when the 'L' or 'P' dot was lighter than the internal positive control the sample was considered to contain less than 10^3 cells ml^{-1} in the original sample. When the 'L' or 'P' dot was darker than the internal positive control the sample was considered to contain greater than 10^3 cells ml^{-1} in the original sample. Based on the intensity of the dots, the assay is semiquantitative. The assay is able to detect 25 *Legionella* species.

Legionella culture and microbial analyses

All water samples were analyzed for *Legionella* by culture. One hundred-milliliter samples were filtered through 0.2-mm polyethersulfone-based membrane filters (Supor filters, Gelman, Ann Arbor, MI, USA), placed in 3 ml of filter-sterilized deionized water and vortexed for 60 s to dislodge any trapped bacteria. Following concentration, samples were subjected to acid treatment to eliminate non-*Legionella* organisms [5]. After acid treatment, 0.1 ml was spread plated in duplicate onto selective and non-selective media. *Legionella* samples were cultured on buffered charcoal yeast extract agar amended with alpha-ketoglutarate (BCYEα) alone or amended with glycine (3 g L^{-1}) and the following antibiotics: vancomycin (5 mg L^{-1}), polymyxin B (100 IU ml^{-1}), and cyclohexamide (80 mg L^{-1}) (BCYEαGVPC). Plates were incubated from 5–15 days at 37°C with 2.5% CO_2 in a humid environment. A sequential culturing method [21] was also employed in an attempt to improve the recovery efficiency of *Legionella* species. Colonies exhibiting *Legionella* morphology were transferred to BCYEα with and without cysteine; colonies which were unable to grow without cysteine were further tested by immunofluorescent staining.

Suspect colonies were placed in 5 ml of filter-sterilized water and 20 ml of each sample was added to one well of an eight-well toxoplasmosis slide (Bellco Biotechnology, Vineland, NJ, USA), air dried, and heat fixed. Next, 20-ml aliquots of a Poly II conjugate were added to each well. The conjugate contained antisera against: *L. pneumophila* serogroups 1–7 and *L. bozemaneii*, *L. dumoffii*, *L. jordanis*, *L. micdadei*, *L. gormanii*, and *L. longbeachae* serogroups 1 and 2 (Scimedix, Denville, NJ, USA). Following incu-

Table 1 Physical and chemical parameters associated with eyewash stations and emergency showers

Parameter	Eyewash stations				Emergency showers			
	Range	x[a]	s.d.[b]	*n*	Range	x	s.d.	*n*
HPC[c] (CFU ml^{-1}) MF	1.33×10^3–4.26×10^6	7.90×10^5	1.70×10^6	6	1.85×10^3–2.70×10^4	1.45×10^4	9.39×10^3	8
HPC (CFU ml^{-1}) PP	2.60×10^2–5.30×10^3	1.20×10^3	1.50×10^3	12	1.20×10^2–1.20×10^4	3.89×10^3	3.97×10^3	12
AODC[d] (cells ml^{-1})	1.68×10^3–4.06×10^6	4.11×10^5	9.97×10^5	18	3.71×10^3–7.14×10^6	9.98×10^5	2.20×10^6	20
pH	7.57–8.08	7.83	0.16	18	7.26–8.37	7.96	0.31	20
Hardness	146–452	289.47	94.95	17	150–514	296.58	108.11	20
Alkalinity	89–164	134.41	26.43	17	87–158	128.1	24.19	20
Turbidity (NTU)	0.10–1.60	0.43	0.42	17	0.26–2.80	1.92	3.86	20
Conductivity (*mho*-cm^2)	480–730	681	67.7	17	460–930	687	117	20
Total chlorine (ppm)	0.00–0.41	0.10	0.13	18	0.00–0.10	0.01	0.01	20
AOC[e] (μg L^{-1})	15.52–126.34	68.4	45.42	4	62.47–117.87	60	31.02	3

[a]Mean.
[b]Standard deviation.
[c]HPC = heterotrophic plate count by MF, membrane filtration; PP, pour plate method; CFU ml^{-1}, colony forming units per milliliter.
[d]AODC = Acridine Orange Direct Count.
[e]AOC = Assimilable Organic Carbon.

bation at 37°C for 30 min, slides were washed in phosphate-buffered saline (pH 7.6), rinsed in distilled water and air dried. Slides were prepared for microscopy and analyzed under an epifluorescent microscope (Olympus Model BH-2, Lake Success, NY, USA).

Results and discussion

Water quality measurements

Table 1 presents the results and basic statistics of the chemical and physical parameters of the water collected from emergency showers and eyewash stations. Total chlorine levels were undetectable for many samples, with average values of 0.10 ppm for eyewash stations and 0.01 ppm for emergency showers. It is not surprising that chlorine residuals were not detected in many of the stations, since the water often remains in the pipes for extended periods. If the stations are flushed for 5 min, chlorine levels similar to the tap water controls are detected. Mean turbidity measurements were higher in emergency showers (1.92 NTU) than in eyewash stations (0.43 NTU). Higher shower turbidities may be due to the force at which water is expelled, possibly causing debris from the biofilm to slough off, whereas the eyewash samples are expelled upward, against gravity. Visible turbidity (debris and rust) was observed in 15/50 (30%) of all stations. The mean total chlorine level of the tap water controls was 0.9 ppm and the total bacterial count ranged from negative (no growth) to 1.3×10^4 CFU ml^{-1} on R2A media.

Heterotrophic plate counts (HPC) for the eyewash stations were slightly higher by the membrane filtration method than those of emergency showers, however, AODC measurements for both were similar as seen in Table 1. Seven samples were subjected to Assimilable Organic Carbon (AOC) analyses. AOC are carbon compounds which are easily assimilable (utilizable as food source) by waterborne bacteria and may be responsible for bacterial regrowth or increased multiplication of indigenous microorganisms in response to increased nutrient availability. It has been suggested that an AOC concentration of less than 10 mg L^{-1} would be sufficient to maintain biological stability [24]. Mean AOC values of 45 mg L^{-1} (eyewash stations) and 31 mg L^{-1} (showers) were detected in this study, however one eyewash sample had an AOC value of 126.34 mg L^{-1}. Increased AOC values did not always correspond to the highest HPC counts, since the indigenous microorganisms may have been unable to utilize some of the recalcitrant forms of carbon present.

Protozoal and other microorganism identification

Of the fifty samples collected, twenty-six were examined for the presence of protozoa, fungi, yeasts, cilitates, flagellates, and nematodes. Table 2 presents the number of times a sample was positive for various protozoa. By far the most frequent isolate of eyewash stations was *Hartmannella* species, the same was true for emergency showers. Several samples were positive for four different genera of protozoa. Seventy-six percent of the 26 eyewash samples examined were positive for at least one species of protozoa. Examination of a sample from the furnace room floor drain was found to be positive for multiple protozoa (see Table 2). Two showerhead samples were positive for a filamentous

Table 2 Distribution of protozoa in eyewash stations, emergency showers, and furnace room drain

Amoebae sp	Eyewash stations	Emergency showers	Furnace room
Acanthamoeba	1[a]	1	1
Cochliopodium	0	0	1
Hartmannella	7	4	1
Mastigina	1	0	0
Naegleria	0	0	1
Vahlkampfia	2	1	1
Vannella	2	1	1
Vexillifera	1	3	1
Number of samples	12	13	1
Percent positive	76.9%	46.2%	100%

[a]Number positive for amoebae species listed.

fungi identified as *Fusarium moniliforme*. Two other eyewash samples were positive for yeast, while one was positive for nematode eggs and only the floor drain sample was positive for ciliates.

In contrast to another study [23], where a mixture of *Hartmannella* and *Acanthamoebae* were primary isolates of eyewash stations, our study only found *Acanthamoebae* in two out of 25 (8.0%) stations. In previous studies [3,19] *Hartmannella* was the most prevalent protozoa identified, as it was in this study 11/25 (44.0%). Tap water control samples were negative for protozoa, *Legionella* (by PCR and culture), yeast, and fungi.

Legionellae are able to survive and multiply within the vacuoles of certain species of protozoa (trophozite form) until the protozoal cell bursts, however if adverse environmental conditions force the protozoa to encyst, the *Legionella* are shielded until conditions for excystation exist [2,9,25]. In the cyst state protozoa and associated *Legionella* are extremely resistant to temperature extremes, desiccation and disinfection. For example, it has been demonstrated that *L. pneumophila* within *Acanthamoeba polyphaga* can survive exposure to 50 mg L^{-1} of free chlorine [13]. Given the association of protozoa in biofilms combined with the ability to encyst and resist disinfection, eradication efforts are extremely difficult.

Legionella species detection by PCR and culture

The use of the EnviroAmp allowed the detection of *Legionella* species at concentrations as low as 10 cells ml^{-1}. The PCR is semiquantitative, and samples yielding a positive at the same intensity as the internal positive control contain approximately 1000 *Legionella* cells ml^{-1}. Subsequently darker positives contain greater than 1000 cells and faint positives are 'graded' less than 1000 cells or 10–1000 cells ml^{-1}. Table 3 examines the concentration of *Legionella* species in eyewash and emergency shower samples respectively. It is interesting to note that 22% of eyewash samples and 30% of emergency showers are positive for *Legionella* species at concentrations of 10–1000 cells ml^{-1}. *Legionella pneumophila* was also more frequently detected in eyewash stations than in emergency showers and interestingly amoebae were also more frequently detected in eyewash stations (76.9%) than in shower (46.2%). This increased detection may be due to the smaller pipe diameter and increased surface area of the eyewash stations. The number of eyewash stations positive for *Legionella* was slightly greater in this study (94.4%), than previously reported (87.5%) [19]. The previous studies utilized direct fluorescent antibody staining which may have missed *Legionella* present in low concentrations due to shielding of the organisms by debris. Another study of ground, surface and potable waters for the presence of *Legionella* showed similar results in that there was a much higher rate of positives when samples were analyzed by PCR in comparison with culture or DFA [15]. This may be due to the increased sensitivity and increased number of species (over DFA) provided by the PCR assay. Despite inclusion of the sequential culturing method, *Legionella* isolates were only recovered from three samples, one eyewash and two shower. Of the three positive samples, only one (showerhead) was recovered through repeated culturing. This is not surprising given the recent report of inhibition of *Legionella* species by bactericins secreted by HPC bacteria [22]. Legionellae are also known to exist in a viable but nonculturable state when exposed to unfavorable environmental conditions [12].

Non-culturable *Legionella* cells have also been shown to cause legionellosis. In one report by Miller *et al* [18] an outbreak of Pontiac fever at a health resort was traced to a contaminated hot tub. *Legionella* cells were only detected by PCR and DFA, culture results during the outbreak were uniformly negative. Interestingly, about 7 months after the outbreak the sand filters tested culture positive for the same serogroup of *Legionella* that was implicated in the outbreak [18]. In light of this information, even emergency stations which contain dead *Legionella* cells should be flushed and cleaned thoroughly, followed by routine monitoring employing culture and DFA or culture and PCR for the detection of *Legionella*.

The results of this study clearly show that initiation of a routine maintenance program, consisting of bi-monthly flushing, is not sufficient to control microbial contamination of emergency stations. More aggressive disinfection methods including hyperchlorination and heat shock treatments should be investigated to prevent contaminated stations especially in older buildings. In addition, the use of large volume, sterile water, self-contained single-use units should be investigated in high risk areas (industrial chemical plants, university chemistry laboratories).

Table 3 EnviroAmp PCR results of eyewash stations and emergency showers

Legionella sp (No. of cells)	Eyewash stations		Emergency showers	
Concentration	*Legionella* sp	*L. pneumophila*	*Legionella* sp	*L. pneumophila*
PCR Negative[a]	1	6	3	18
10–1000	4	9	6	2
= 1000	7	2	3	0
> 1000	6	1	8	0
No. positive	17	12	17	2
Total No.	18	18	20	20
Percent positive	94.4%	66.6%	85.0%	10.0%

[a]Indicates that the positive and negative controls worked and the samples were not inhibited.

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