Factors Affecting Colonization of *Legionella* sp. in Water Distribution Systems

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Abstract

Repeated and extensive sampling of a cluster of buildings that are in close proximity to the each other indicate that some buildings are chronically colonized with *Legionella* while others continue to be free of this pathogen. All of these buildings receive municipally treated water from a common source. This observation has challenged us to investigate which environmental parameters (e.g., plastic vs copper piping, building age, etc.) and environmental variables (temperature and disinfectant residual) might allow *Legionella* to colonize certain potable water systems and not others. This information can be used to assign risk factors to potable water systems that are part of a Water Management Plan.

Introduction

Chlorine and thermal treatments are the most commonly used methods to control and prevent *Legionella* amplification in building water systems. *Legionella* occurs in water systems at temperatures in the range of 68 ^{0}F to 122 ^{0}F. Elevating the temperature of hot water storage tanks is thought to be an effective measure to control *Legionella* growth in health care facilities and other high risk facilities but studies suggest that the hot water temperature must be greater than 122 ^{0}F at outlets (Bédard et. al, 2016; HSE, 2014). The Veterans Health Administration (VHA) requires that all VHA-owned facilities where patients, residents or visitors stay overnight maintain water temperatures at 124 ^{0} F or higher in hot water circulating systems to inhibit *Legionella* growth (VHA, 2014). However this does not preclude the possibility that hot water emanating from distal sites such as faucets and showers will be within the permissive growth range of *Legionella*.

Chlorine is a disinfectant used by facilities for routine treatment of both hot and cold domestic water. It can be applied to both the cold and hot water distribution system. Chlorine's effectiveness against microorganisms depends on its concentration in the system and the retention time.

In this study, we examined the effects of water temperature, disinfectant residual levels, and water use frequency on the presence of *Legionella* in a large mid-west hospital's potable water system. Only hot water systems were sampled in this project. These systems are re-circulating in nature. Therefore, if a biofilm is present in one location, it can colonize the entire distribution system through dispersal mechanisms and cause contamination of distal sites. Cold water systems, on the other hand, flow unidirectionally.

Materials and Methods

Potable Water Sample Collection

One liter (1 L) potable first draw hot water sample from faucets were collected in sterile wide-mouth screw cap polypropylene plastic bottle containing 150-200mg sodium thiosulfate preservative.

Swab Sample Collection

If swab and water sample were required from the same device, water samples were collected prior to swabbing any surface. A sterile swab (TransPorter, Hardy Diagnostics) was used to sample biofilms.

Preparation of Samples for Bacteriological Examination

Filtration of Potable Water Samples

Five hundred ml (500 ml) of each potable water sample was filter-concentrated using a 47-mm filter funnel assembly disinfected with 80% isopropyl alcohol between uses and containing a sterile 0.22 μm polycarbonate filter. After filtration, the filter was removed aseptically from the holder with sterile filter forceps, folded to the outside, and placed into a sterile, 50-ml centrifuge tube containing 5ml of sterile Butterfield's buffer. The centrifuge tube was then vortexed for one minute at maximum speed to elute bacteria from the filter.

Swab Samples

The surface to be swabbed was moistened with either water from the device being sampled or the sterile buffer inside the sterile swab tube. When possible, about 2 square inches of the surface was swabbed using a back and forth motion. The swab was then placed into a sterile tube containing 1 ml of buffer.

Acid Treatment

Because water samples may contain high concentrations of non-*Legionella* bacteria, it was necessary to use a selective procedure to reduce their numbers before culture (CDC, 2005). Two hundred (200) μl of the vortexed suspension was placed into a sterile 1.5 ml centrifuge tube containing 200 μl of acid buffer. The suspension was then incubated for 5 minutes at room temperature. For swab samples, 100 μl of the suspension was placed into a sterile 1.5 ml centrifuge tube containing 100 μl of acid buffer and treated as described above.

Media for *Legionella* Growth and Isolation

Buffered charcoal yeast extract (BCYE) agar containing 0.1% alpha-ketoglutarate was used as the base medium used for the recovery of *Legionella* (1). Two types of selective BCYE agar were used in the processing of the samples. The first was designated BCYE complete with antibiotics (purchased from Hardy Diagnostics); the second, BCYE complete without antibiotics.

Plating of Samples

Plates (described above) were inoculated with 0.2 ml of either acid-treated or non-acid treated suspension and distributed over the agar surface with a plastic spreader. They were then incubated at 36 ^{o}C in a humidified incubator for 14 days at a minimum humidity of 95%.

Examination of Cultures for *Legionella*

Plates were examined after 72 to 96 hours of incubation for *Legionella*. Suspect *Legionella* colonies were streaked onto BCYE agar plate without L-cysteine and antibiotics, and a positive control BCYE agar plate without antibiotics. The plates were incubated for 24-48 hours. Colonies that grew on BCYE agar, but not BCYE agar without L- cysteine, were considered to be presumptive *Legionella* species and later serotyped using the Dry Spot ™agglutination test (Oxoid, Dardilly, France) or direct fluorescent antibody (MTech).

Preparation of Samples for Analysis by Real-Time Polymerase Chain Reaction (RT-PCR)

DNA Extraction

DNA was extracted from swab samples using the Aquadien™ Bacterial DNA Extraction and Purification Kit (Bio Rad). One hundred (100) µl of the swab sample was added to a cryotube containing 2 ml of R1 solution. The contents were mixed by vortexing and incubated at 95°C for 15 min. The tube was then vortexed for 20 sec. and left at room temperature for 20 min. Five hundred (500) µl of the supernatant was added to the purification column contained within a collector vial and centrifuged for 10 min. at 6000 x g. The collector vial was emptied and an additional 500 µl of the supernatant was added to the purification column. The tube was centrifuged for 10 min. at 6000 x g. Fifty (50) µl of R2 solution was added to the purification column and the collector vial was discarded. The purification column was covered with a new clean collector vial, turned upside down and centrifuged at 1000 x g for 3 min. The purification column was discarded. DNA extracts were stored at - 20°C until they were used for qPCR assays.

Quantitative PCR (qPCR) Analysis

All qPCR assays were performed using a 7900 HT Fast Real-Time Sequence Detector (Applied Biosystems, Foster City, CA). Reaction mixtures (20 µl) contained 10 µl 2X qPCR Master Mix (Applied Biosystems), TaqMan Environmental Master Mix 2.0 for qPCR with 0.08 µmol/l TaqMan probe (final concentration), 0.2 µmol/l primers and 2 µl of template DNA. The primers and probes used in the assay were as described in Lu et. al (2015). The sample was then held for 10 min at 95°C to denature the template DNA. The following quantification cycling protocol was used: 40 cycles at 95°C for 15 s and 55°C for 30 s with an extension at 72°C for 30 s, and a final hold at 72°C for 5 min. In addition, the TaqMan Exogenous Internal Positive Control Reagents (a VIC-labelled probe) manufactured by ABITM (Life Technology) was also used as a secondary confirmation. The baseline cycles were set from three to 15 and the threshold value at the point where fluorescence exceeded ten times the standard deviation of the mean baseline emission. According to this standard, a setting of threshold 0.2 was used.

Free Chlorine

Free chlorine was determined by using an EPA approved method based on the use of DPD reagent. A kit sold by Hach Company uses a DR890 spectrophotometer and a DPD reagent designed to test for free chlorine in 10 ml samples.

Results and Discussion

The growth of *Legionella* within a facility's plumbing system is influenced by factors such as the system's plumbing materials, water temperature from the hot water system, and disinfectant residual concentrations. In this study, our principal hypothesis was that the colonization of a large hospital facility by *Legionella* is dependent on water temperature, free chlorine residual concentration at the distal sampling site of a building's plumbing system and use frequency of the site. We also hypothesized that *Legionella* colonization of a site (such as a point-of-use outlet) is promoted by the presence of components made of materials such as plastic and rubber. In the following sections, we discuss our findings within the context of our hypotheses.

Effects of Temperature of Hot Water Systems and Free Chlorine Concentration on Colonization of Hospital Buildings by *Legionella*

Buildings were designated as positive for *Legionella* if any of the building locations tested were positive for *Legionella* by culture. Conversely, buildings were designated as negative for *Legionella* spp. if all of the building locations tested negative for *Legionella* by culture. Free chlorine delivered by the municipal public water supply is the only source of disinfectant in both the hot and cold water systems. The first draw average temperatures of the two building types were more significant ($p=1.7 \times 10^{-4}$) than the average temperatures of the two-minute purge samples ($p=3.1 \times 10^{-2}$; Table 1). It is interesting that even though the first draw and two-minute purge temperatures for the two building types were significant ($P<0.05$), the average temperatures (95.08 °F to 111.4 °F for buildings positive for *Legionella,* and 90.87 °F to 109.93 °F for buildings negative for *Legionella*) are within the temperature range that promotes growth of this organism. Furthermore, the first draw mean temperature of buildings positive for *Legionella* was 95.08 °F. According to the Centers for Disease Control (CDC), the optimum temperature for the growth of *Legionella* is 95 °F which is in agreement with our results (Fields et. al., 2002). Therefore, the temperature of the hospitals' hot water systems is conducive to growth of *Legionella*.

Table 1: Effect of Temperature on *Legionella* Colonization of Hospital Buildings in Hot Water Systems

Prevalence of *Legionella*	Number of Observations	First Draw, °F Mean (Range)	2-Minute Purge, °F Mean (Range)
Buildings positive for *Legionella*	374	95.08 (55.4-136)	111.4 (65.3-140.5)
Buildings negative for *Legionella*	491	90.87 (58-131)	109.93 (65-150)
P value		$1.7X10^{-4}$	3.1×10^{-2}

The mean free chlorine residuals of first draw (0.18 mg/L and 0.26 mg/L for *Legionella* positive and negative buildings, respectively) and 2-minute purge samples (0.26 mg/L and 0.32 mg/L for *Legionella* positive and negative buildings, respectively) were consistent with residual levels that would be expected in a public water supply system (Table 2). However, the first draw ($p=1.2X10^{-4}$) and 2-minute purge ($p=8.0X10^{-3}$) mean free chlorine concentrations of the two building types were significant ($P<0.05$). These results indicate that *Legionella* may not be as tolerant to chlorine as other studies suggest. The 2-min purge free chlorine residual mean for buildings positive for *Legionella* spp. (0.26 mg/L) is identical to the first draw free chlorine residual mean for buildings negative for *Legionella* spp. (0.26 mg/L). This indicates that microbicidal effect of free chlorine is significantly effective even at a relatively low concentration of 0.26 mg/L. A first draw sample is more representative of water people are exposed to. This further emphasizes the importance of maintaining a free chlorine concentration of 0.26 mg/L or greater in the hot water of a building's distribution system in order to prevent amplification of *Legionella*.

Table 2: Effect of Free Chlorine Concentration on *Legionella* Colonization of Hospital Buildings in Hot Water Systems

Prevalence of *Legionella*	Number of Observations	First Draw, mg/L Mean (Range)	2-Minute Purge, mg/L Mean (Range)
Buildings positive for *Legionella*	369	0.18 (0-0.1.8)	0.26 (0-1.31)
Buildings negative for *Legionella*	488	0.26 (0-1.37)	0.32 (0-1.37)
P value		$1.2X10^{-4}$	$8.0X10^{-3}$

Effects of Temperature of Hot Water Systems and Free Chlorine Concentration on Colonization of Building Sites by *Legionella*

The term (site) is used to describe any terminal hot water outlet and for all practical purposes refers to hot water faucets. The minimum detection level for a positive site was 0.05 CFU/ml and no distinctions or comparisons are made in the concentrations of *Legionella* obtained from a site. A site that yielded no detectable *Legionella* (i.e., below the minimum detection limit of 0.05 CFU/ml) is designated as Below Detectable Limit (BDL).

The mean temperatures of first draw samples were similar for sites positive and negative for *Legionella* but not for the 2 minute purge samples (Table 3). As discussed previously, the average temperatures (91.58 °F to 106.7 °F for sites positive for *Legionella,* and 90.87 °F to 109.93 °F for sites negative for *Legionella*) are within the temperature range that promotes growth of this organism. Therefore, the temperature of the hospital's hot water system is going to be conducive of *Legionella* spp. since raising the water temperature to 140 °F or greater is not a practical solution because of the possibility of scalding.

Table 3: Effect of Temperature on *Legionella* Colonization of Sampling Sites in Hot Water Systems

Prevalence of *Legionella*	Number of Observations	First Draw, °F Mean (Range)	2-Minute Purge, °F Mean (Range)
Sites positive for *Legionella*	112	91.58 (69.2-121.9)	106.7 (70-134)
Sites negative for *Legionella*	491	90.87 (58-131)	109.93 (65-150)
P value		$4.38X10^{-1}$	$3.9X10^{-2}$

The mean chlorine residuals of first draw (0.12 mg/L and 0.26 mg/L for *Legionella* positive and negative sites, respectively) and 2-minute purge samples (0.18 mg/L and 0.32 mg/L for *Legionella* positive and negative sites, respectively) were consistent with residual levels that would be expected in a public water supply system (Table 4). However, the first draw ($p=8.15X10^{-10}$) and 2-minute purge ($1.82X10^{-7}$) mean free chlorine concentrations of the two site types were significant ($P<0.05$). These results also suggest the importance of maintaining a free chlorine concentration of 0.26 mg/L or greater throughout a building's hot water system in order to prevent amplification of *Legionella*.

Table 4: Effect of Free Chlorine on *Legionella* Colonization of Sampling Sites in Hot Water Systems

Prevalence of *Legionella*	Number of Observations	First Draw, mg/L Mean (Range)	2-Minute Purge, mg/L Mean (Range)
Sites positive for *Legionella*	113	0.12 (0-0.79)	0.18 (0-0.94)
Sites negative for *Legionella*	488	0.26 (0-1.37)	0.32 (0-1.37)
P value		$8.15X10^{-10}$	$1.82X10^{-7}$

Effects of Temperature of Hot Water Systems, Free Chlorine Concentration, and Use Frequency on the Colonization of Building A by *Legionella*

The effects of temperature, free chlorine, and use frequency on the colonization by *Legionella* was examined in Building A which is prone to high rates of *Legionella* colonization (Table 5). There was not a significant difference in temperature between the north (high use) and south (low use) ends of the building with regard to colonization by *Legionella* ($p=8.2X10^{-3}$ for first draw and $p=4.24X10^{-2}$ for 2-minute purge). As discussed previously, the mean temperatures of first draw and 2-minute purge samples for the north (high use) and south (low use) ends of Building A are within the temperature range that promotes growth of *Legionella* spp.

Table 5: Temperature of Hot Water Systems and Use Frequency Affect *Legionella* Colonization of Building A

Use Frequency of Building A	*Legionella* Positive Sites	Number of Temperature Observations	First Draw, °F Mean (Range)	2-Minute Purge, °F Mean (Range)
North end (high use)	2 out of 11	10	109.36 (80.6-124.5)	119.30 (108-124.4)
South end (low use)	19 out of 43	30/15*	96.48 (62-121.9)	111.52 (73-124)
P value			$8.2X10^{-3}$	$4.24X10^{-2}$

* Number of observations for first draw and two minute purge samples were 30 and 15, respectively.

The mean chlorine residuals of first draw (0.672 mg/L and 0.197 mg/L for high use and low use sites, respectively) and 2-minute purge samples (0.650 mg/L and 0.350 mg/L for high use and low use sites, respectively) were consistent with residual levels that would be expected in a public water supply system (Table 6). However, there was a significant difference in both the first draw ($p=6.87X10^{-6}$) and two-minute purge ($p=4.65X10^{-3}$) average free chlorine concentrations between the north (high use) and south (low use) ends of the building (Table 6).

Based on these results, we can conclude that in Building A, the high use areas have lower *Legionella* spp. counts, higher hot water temperatures and higher free chlorine residual concentrations. Conversely, the low use areas in Building A have higher *Legionella* spp. counts, lower hot water temperatures and lower free chlorine residual concentrations.

Table 6: Free Chlorine of Hot Water Systems and Use Frequency Affect *Legionella* Colonization of Building A

Use Frequency of Building	*Legionella* Positive Sites	Number of Free Chlorine Observations	First Draw, mg/L Mean (Range)	2-Minute Purge, mg/L Mean (Range)
North end (high use)	2 out of 11	10	0.672 (0.12-0.86)	0.650 (0.08-0.81)
South end (low use)	19 out of 43	30/15	0.197 (0-1.15)	0.304 (0.01-1.3)
P value			$6.87\ X10^{-6}$	$4.65X10^{-3}$

* Number of observations for first draw and two minute purge samples were 30 and 15, respectively.

Examination of *Legionella* Colonization of Fixtures in Building A by Culture Method and Real Time Polymerase Chain Reaction (RT-PCR)

Swab samples of supply line fixtures in Building A were examined by culture and RT-PCR methods (Tables 7-9). Supply lines tested in a previous study had been implicated as the source of *Legionella* spp. in the water samples (Coughlin et. al., 2016). Therefore, we wanted to determine if the hot water supply lines in this study could be potential sources of contamination of water samples.

In Table 7, the highest number of *Legionella pneumophila* SG1 colonies, as determined by culture method, was observed in the blended or mixed water samples, followed by hot and cold water samples. A colony forming unit (CFU) is indicative of a single bacterium that has replicated to sufficient density to be visible on an agar plate. The higher percent of positive *Legionella* samples encountered in the blended waters compared to both the cold and hot water samples may indicate that water temperature at these locations is more conducive to *Legionella* growth. Furthermore, the non-metallic materials of construction present in an aerator are inherently more suitable for microbiological growth due to their hydrophobicity biodegradability and ability to retain water. It was suspected that *Legionella* spp. was not detected in the cold and hot flex hose supply lines because of the overgrowth of heterotrophic bacteria. Therefore, these samples were also tested by RT-PCR for the presence of *Legionella* spp. Only the hot flex hose supply line tested positive for *Legionella* spp. by RT-PCR.

Table 7: Detection of *Legionella* spp. in Swab Samples of From a Faucet with Separate Control Handles for Hot and Cold Water

Sample	Temp.	*Legionella pneumophila* SG1 (CFU/swab)	*Legionella spp.* (CFU/swab)	*Legionella spp. by* RT-PCR (Presence/ Absence).
Flex hose supply line	Cold	ND	ND	Absent
Copper pipe supply line	Cold	BDL	BDL	NT
Copper body of cartridge	Cold	BDL	BDL	NT
O-ring for cartridge seat	Cold	2	BDL	NT
O-ring for cartridge seal	Cold	1	BDL	NT
Flex hose supply line	Hot	BDL	BDL	Present
Copper pipe supply line	Hot	94	BDL	NT
Copper body of cartridge	Hot	BDL	BDL	NT
O-ring for cartridge seat	Hot	94	BDL	NT
O-ring for cartridge seal	Hot	BDL	BDL	NT
Faucet (upstream of aerator)	Mix	14	BDL	NT
Aerator water side	Mix	10	BDL	NT
Aerator gasket	Mix	200	BDL	NT
Aerator air side	Mix	100,000	BDL	NT

BDL: Below Detection Limit
ND: *Legionella* spp. colony counts were non-determinable due to overgrowth from competing bacteria.
NT: Not Tested

The results from a faucet with a single control handle for hot and cold water are presented in Table 8. All of the samples where *Legionella* spp. was non-determinable by culture method tested positive for *Legionella pneumophila* SG1 by RT-PCR. These results suggest that O-rings facilitate the growth of *Legionella* spp. especially in hot water systems.

Additionally, the ability to culture *Legionella* spp. from swab samples is often hindered by the overgrowth of heterotrophs. Therefore, this study shows that PCR, in conjunction with swab samples, is a more suitable method to detect *Legionella* spp. in biofilms.

Table 8: Detection of *Legionella* spp. from a Faucet with a Single Control Handle for Hot and Cold Water

Sample	Temp	*Legionella pneumophila* SG1 (CFU/swab)	*Legionella spp.* (CFU/swab)	*Legionella pneumophila* SG1 (GU/swab)
Copper pipe supply line	Cold	BDL	BDL	NT
Cartridge seat	Cold	ND	ND	211
O-ring for cartridge seat	Cold	BDL	BDL	NT
Copper pipe supply line	Hot	BDL	BDL	NT
Cartridge seat	Hot	ND	ND	233
O-ring for cartridge seat	Hot	40	BDL	NT
Copper pipe discharge	Mix	BDL	BDL	NT
Cartridge seat	Mix	BDL	BDL	NT
O-ring for cartridge seat	Mix	60	BDL	NT
Aerator screen: air side	Mix	ND	ND	17
Aerator screen: water side	Mix	ND	ND	1110
Aerator O-ring	Mix	ND	ND	885

GU: Genomic Unit is a calculated value that is indicative of a single bacterium that has had its DNA amplified by PCR.
BDL: Below Detection Limit
ND: *Legionella* spp. colony counts were non-determinable due to overgrowth from competing bacteria.
NT: Not Tested

The results from on-site swabbing of taps are presented in Table 9. Three of the five samples where *Legionella* spp. was non-determinable by culture method tested positive for *Legionella pneumophila* SG1 by RT-PCR. The three samples that tested positive were O-rings from hot and mixed water systems suggesting that these gaskets facilitate the growth of *Legionella* spp. These results also show that PCR, in conjunction with swab samples, is a more suitable method to detect *Legionella* spp. in biofilms. It is also possible that all samples that were below detection limit (BDL) by culture could have been positive by PCR for *Legionella* spp. However, BDL samples were not tested by PCR because of resource and time issues.

Table 9: Detection of *Legionella* spp. in Taps by On-Site Swabbing

Sample	Temp	Description	*All Legionella spp.* (CFU/ml)	*All Legionella spp.* (CFU/swab)	*Legionella pneumophila* SG1 (GU/swab)
A1	Hot	First Draw Water	BDL		
A2	Hot	O-ring for cartridge seat		ND	100
A3	Hot	Copper pipe supply line		BDL	NT
A4	Cold	O-ring for cartridge seat		ND	BDL
A5	Cold	Copper pipe supply line		BDL	NT
A6	Mix	Aerator O-ring		BDL	NT
B1	Hot	First Draw Water	BDL		
B2	Hot	O-ring for cartridge seat		ND	151
B3	Hot	Plastic pipe supply line		BDL	BT
B4	Cold	O-ring for cartridge seat		ND	BDL
B5	Cold	Plastic pipe supply line		BDL	NT
B6	Mix	Aerator O-ring		ND	27
C1	Hot	First Draw Water	1 CFU/ml		
C2	Hot	O-ring for cartridge seat		BDL	NT
C3	Hot	Plastic pipe supply line		BDL	NT
C4	Cold	O-ring for cartridge seat		BDL	NT
C5	Cold	Plastic pipe supply line		BDL	NT
C6	Mix	Aerator O-ring		BDL	NT
D1	Hot	First Draw Water	BDL		
D2	Hot	O-ring for cartridge seat		BDL	NT
D3	Hot	Plastic pipe supply line		BDL	NT
D4	Cold	O-ring for cartridge seat		BDL	NT
D5	Cold	Plastic pipe supply line		BDL	NT
D6	Mix	Aerator O-ring		BDL	NT

GU: Genomic Unit is a calculated value that is indicative of a single bacterium that has had its DNA amplified by PCR.
BDL: Below Detection Limit
ND: *Legionella* spp. colony counts were non-determinable due to overgrowth from competing bacteria.
NT: Not Tested

Summary Points

1) PCR is a more sensitive technique, as compared to culture method, for examining *Legionella* spp. in biofilms.
2) Swab samples from aerators are better indicators of *Legionella* spp. colonization of a distribution system in comparison to water samples.
3) Hot water systems are more conducive to growth of *Legionella* spp. compared to cold water systems.
4) The ideal method for controlling *Legionella* in hot water systems is a temperature of 124 ^{0}F as recommended by the CDC and VHA and a minimum chlorine concentration of 0.26 ppm.

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Acknowledgements

The authors would like to acknowledge Steve Eisele, Steven Durr, Carl Groenewegen, and Joe Patterson of Weas Engineering for their help with sample collection.