Sampling Strategies and Test Methods for the Detection of *Legionella* in Potable Water Systems

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Abstract

The adoption of ASHRAE 188 has resulted in the need to validate Water Management Programs (WMP) by testing the potable water for the presence of *Legionella*. Professional and government organizations such as the American Industrial Hygiene Association, the CDC and OSHA provide some guidance as to test frequency and actionable concentrations of *Legionella* in a WMP. Data from several studies is presented, which deal with key remaining issues related to validation of a WMP and include identification of appropriate sample locations, the number of samples that should be tested and when PCR ought to be considered as an alternative test method to conventional culture techniques.

Introduction

Since ASRAE 188 was ratified in 2015, there is now a need to establish guidelines and criteria for implementing a validation program which are necessary components of a Water Management Program. An intensive environmental surveillance of a major mid-west hospital's potable water system in 2015 provided an opportunity to address some basic issues associated with validation programs. Specifically, the issues of which types of water samples to collect (hot, cold, first draw, and prolonged purge) and where to collect them (point of use, system mains) was addressed. The use of PCR as an alternative to culture methods were also investigated since PCR offers the advantage of speed, sensitivity and objectivity compared to culture analysis.

Materials and Methods

Sample Collection
One liter (1 L) potable water sample from each site was collected in sterile wide-mouth screw cap polypropylene plastic bottle containing 150-200 mg sodium thiosulfate preservative.

Preparation of Samples for Bacteriological Examination

Filtration
Five hundred mL (500 mL) of each sample was filter-concentrated using a 47-mm filter funnel assembly containing a 0.20 μm polycarbonate filter. After filtration, the filter was removed aseptically from the holder with sterile 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.

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. One (1.0) mL of the vortexed suspension was placed into a sterile 1.5 mL centrifuge tube containing 1.0 mL of pH 2.0 acid buffer [1]. The suspension was then incubated for 5 minutes at room temperature before spreading on the appropriate Petri plate.

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 Oxoid™ GPVC selective supplement antibiotics; the second, BCYE complete without antibiotics.

Plating of Samples

Plates (described above) were inoculated with 0.2 mL of either an acid-treated or non-acid treated suspension and distributed over the agar surface with a plastic spreader. They were then incubated at 37 ˚C in a humidified incubator for 14 days.

Examination of Cultures for *Legionella*

Plates were examined after 4-8 days of incubation for *Legionella*. Suspect *Legionella* colonies were streaked onto BCYE agar plate without L-cysteine, and a positive control BCYE agar plate. 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 a latex agglutination test (Oxoid, Dardilly, France) or direct fluorescent antibody (Pro Lab Diagnostics, Round Rock, TX).

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

DNA Extraction

DNA was extracted from filtered water samples using the Meta-G-Nome DNA Isolation Kit (Epicenter, Madison, WI). Water samples were filtered through a pre-sterilized 0.20 µm polycarbonate filter membrane (Millipore). The membrane was then removed and cut into two pieces and placed in of a 50 mL sterile conical tube. One (1) mL of filter wash buffer containing 0.2% Tween 20 was added to the tube containing the filter pieces and vortexed intermittently for 2 minutes. This cell suspension was transferred to a clean micro-centrifuge tube and centrifuged at 14,000 x g for 2 minutes to pellet the cells. The pellet was re-suspended in 300 µL of TE buffer, followed by addition of 2 µL of Ready-Lyse Lysozyme Solution and 1 µL of RNase A. The contents were mixed by vortexing and incubated at 37 °C for 30 minutes. This step was followed by addition of 300 µL of Meta-Lysis Solution (2X) and 1 µL of Proteinase K to the tube and mixed by vortexing. Tubes were then incubated at 65 °C for 15 minutes then transferred to an ice bath cool the sample to room temperature

(for 3-5 minutes) after which 350 µL of MPC Protein Precipitation Reagent was added to the tube and mixed by vortexing. The debris was pelleted by centrifugation for 10 min. at 14,000 x g. The supernatant was transferred to a clean micro-centrifuge tube followed by addition of 570 µL of isopropanol to the supernatant and inverted several times to mix. DNA was pelleted by centrifugation for 10 minutes at 14,000 x g. Isopropanol was decanted without dislodging the DNA pellet followed by the addition of 500 µL of 70% ethanol and centrifugation for 5 min. at 14,000 x g. The ethanol was decanted and the pellet air-dried for 8 min. at room temperature after which 50 µL of TE buffer was added. DNA extracts were stored at approximately -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). (Applied Biosystems, Foster City, CA). Reaction mixtures (20 µL) contained 10µ L of 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. [2]. 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 fluorescence value ten times the standard deviation of the mean baseline emission. According to this protocol, a threshold 0.2 ΔRn was used.

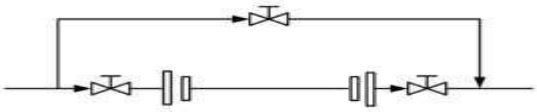
Free Chlorine

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.

Biofilm Bypass Manifold

A device was manufactured called a Biofilm Bypass Manifold (BBM) that was installed in 6 buildings on both the cold and hot water mains. The main flow of water was through the straight portion of the manifold which also contained a copper insert with threaded unions so that the copper insert could be removed, swabbed and reinserted into the BBM. The BBM permits sampling of water and biofilm without interrupting flow to point of use devices (POU). The copper insert is made from the original piping in the mains water supply. The bypass component of the manifold is maintained partially open during times of normal flow to prevent stagnation from occurring throughout the BBM.

Figure 1: Biofilm Bypass Manifold



Results

Table 1: Presence of *Legionella* in Hot Vs Cold Water First Draw Water Samples

	n (%)	Free Chlorine mg/L			Temp °F			p	
		Range	Avg.	SD	Range	Avg.	SD	Temp	Free Chlorine
Hot Positive	35 (43)	0-1.11	0.23	0.33	71-112	91	13.2		
Hot Negative	47 (57)	0-0.94	0.20	0.25	71-115	95	13.6		
Cold Positive	25 (38)	0-1.29	0.67	0.52	70-101	78	7.7		
Cold Negative	40 (62)	0-1.18	0.64	0.45	71-85	77	3.6		
Cold Negative vs Cold Positive								0.245	0.887
Hot Negative vs Hot Positive								0.197	0.778
Total Cold vs Total Hot								$1.77X10^{-13}$	$2.10X10^{-9}$

Free chlorine delivered by the municipal public water supply is the only source of disinfectant in both the hot and cold water systems. The range of free chlorine in both the hot and cold water in Table 1 is expansive and for the most part, is overlapping. Nevertheless, the difference between the average free chlorine values for both the hot and cold waters is highly significant ($p=2.10x10^{-9}$). In spite of this highly significant difference, there is little difference between the percent of hot samples positive for *Legionella* (43%) versus the number of cold samples testing positive for *Legionella* (38%).

Like free chlorine, the temperature range of the hot and cold water samples is expansive and overlapping and the difference between the average temperatures of the two systems is also highly significant ($1.77x10^{-13}$). It is therefore interesting that neither the free chlorine nor the temperature differences in these two systems had any apparent effect on the presence of *Legionella spp*. therein.

Noting that the temperature and free chlorine range within the hot and cold water systems is so expansive, it was informative to determine if these parameters affected the incidence of *Legionella spp*. within a given system. The average free chlorine and temperature for samples testing positive for *Legionella* is essentially the same as for those samples testing negative for both the hot (p = 0.197 and 0.778 respectively) and cold water (p = 0.245 and 0.887 respectively) systems. Therefore, for first draw samples, the free chlorine concentrations and temperature differences within each of these two systems would not be expected to affect the occurrence of *Legionella*.

Table 2: First Draw vs 2 Minute Purge of Sink Faucets

	First Draw						2 Minute Purge			
	Cold		Hot		Blended by Mixing Valve		Cold		Hot	
	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR
n	66	64	87	81	7	7	13	11	16	10
Positive	13	16	18	28	4	3	2	3	3	4
Percent	20	25	21	35	57	43	15	27	19	40

A PCR sample testing positive for any of the three targets was deemed positive for *Legionella* and any samples that produced *Legionella*-like colonies on BCYE agar but failed to grow when sub-cultured on BCYE agar less cysteine was deemed culture positive. The data in Table 2 reveal that there is virtually no difference in the number of positive samples collected from first draw sink faucets unless the cold and hot water lines entering the faucet are delivered via a mixing valve. Amongst cold and hot first draw samples, PCR appears to be a more sensitive indicator of *Legionella* contamination than culture. The number of blended samples is low and therefore skews the apparent lower sensitivity of PCR versus the culture method. However, the higher percent of positive *Legionella* samples encountered in the blended waters compared to both the cold and hot water samples may indicate that this type of valve is inherently more prone to *Legionella* contamination than both unblended hot and cold water.

The relatively low number of 2 minute purge samples probably exaggerates the ability of PCR to detect the presence of *Legionella* compared to the culture method since there is only a difference of one positive sample between PCR samples and culture samples in both the hot and cold water. This bias is further skewed by having a low number of 2 minute purge samples. Overall, there appears to be little significant

difference between the number of positive samples, both hot and cold, when comparing first draw to the 2 minute purge.

Table 3: *Legionella pneumophila* Serogroup 1 isolated from Sinks by Swab and Water Samples

Location	Cold Water		Hot Water		Plumbing Surfaces: CFU/Swab			
	First Draw CFU/mL (Temp)	Supply Line CFU/mL (Temp)	First Draw CFU/mL (Temp)	Supply Line CFU/mL (Temp)	Aerator	3/8 inch Feed Line		Material
						Cold	Hot	
1339-T8	ND	ND	100 (85)	10 (105)	100	310	200,000	Plastic
1339-T6	ND	ND	26 (73)	23 (104)	60	BDL	200,000	Plastic
1339-T4	ND	ND	11 (108)	4 (114)	10	BDL	40	Plastic
1339-T3	10 (67)	BDL (59)	ND	ND	20	BDL	BDL	Plastic
1339-T2	ND	ND	100 (88)	100 (94)	80	200,000	200, 000	Plastic
1339-TR	ND	ND	23 (88)	32 (98)	120	60	80	Plastic
1321	ND	ND	100	8	1000	BDL	10	Plastic
1333-1	BDL (95)	20 (71)	BDL (75)	BDL (117)	ND	BDL	BDL	Metal
1324-1	BDL (69)	BDL (66)	BDL (102)	BDL (121)	ND	BDL	BDL	Metal

ND: No Data

Unlike all other samples collected during this study, the samples in Table 3 were collected in the fall months as opposed to the summer months of 2015. Supply line samples were collected by removing the 3/8 inch feed line connecting it to the sink taps and discharging a liter of water from the supply line and then collecting another liter of water from the supply line for microbiological analysis. Due to small number of samples, it is not possible to accurately compare the effects of hot and cold water to the concentration of *Legionella pneumophila* serogroup 1 in this table. However, there does appear to be a correlation between the presence *Legionella pneumophila* serogroup 1 in hot first draw water samples and hot water samples collected from the supply line. This observation is in contrast to the absence of *Legionella pneumophila* serogroup 1 in the only cold water supply line sample but without other cold water samples to compare, the significance of this result is uncertain. As noted previously, there is a large variation in the temperature from first draw samples.

The highest colony counts were from the inside of the 3/8 inch flexible plastic tubing. The aerator and the soft plastic that composes the 3/8 inch feed lines appear to be highly associated with the presence of *Legionella pneumophila*, presumably existing as an adherent biofilm. With the exception of location 1339-T3 every 3/8 inch plastic feed line servicing the hot water taps possessed a *Legionella* biofilm. Three of 3/8 inch lines had as much as 200,000 CFU/swab. This is in sharp contrast to the absence of

any detectable *Legionella* biofilm on 3/8 inch feed lines that are made of copper (locations 1333-1 and 1324-1). Not only were these two locations devoid of detectable *Legionella*, the water servicing these locations had few (1331-1, 20 CFU/mL) or no detectable *Legionella* present.

Table 4: System Water Biofilm and Planktonic *Legionella*

Building	Sample	Temp.	BBM		POU: positives/total	
			Culture	PCR	Culture	PCR
1A	Water	Hot	0	0	0/7	1/9
		Cold	0	0	1/9	0/9
	Swab	Hot	0	0		
		Cold	0	0		
2B	Water	Hot	0	+	0/6	1/6
		Cold	0	0	0/5	0/5
	Swab	Hot	0	0		
		Cold	0	0		
3C	Water	Hot	+	+	1/2	0/2
		Cold	0	0	1/2	1/2
	Swab	Hot	0	0		
		Cold	0	0		
4E	Water	Hot	+	+	1/1	1/1
		Cold	+	+	ND	ND
	Swab	Hot	+	+		
		Cold	0	0		
5R	Water	Hot	0	0	1/7	1/9
		Cold	0	0	1/9	0/9
	Swab	Hot	0	0		
		Cold	0	0		
6S	Water	Hot	0	0	2/17	10/17
		Cold	0	0	2/11	5/11
	Swab	Hot	0	0		
		Cold	0	+		

Biofilm Bypass Manifolds (BBM) were installed on the hot and cold water mains in six buildings as identified in Table 4. They were installed on the hot water return lines and at a location furthest from the point of entry on the cold water main line. These locations were chosen to maximize the likelihood of obtaining a positive result, i.e., lowest hot water temperature and lowest disinfectant level in the cold water main line. The copper insert was created by cutting into the original copper pipe and using this section to form a replaceable copper insert. The copper insert was integrated into the system by affixing it to unions on both ends. The copper inserts were swabbed immediately after the pipe was sectioned and before it was modified to become a

replaceable insert. Water samples were also collected at various point of use locations throughout these buildings and from the BBM itself.

The copper inserts from the BBM's were for the most par devoid of biofilm. Biofilm was identified in the hot copper insert in building 4E by culture and PCR and the cold water copper insert in building 6S by PCR. Therefore, out of 12 swabs, only 3 were positive for *Legionella*. Of the 3 positive swabs, the associated BBM water samples tested positive only in hot water from building 4E. Conversely, hot water from the BBM in building 3C and cold water from the BBM in building 4E tested positive for *Legionella* but was not associated with any biofilm from their respective BBM copper inserts.

It is not possible to make a correlation between the presence of *Legionella* in the water taken from the BBM (buildings 2B, 3C and 4E) and the presence of *Legionella* in POU locations associated with the BBM since the number of POU samples were very low. However, in building 6S, 17 hot water POU samples and 11 cold water POU samples were taken. Based on PCR tests, there was a significant incidence of *Legionella* in the cold and hot POU samples but none was found in the cold and hot mains.

Table 5: Detection of *Legionella* spp. by PCR and Culture in Water and Swab Samples

	PCR Positive Culture Positive	PCR Positive Culture Negative	PCR Negative Culture Positive	PCR Negative Culture Negative
Hot Water	9	16	8	40
Cold Water	14	20	7	50
Hot Line Swab	2	1	1	9
Cold Line Swab	1	2	0	10
Total	26 (14)	39 (21)	16 (8)	109 (57)

Bracketed numbers refer to percentage

The data in Table 5 indicate that PCR is a more sensitive method of detecting *Legionella* spp. than culture methods. The total number of PCR positive samples totaled 35% (21+14) compared to 22% (14+8) for culture methods. Furthermore, the number of "PCR Positive Culture Negative" samples was 21% versus 8 % for the converse situation. Of the total number of positive samples (26+39+16), PCR and culture were in agreement at a frequency of 32% (26/81) primarily because of the high proportion of "PCR Positive Culture Negative" samples. Negative samples for both PCR and culture reflect the overall propensity of the sample group to be negative for *Legionella* spp.

Table 6: Correlation of CFU/ml and GU/ml

	Pearson Product Moment Correlation Coefficient (r)					
	Legionella species: 16S Gene		*Legionella pneumophila*: mip Gene		*Legionella pneumophila* sg 1: wzm Gene	
CFU/mL	n	r	n	r	n	r
<1.0	52	-0.110	40	-0.087	29	-0.419
1-10	17	+0.455	11	-0.065	18	+0.674
11-100	10	+0.207	9	+0.518	8	+0.290
>100	2	ID	2	ID	2	ID

ID: Insufficient Data

A genomic unit (GU) is a calculated value that is indicative of a single bacterium that has had its DNA amplified by PCR. Likewise, a colony forming unit (CFU) is indicative of a single bacterium that has replicated to sufficient density to be visible on an agar plate. Either metric can be used as an indicator of population density and under ideal circumstances, should at least be highly correlated. Since the culture method of enumerating *Legionella* is still considered the "Gold Standard", Table 6 correlates CFU/ml to GU/mL comparing a precise GU/ml values to a range of CFU/mL. The "r" value was calculated only for samples that had a detectable GU/mL or CFU/mL. For a correlation to be highly significant, an absolute r value greater than 0.50 is expected. There are only two occasions when r>0.50, when the *Legionella pneumophila* was present at 11-100 CFU/ml and *Legionella pneumophila* serogroup 1 was present at 1 - 10 CFU/ml. However, these correlations appear randomly dispersed throughout the table.

Table 7a: PCR and Culture Agreement for all Samples Collected

	Total Culture Positive		Total PCR Positive		Matched Culture and PCR Positive	
	n	%	n	%	n	%
Legionella spp. (16S)	51	100	75	100	35	67
Legionella pneumophila (mip)	45	88	45	60	11	24
Legionella pneumophila sg 1(wzm)	42	82	24	32	10	24

A total of 239 samples were processed by PCR and culture and as noted in Table 7a, there are significant differences between the ability of culture and PCR to detect the presence of *Legionella*. PCR is better able to detect the presence of non-*pneumophila* species of *Legionella* (75 vs 51) but the sensitivity of PCR decreases as the identity of

Legionella becomes more precise. It is usual for PCR to detect a greater number of positives compared to culture techniques since PCR is able to detect Viable But Non Culturable (VBNC) organisms. However, the phenomenon of VBNC does not appear to present itself when positive samples are compared at the species and serogroup level. Interestingly, the total number of positives at the species level is the same for both PCR and culture (45 and 45). However, PCR and culture agree with only 11 of these 45 samples which is equal to a 24% match. At the serogroup level, PCR was inferior to culture in its ability to detect serogroup 1. When referenced to the total number of positives by culture, this also equaled a 24% match. The best match of PCR and culture occurred at the genus level, i.e., 67%.

Table 7b: Amplicon Agreement

		mip (n)		wzm (n)	
		Present	Absent	Present	Absent
16S (n)	Present	40	37	23	55
	Absent	9		2	
wzm (n)	Present	17	15		
	Absent	35			

The poor match between culture and PCR positive samples could be a result of the PCR primers not correctly identifying or annealing to the intended amplicon. One method of determining the veracity of a PCR result is to compare the performance of amplicons that constitute sub-sets of each other. By definition, a member of a subset will be present in the superset. Therefore, when detected, the wzm amplicon should also be associated with the detection of mip and 16S amplicons. In Table 7b, wzm amplicon was associated with the 16S amplicon 42% of occasions (23/55) and 49% of occasions (17/35) with the 16S amplicon. Theoretically, the percent association should have been the same for both. More problematic than this 7 percentage point difference is that mip was absent in 15 occasions when wzm was present. This alludes to either a negative PCR bias for detecting the presence of mip or a false positive bias for detection of wzm. In view of the total number of *Legionella pneumophila* sg1 detected by culture as constituting 87% of all *Legionella* spp., and that 93% (42/45) of these were serogroup 1, it appears that the PCR conditions used in this study under represent the total number of *Legionella pneumophila* present. In an analogous manner, wzm also appears to be under representing the population of *Legionella pneumophila* serogroup 1.

Summary

Environmental surveillance of a hospital for *Legionella* immediately begs the question as to where and how these samples should be taken. The data collected from a survey

of a large mid-western hospital has provided some guidance on this matter. In a hospital setting the matter of primary concern is to choose locations that have the highest probability of affecting patient health. Therefore, water obtained from rooms accommodating patients in ICU's and transplant wards are highly desirable and conversely, water from public washrooms have comparatively minimal value.

It is commonly thought that hot water POU locations are more likely to harbor *Legionella* than cold water [3]. Neither cold nor hot water present ideal growth conditions for *Legionella* so presumably it is the lack of disinfectant in the hot water that accounts for its prevalence in these systems. The data collected in this study indicates that although the differences in disinfectant levels between hot and cold water systems is substantial, this did not correlate with the number of POU locations testing positive for *Legionella*. This contrary result may be due to the time of year during which these samples were collected, i.e., the summer months. It is typical that Legionellosis reaches its highest levels in the summer months [4] and this may be a consequence of the potable water coming closer to the optimum growth temperature of *Legionella*. A follow-up study is in progress to determine if a correlation exists between colonization of POU devices by *Legionella* and the temperature and free chlorine residual after a two minute purge. It is suspected that environmental conditions after a two minute purge might be more predictive of the microbial ecology of a POU device. Therefore devices that are chronically deficient in disinfectant concentration and maintained at a temperature conducive to *Legionella* growth would be more likely to be culture positive after a 2 minute purge than first draw samples collected from the same device.

Whether a sample is collected immediately upon opening a valve (first draw) or purged to remove stagnant water could affect the microbiology of the water. Due to stagnation, first draw samples would be expected to contain less disinfectant and therefore higher concentrations of bacteria than samples collected after a purge. Furthermore, loosely adhering biofilm would be sloughed within the first few seconds of opening a valve and the bacteria therein would go by unnoticed if a purged sample were to be collected. Despite the apparent propensity for obtaining more positive samples from a first draw sample as compared to a purge is that the purge sample provides information upstream of the valve. In this survey, we investigated and compared the microbiology of the water immediately upstream of the valve by removing the tubing that connects POU devices to the main supply line. Thus any residual affect that the valve might have on the perceived quality of the main supply water is obviated. In our study, we determined that water obtained from the first draw was very similar to that obtained directly from the main line. This result may be anomalous because many of the samples were obtained from a building that was largely unoccupied and therefore stagnation of even the cold and hot water mains was likely. Nevertheless, the highest concentrations of *Legionella* were obtained from plastic 3/8 inch lines that connect the supply line to the valve. Aerators attached to the faucet were also contaminated with *Legionella* but this was not a surprising result (5). Interestingly, only the plastic 3/8 inch connector lines were heavily contaminated. Copper 3/8 inch connector lines were essentially free of *Legionella*.

Whether it is the hydrophobic nature of the plastic substrate, its ability to adsorb organics or a biodegradable component within the plastic lines that support bacterial growth is not known but these types of connectors should become highly suspect in future environmental surveillance programs.

One of the desired outcomes from installing a BBM is that these devices might reduce or eliminate the need to obtain multiple POU samples. This would be the case if the mains, both hot and cold, are the primary source of *Legionella* and not simply acting to seed POU devices with low levels of *Legionella*. The samples obtained from the BBM's reveal that the hot and cold water mains, the water therein and the pipe surfaces, are seldom contaminated with *Legionella*. It would appear that the hot and cold water mains are probably carriers of low levels of *Legionella* that are transported to terminal POU devices where they can achieve higher concentration by growing biofilms on materials more able to support bacterial growth, i.e., soft plastic components. Once a POU device becomes contaminated with a *Legionella* biofilm, it is possible that the biofilm could grow and extend upstream of the POU device and in the case of a recirculating hot water system, seed many other POU devices.

The use of PCR presents an opportunity to reduce the time line for obtaining surveillance data from weeks to hours. PCR can also eliminate the subjectivity of interpreting colony morphology and serological reactions. PCR is not new and there are accepted protocols for using it as an alternative to culture methods. Largely because PCR can overestimate the number of *Legionella* in a sample because of its ability to amplify dead and VBNC organisms, the gold standard for *Legionella* continues to be the culture method. But because the advantages of PCR are so important, samples collected during this survey were subjected to both PCR and culture analysis to determine if PCR can provide an equitable alternative to culture. As was expected, the total number of positive samples was greater when PCR was used and opposed to culture. This was the case when all *Legionella* species were tested by using a primer/probe specific to the 16S DNA. The mip gene was able to detect the presence of *Legionella pneumophila* at the same frequency as the culture method. However, this frequency was not associated with a perfect match of test results for a given sample. In fact a mismatch was more likely to occur with all targets except at the genus level. Given that PCR was able to detect *Legionella* more often than by culture, it was expected that all PCR samples would be matched with its corresponding culture result. This however was not the situation. It would appear therefore that the apparent greater sensitivity of PCR to detect the presence of *Legionella* (at the genus level in this study) is not solely due to the presence of VBNC bacteria. It may be that there are PCR inhibitors in some water samples.

Although the infectious dose of *Legionella* is not known and would likely vary depending upon the pre-existing health of an exposed individual and the exact strain of *Legionella*, the probability for infection to ensue will be dependent upon the number of bacteria inhaled. Therefore, a method of quantifying the number of *Legionella* in a sample is an important metric that can be used in determining a course of action to be taken following an environmental survey. Real time qPCR permits the

quantification of an amplified target. The 16S target can occur in multiple copies in a bacterial cell (6) and therefore it is inherently difficult to correlate the number of GU's derived by its amplification to a corresponding CFU count. However, the genes coding for the mip gene and the expression of serogroups, are present at one copy per genome [7, 8]. Therefore, there should be a correlation between CFU's and GU's when mip and wzm are compared to CFU's. Such a correlation however was not observed at low and high concentrations of viable *Legionella*. This lack of correlation is not reported in the literature and suggests that the extraction/purification method and or the PCR parameters require further refinement.

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