Evaluation of a most probable number method for the enumeration of *Legionella pneumophila* from North American potable and nonpotable water samples

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ABSTRACT

This study compares the performance of a novel most probable number (MPN) method (Legiolert™/Quanti-Tray®) with *Standard Methods for the Examination of Water and Wastewater* 9260 J for the enumeration of *Legionella pneumophila* from potable and nonpotable waters. Data from the study showed that Legiolert exhibited higher sensitivity for the detection of *L. pneumophila* for potable water and equivalent sensitivity for nonpotable water. The Legiolert medium had a high specificity with no false positive signals reported for either water type. The new method represents a significant improvement in usability and accuracy in the enumeration of *L. pneumophila*.

Key words | culture, Legiolert, *Legionella*, legionnaires', quantification, SM9260 J

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INTRODUCTION

Following the outbreak of legionnaires' disease in Philadelphia in 1976 (Fraser *et al.* 1977), *Legionella pneumophila* has emerged as an opportunistic pathogen of growing global concern. An increasing incidence in the annual number of cases has been reported by both the Centers for Disease Control (Adams *et al.* 2015; Garrison *et al.* 2016) and the European Centre for Disease Prevention and Control (Beauté *et al.* 2016). A number of large outbreaks associated with both potable and nonpotable water have demanded attention, increased public awareness, and stimulated the publication of new guidance documentation such as the CDC toolkit (Messonnier *et al.* 2016) and ASHRAE 188 (ASHRAE 2015), and in some cases the passing of legislation mandating routine monitoring and a risk management plan (New York State Department of Health 2016). Technical actions vary globally but are all based upon a quantitative assessment of the presence of *Legionella* in a given system, whether the system comprises a potable, premises plumbing network or a cooling system. For example, action limits in cooling towers in New York state start at 20 CFU/mL for review of the treatment program and initial disinfection but escalate to system decontamination with a halogen-based compound once the concentration exceeds 1,000 CFU/mL. For New York, action decisions and recommendations are based on quantification from traditional spread-plate culture methods conducted by a laboratory approved by the New York State Environmental Laboratory Program (ELAP). Likewise, Québec regulations stipulate the *Legionella pneumophila* concentration in cooling towers must be <10 CFU/mL or actions must be taken (Gazette Officielle du Québec 2014); similar limits exist in potable samples from 'covered facilities' (hospitals and health care facilities), with the caveat that a percentage of outlets in a given system all exceed the minimum action limit. In 2011 the German Federal Ministry of Health published the

doi: 10.2166/wh.2017.118

German drinking water ordinance imposing strict action limits of 1 CFU/mL for total *Legionella* (all *Legionella* species), and ISO 11731-2 (2004) was deemed as the regulatory standard culture method (German Federal Ministry of Health 2011).

Several new quantitative methods have been introduced in recent years in order to facilitate routine testing, but culture-based methods continue to be the most rigorous and are therefore considered the gold standard by which other methods are judged. One version of the plate culture method is *Standard Methods for the Examination of Water and Wastewater* 9260 J: *Legionella*. The SM9260 J method allows for several procedural options around a common theme of the use of selective media for isolating *Legionella*. One medium invoked in SM9260 J, which was employed in this study, is CCVC, a medium which is based upon buffered charcoal yeast extract (BCYE) nonselective medium and fortified with multiple selective agents (colistin, cephalothin, vancomycin, and cycloheximide; APHA/AWWA/WEF 2007). In this embodiment of SM9260 J a sample is either analyzed directly or after concentration, which is achieved by concentration by membrane filtration through a 0.2 μm pore size membrane filter and subsequent resuspension in diluent. Either suspension is then plated on selective CCVC agar with or without a short treatment with an acid solution, and incubated at 35 °C for 7 days. Presumptive colonies are then sub-cultured for confirmation.

Legiolert™, an alternative, MPN-based method for quantifying *L. pneumophila*, has been developed. Legiolert is delivered as a powdered reagent in a blister pack and utilizes fixed protocols dictated by the sample type. For potable and related water matrices, Legiolert is designed to test 10 ml of sample and, for nonpotable waters, Legiolert is designed to test 0.1 mL of sample. For potable water, Legiolert/Quanti-Tray® is incubated at 39 °C ± 0.5 °C and at 37 °C ± 0.5 °C for nonpotable water, both with ≈80% relative humidity for 7 days. When *L. pneumophila* are present in a water sample and tested using Legiolert, they produce any combination of brown color and turbidity, and represent a confirmed detection result. Enumeration is achieved by the most probable number (MPN) technique. Legiolert utilizes IDEXX's selective media formulation to detect exclusively *L. pneumophila*, with no characterized bias for specific serogroups.

We report here the outcome of a comparison study between Legiolert and *Standard Methods for the Examination of Water and Wastewater* 9260 J for the enumeration of *L. pneumophila* from naturally contaminated potable water samples and nonpotable water samples, the latter of which primarily comprised cooling towers and cooling systems. We were interested in comparing this outcome with a previous comparison of Legiolert vs. ISO-11731-2 that was conducted with potable samples in Germany in which Legiolert exhibited greater sensitivity for the quantification of *L. pneumophila* (Sartory *et al.* 2017).

MATERIALS AND METHODS

Legiolert/Quanti-Tray description and procedure

All Legiolert materials were from IDEXX Laboratories, Inc. The Legiolert method consists of a blister pack containing reagent powder and the Legiolert Quanti-Tray growth and quantification platform. For the nonpotable water application, a pretreatment solution is included. The potable water application was carried out as follows. A blister pack of Legiolert powder reagent was added to 90 mL of sterile deionized water and mixed thoroughly. Following brief agitation of the potable water sample 10 mL was added to the reagent mixture and agitated. The contents were sealed into a Legiolert Quanti-Tray as described below. The nonpotable water application was carried out as follows. First, a multi-dose pretreatment reagent was reconstituted by adding 100 mL of sterile deionized water to a vessel containing powdered pretreatment reagent. For each test the sample was agitated and an aliquot of 0.2 mL was added to 0.2 mL of pretreatment solution in a microfuge tube. The mixture was vortexed hard for ~5 seconds and incubated at room temperature for 60 seconds. A 0.2 mL aliquot from the reaction tube was then immediately transferred to the 100 mL vessel containing reconstituted Legiolert reagent. The contents were agitated and sealed into a Legiolert Quanti-Tray as described below.

Completed sample mixtures were poured into the Legiolert Quanti-Tray and immediately sealed in a Quanti-Tray SealerPLUS. Sealed trays were incubated paper side down (wells facing upwards) at 39 ± 0.5 °C in a humidified

environment for potable water samples and at 37 ± 0.5 °C in a humidified environment for nonpotable water samples. Humidity was generated by addition of a water reservoir to the lowest shelf of each incubator. As recommended by the manufacturer, adequate humidity was assessed by confirming that weight loss over the incubation period was ≤10%. To accomplish this, filled and sealed Quanti-Trays were weighed immediately after sealing and again following 7 days of incubation, and the resulting difference was calculated and compared with the initial weight. Quanti-Trays were analyzed after 7 days for the presence of brown color and/or turbidity.

The specificity of Legiolert was analyzed by performing secondary confirmations on at least 25% of all positive wells observed for each sample. Confirmations were performed using BCYE and tryptic soy agar with 5% sheep blood (BA) by the following procedure. For each positive well the sampling area on the paper side of the Quanti-Tray was identified and a razor was cleaned using a disposable alcohol wipe. The razor was used to cut a small opening in the paper above each well to be sampled and 5 µl was transferred from each well to both a BCYE plate and a BA plate. A 3-zone streak was performed for each aliquot on each plate and plates were incubated for 2–4 days at 36 ± 2 °C with humidity. Incubation time was variable based on recovery time for individual isolates to yield clear morphology and accurate confirmation. Following incubation, isolates were regarded as *L. pneumophila* if they grew on BCYE but failed to grow on BA irrespective of any additional non-*Legionella* isolates present.

SM9260 J procedures

The *Standard Methods for the Examination of Water and Wastewater* 9260 J procedure was carried out using CCVC selective medium. For potable water, samples were agitated and 250–1,000 mL of water was filtered through a 0.2 µm polycarbonate filter, after which the filter was transferred with sterile forceps to a 50 mL conical tube containing 5 mL of sterile water and vortexed hard for 30 seconds to dislodge bacteria from the filter. Filtrate was direct plated to each of two CCVC plates and one BCYE plate by spreading 0.1 mL using a sterile glass rod. For the nonpotable application, samples were first agitated then 0.1 mL was direct plated to one BCYE and one CCVC plate. In parallel, the sample was acid treated with the acid reagent described in SM9260 J (0.2 M HCl/KCl, pH 2.0). One milliliter of sample was added to 1 mL acid buffer, incubated for 15 minutes at room temperature, and neutralized by the addition of 1 mL of alkaline neutralizer solution (0.1N KOH). Treated sample was plated by spreading 0.3 mL of the neutralized mixture to one BCYE and one CCVC plate. Note that this volume plated is the equivalent of 0.1 mL of the original, untreated sample. Plates for all water types were incubated at 35 ± 2 °C with humidity and were examined on days 3 and 7 for the presence of presumptive *Legionella* colonies. Presumptive colonies, those that exhibited the typical ground glass appearance of *Legionella* on BCYE media, were confirmed by sub-culturing to both BCYE and BA. In addition to the standard protocol outlined in SM9260 J, presumptive *Legionella* isolates were further screened for fluorescence when exposed to ultraviolet light to determine if isolates were *L. pneumophila* species or non-*pneumophila* species of *Legionella*, as many non-pneumophila species are known to exhibit auto fluorescence, differentiating them from *L. pneumophila*. Data from fluorescent isolates was filtered from the comparative data analysis in order to compare sensitivity of both methods for *L. pneumophila* isolates. Isolates with ambiguous reactions were further analyzed by latex agglutination or direct fluorescent antibody microscopy to confirm the species.

Data analysis

For all data resulting from method SM9260 J the highest count for all parallel plate conditions tested was used for comparisons and was termed the 'best' condition. To compare the sensitivity of the two methods, the comparative *L. pneumophila* paired count data was analyzed using each of two statistical comparisons. The data was analyzed by a standard two-tailed t-test, but an evaluation revealed that the data did not follow a normal distribution. Therefore, the Wilcoxon signed rank test, which is a more appropriate test for non-normal, nonparametric data, was used as described (Oshiro 2010). Both results are presented. Potable water data was all normalized to be expressed per 10 mL of initial sample volume.

The data were also analyzed by a McNemar's binomial test (McNemar 1947) to evaluate differences between the frequencies of positive and negative samples by the test methods for the recovery of *L. pneumophila*.

RESULTS

Water sample composition

A diverse array of potable and nonpotable water samples were analyzed over the period April to September 2016. Potable water samples comprised diverse premises potable water and related systems including, but not limited to, hot water taps, showers, fountains, eyewash stations, ice machines, holding tanks, and drinking fountains. The samples were taken from domestic buildings including, but not limited to, offices, medical buildings, nursing homes, sports facilities and rehabilitation centers. Nonpotable water samples were primarily cooling towers. Ten milliliter aliquots of potable water samples were analyzed by Legiolert and compared with variable volumes ranging from 250 to 1,000 mL analyzed by SM9260 J. One hundred microliter aliquots of nonpotable water samples were also analyzed using the Legiolert nonpotable protocol and compared with the same volume analyzed by SM9260 J. Data was generated for both sample types on confirmed *L. pneumophila* isolated by both methods.

Comparative recovery in potable water samples

Of 491 US potable water samples analyzed 74 yielded data pairs with at least one nonzero value. As shown in Table 1, counts from Legiolert ranged from 0 to 1,460 MPN 10 ml^{-1} (mean 37.4), and from 0 to 50 cfu 10 ml^{-1} (mean 5.3) from SM9260 J method. The majority of paired results (64 out of 74, i.e. 86.5%) were from a SM9260 plate count range of 0 to 10 cfu, but were encompassed within a count range of 0 to 172 MPN by Legiolert. This difference in range of counts and sensitivity was also reflected in ranges of SM9260 J counts. Indeed, Figure 1 shows that in eight samples with counts >25 Legiolert MPN Legiolert showed dramatically higher results than the corresponding result from SM9260 J. This result suggests that Legiolert may be able to more accurately report the number of *Legionella* found at higher concentrations. However, since very few data points contribute to this condition more samples with high levels of *Legionella* would need to be tested to further examine this trend.

Table 1 | Comparative count binning of *Legionella pneumophila* counts from potable samples by SM9260 J and Legiolert/Quanti-Tray based on ranges of counts by SM9260 J for nonzero data pairs

Count range SM9260 J cfu 10 ml^{-1}	Number of samples	Comparative count range Legiolert MPN 10 ml^{-1}
0–10	64	0–172
11–20	6	2–39
21–30	1	42
31–40	2	522–1,460
>40	1	159

The outcomes of the statistical T-test and Wilcoxon signed rank analysis of the paired count data are presented in Table 2A and a bivariate analysis of the raw data pairs in Figure 1. A Wilcoxon signed rank test revealed higher sensitivity (prob > |S| = <0.0001, significance level = 0.05). A two-tailed t-test showed no statistical difference (prob > |t| = 0.120). Since many samples analyzed had low spread plate counts, with 86.5% having 10 or fewer colonies on any single plate, more samples would need to be analyzed to confirm that higher recovery by Legiolert is also observed over the full plate counting range of 30–300 colonies typically used as a method comparison guideline for traditional spread-plate methods.

The outcome of the McNemar's analysis is presented in Table 2B. This analysis showed no statistical difference ($p = 1.0000$), suggesting that, though there appears to be a sensitivity difference for quantification, both methods appear to be equally sensitive for determining presence/absence of *L. pneumophila*.

Comparative recovery in nonpotable water samples

Of 846 US nonpotable water samples analyzed 49 yielded data pairs with at least one nonzero value. As shown in Table 3, counts from Legiolert ranged from 0 to 240 MPN/0.1 mL (mean 14.6), and from method SM9260 J from 0 to 100 cfu/0.1 mL (mean 9.4). The majority of paired results (40 out of 49, i.e. 81.6%) were from SM9260 J plate counts

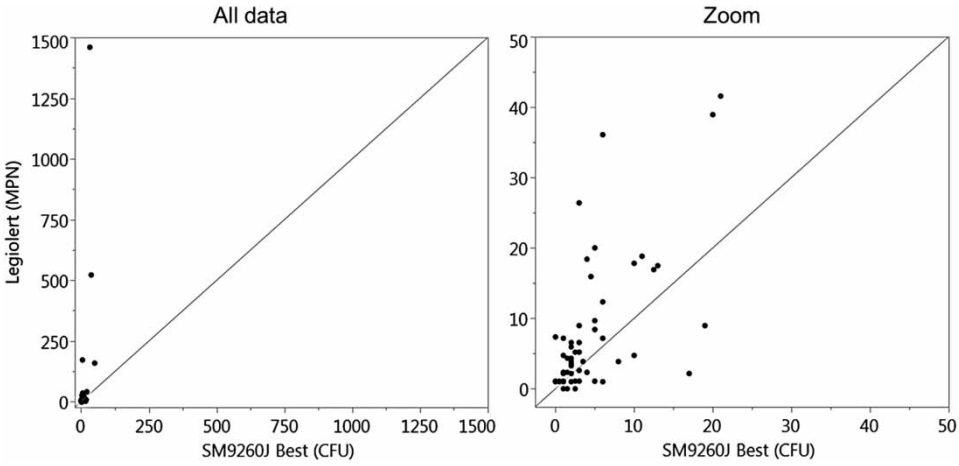

Figure 1 | Bivariate analysis of Legiolert (MPN) by SM9260 J best (CFU) for 10 mL potable water.

range of 0 to 10 cfu but were encompassed within a count range of 0 to 42 MPN by Legiolert. As with the potable comparison, other groupings of SM9260 J counts yielded variances revealing higher sensitivity for Legiolert, but only an examination of additional samples with higher *L. pneumophila* concentrations would reveal a true correlation between higher Legiolert sensitivity at those concentrations. The outcomes of a statistical T-test and Wilcoxon signed rank analysis of the paired count data is presented in Table 4 and a bivariate analysis of the raw data pairs in Figure 2. A two-tailed Wilcoxon Signed Rank test revealed equivalent sensitivity (prob > |S| = 0.728, significance level = 0.05). A two-tailed t-test similarly showed no statistical difference (prob > |t| = 0.201).

The outcome of the McNemar's analysis is presented in Table 4B. This analysis showed no statistical difference (p = 0.6831), agreeing with the statistical evaluation for quantification. Both methods were equally sensitive for determining presence/absence of *L. pneumophila*.

Table 2 | Outcome of statistical analysis of the paired *Legionella pneumophila* counts from 10 ml potable water samples by Legiolert/Quanti-Tray and SM9260 J

A. Quantitative statistics

Statistical analysis	Parameter	vs. SM9260 J Best data
Wilcoxon signed rank test	Prob > |S|	< 0.0001
	Prob > S	< 0.0001
	Prob < S	1
Matched pairs T-test	N	74
	Prob > |t|	0.120
	Prob > t	0.060
	Prob < t	0.940

$p < 0.05$ indicates a significant difference

B. Presence/absence statistics

		Legiolert +	Legiolert −	
SM9260 J	+	67	3	70
	−	4	417	421
		71	420	491
		$p = 1.0000$		

False positivity

One differentiation of the two methods is that the MPN counts from Legiolert are designed as confirmed counts

Table 3 | Comparative count binning of *Legionella pneumophila* counts from nonpotable samples by SM9260 J and Legiolert based on ranges of counts by SM9260 J for nonzero data pairs

Count range SM9260 J CFU 0.1 ml^{-1}	Number of samples	Comparative count range Legiolert MPN 0.1 ml^{-1}
0–10	40	0–42
11–25	3	5–142
26–50	5	11–31
51–75	0	N/A
>75	1	240

Table 4 | Outcome of statistical analysis of the paired *Legionella pneumophila* counts from 0.1 ml nonpotable water samples by Legiolert and SM9260 J

A. Quantitative statistics

Statistical analysis	Parameter	vs. SM9260 J Best data
Wilcoxon signed rank test	Prob > |S|	0.728
	Prob > S	0.636
	Prob < S	0.634
Matched pairs T-test	N	49
	Prob > |t|	0.201
	Prob > t	0.100
	Prob < t	0.900

$p < 0.05$ indicates a significant difference

B. Presence/absence statistics

		Legiolert		
		+	−	
SM9260 J	+	43	3	46
	−	3	797	800
		46	800	846
		$p = 0.6831$		

not requiring additional confirmation steps. For the purposes of this study a fixed percentage of all positive wells were sampled for confirmation by sub-culture to BCYE to estimate the rate of true and false positive results. For the potable water analysis 199/199 wells analyzed were confirmed *Legionella* by sub-culture, resulting in a false positivity value of <0.5% and a specificity of 100%. Similarly, for the nonpotable water analysis 106/106 wells analyzed were confirmed *Legionella* by sub-culture, resulting in a false positivity value of <0.9% and a specificity of 100%.

DISCUSSION

HP Environmental Inc. (HPE) has conducted in-house evaluations of other similar IDEXX products (i.e. Enterolert, Pseudalert, and Colilert). To be useful at HPE, diagnostic environmental methods must be accurate, simple, and affordable for the population for which they are intended. They must also provide results in a timely manner to institute effective control measures in buildings or for treatment of patients. In general, we find that the Legiolert protocols and ease of use meet our expectations for performance and have followed the same well-designed line as previous IDEXX products using the Quanti-Tray platform.

Operational characteristics include the time taken to perform the test, its technical simplicity or ease of use, user acceptability, and stability of the test under user conditions. The ease of use will depend on the practicality of acquiring and maintaining the equipment required to perform the test, the time and difficulty in training staff, and the ability of users to interpret the results of the test correctly. All of these characteristics are important for

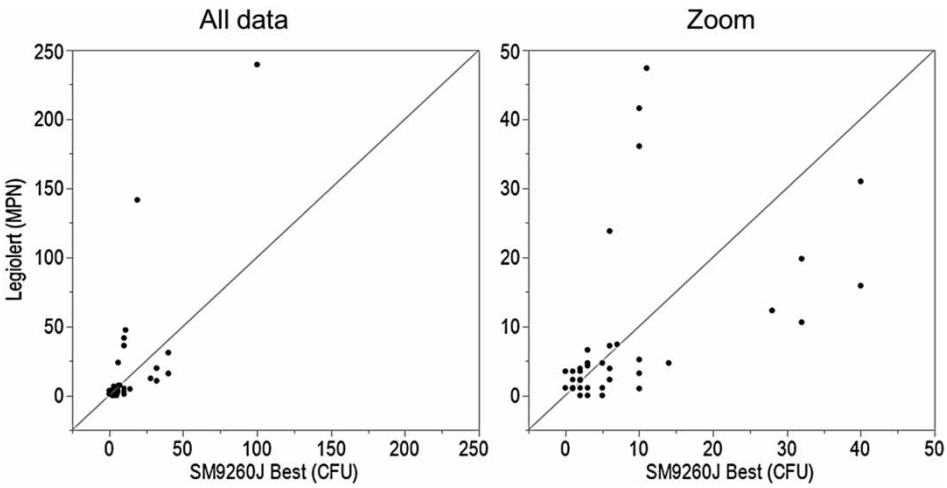

Figure 2 | Bivariate analysis of Legiolert (MPN) by SM9260 J best (CFU) for 0.1 mL nonpotable water.

determining the settings in which Legiolert can be used. We found staff training on Legiolert to be easy and we found that the time taken to perform the Legiolert assay is greater than 50% less than the SM9260 J protocol presently employed in our laboratory.

It was perhaps not surprising that Legiolert exhibited statistically greater sensitivity for quantifying *L. pneumophila* from potable waters than SM9260 J, since a similar study comparing Legiolert with a BCYE-based membrane filtration method (ISO-11731-2) also reported greater Legiolert sensitivity (Sartory *et al.* 2017). In the current study, a simplified protocol in which a 10-fold dilution is directly examined was used in favor of the earlier 100 mL protocol analyzed by Sartory *et al.* in which the sample hardness is measured and subsequently neutralized. The dilution appears to circumvent chemical interferences that the water matrix may introduce without sacrificing overall sensitivity.

Notably, on at least six occasions, we found Legiolert to perform better than the SM9260 J protocol when competing bacteria are in the initial sample. This was observed primarily with cooling tower samples that are expected to have higher levels of bacterial contamination. With such samples, we observed that CCVC media was susceptible to spreading organisms (e.g. *Proteus* and *Pseudomonas* spp.), which prevented the plate from being readable. Acid treatment or retesting of the samples with serial dilutions up to 1:1000 helped but did not eliminate the problem. The Legiolert Quanti-Trays were consistently clean with no contamination issues. In three cases, Legiolert gave a positive result that was not detected by the SM9260 J protocol.

There could be several reasons for differences in recovery between MPN methods and plate methods. Microbiologically, formulation differences can have a significant impact on recovery of microorganisms both from the nutritional composition and from the addition of multiple selective agents. For BCYE-based methods the selective agents added to the medium can vary widely from CCVC, a reflection of differing opinions and data supporting the benefits of specific, alternate formulations. Furthermore, some microorganisms may simply be better suited to propagation in a liquid milieu rather than on solid medium, an idea that has been observed with some bacteria (Ahn *et al.* 2014), though this has not been directly explored with *Legionella pneumophila*. More practically, an MPN device does not experience proximity effects as petri plates do, since each test within an MPN device operates independently of the others. This allows for a linear counting range up to the theoretical maximum by bypassing the deleterious effects of growth inhibition by other waterborne organisms, which has been observed for *Legionella* previously (Cotuk *et al.* 2005; Paszko-Kolva *et al.* 1993). Acceptable counting limits of 300 CFU on standard 90 mm petri plate tests are in place not only to ensure adequate resolution and accuracy, but also to minimize the negative impacts of lack of nutrient availability or waste product accumulation.

For Legiolert, confirmatory sub-culturing revealed very high selectivity with no false positive signals reported for Legiolert. This supports the claim that Legiolert is a confirmed test not requiring that any subcultures be employed for its routine use. Another study with Legiolert with potable samples revealed some false positive signals, with rates less than 4% reported (Sartory *et al.* 2017). Though we did not report the specific serogroups of *L. pneumophila* detected by Legiolert in this study, we were able to perform serogroup assessments using latex agglutination with material extracted directly from positive Quanti-Tray/Legiolert wells.

CONCLUSIONS

The need for accurate and reliable quantification of *Legionella* to conform to growing guidance and legislation internationally has stimulated the development of rapid tests based on molecular technologies such as PCR, antibody capture, and lateral flow. Each are designed to improve on existing culture-based technology by either reducing the time to results or increasing sensitivity. None, however, provides the ability to consistently, accurately, and easily perform routine monitoring in conjunction with a prescribed risk management plan as advised by guidance documents. Though BCYE-based culture methods like SM9260 J are considered the gold standard, they represent a level of subjectivity and difficulty that is problematic for routine monitoring and do not easily differentiate the different species of *Legionella* in the environment, whether they are of significant concern or not. In situations where speciation information is desired, an add-on test requiring extra time, effort, and materials must be performed. Since

Legiolert is *L. pneumophila*-specific, the importance of quantifying species of *Legionella* other than *Legionella pneumophila* (*L.* spp.) is something we had to consider when comparing the differential performance of Legiolert vs. SM9260 J. Environmentally, *L. pneumophila* is the dominant species of *Legionella* in both potable and nonpotable water, generally greater than 80% of all environmental *Legionella* strains isolated (Doleans *et al.* 2004; Lee *et al.* 2010; Lin *et al.* 2011). A similar trend showing the dominance of *L. pneumophila* species was observed in this study as well, though to a slightly lesser degree: of all the samples analyzed by SM9260 J 3.0% (40/1337) contained at least one *L.* spp. compared with 9.2% (123/1337) *L. pneumophila* in the same samples. However, in comparing relative environmental abundance of *L.* spp. to *L. pneumophila* to the clinical speciation profile, the risk for illness can be overwhelmingly attributed to *L. pneumophila*, particularly serogroup 1 (Beauté *et al.* 2016). Furthermore, it has been proposed that the presence of other species like *Legionella anisa* found in water systems does not constitute grounds for remediation due to the questionable risk (Lin *et al.* 2011). For these reasons, Legiolert appears to be an appropriate test system for assessing risk in building systems.

In conclusion, this study has investigated a novel MPN method (Legiolert/Quanti-Tray) for the enumeration of *L. pneumophila*. The method was compared with method SM92060 J for both potable and nonpotable waters. The Legiolert method was found to be more sensitive than SM9260 J for potable water and equally sensitive to SM9260 J for nonpotable water. In this study, only *L. pneumophila* were recovered, indicating a very high specificity and supporting the claim that Legiolert generates a confirmed result. HPE also found the test very simple to use and interpret and concludes that Legiolert will significantly add to the reliability of testing for *L. pneumophila* from drinking water and related samples.

ACKNOWLEDGEMENTS

The generous contribution of IDEXX Laboratories Inc. in the provision of Legiolert reagent, Quanti-Tray and other materials is gratefully acknowledged. This work was supported by IDEXX Laboratories, Inc. IDEXX Laboratories, Inc. aided in the study design, data analysis, and in preparation of the report.

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First received 9 May 2017; accepted in revised form 8 October 2017. Available online 6 November 2017