

Monitoring *Legionella* to Address a Growing Problem

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ABSTRACT

Legionnaires' disease is on the rise around the world, including a 286% increase in the U.S. since 2000. This paper discusses reasons for the increase in incidence, particularly changes in our drinking water infrastructure. The need for monitoring *Legionella* in water samples is stressed, and monitoring methods are reviewed and compared. They include culturing, PCR, and rapid antibody-based tests.

INTRODUCTION

July 27, 2016, marked the 40th anniversary of the beginning of the first recorded outbreak of Legionnaires' disease (a deadly pneumonia) in Philadelphia (Sanford 1979). Since its identification, *Legionella* infection rates have been growing worldwide (Parr *et al.* 2015). New modes of transmission have arisen, and source water supplies and drinking water infrastructure (in addition to premise plumbing) have been implicated in the growth and spread of *Legionella*. These factors emphasize the need for broader and more frequent monitoring of *Legionella* using appropriate methods.

Greater than 60 species of *Legionella* have been identified, many of which are known to cause disease (Muder & Yu 2002). The species *L. pneumophila* is responsible for a majority of, though not all, disease-causing infections. *Legionella* is known to occur in hot water systems because it prefers warmth, water, protozoa, biofilm, and iron. However, it can survive and grow in a much broader range of temperatures and conditions (USEPA 2015a).

Legionellosis has a mortality rate of 5-30% when treated (CDC 2011; WHO 2014; Abdel-Nour *et al.* 2013). Several high-profile outbreaks have recently occurred in the U.S., including one at McLaren hospital in Flint, Michigan (9 deaths between 2014-2015); and another at Opera House Hotel in South Bronx last year (12 deaths) that resulted in the New York State regulation requiring monitoring of cooling towers for *Legionella*. From 2000 to 2014, there was a 286% increase in reported cases in the U.S. (CDC 2016a, 2016b). Much of this increase can be attributed to more frequent clinical testing, as well as improved diagnosis: for example, greater breadth of species and serogroup detection in clinical samples (Mercante & Winchell 2015). Also, an ageing and less healthy human population translates to greater numbers of susceptible individuals. In addition, continued industrialization and deteriorating infrastructure provide more bacterial breeding environments and avenues of transmission.

IMPLICATIONS FOR DRINKING WATER

Industry-related sources (cooling towers, tanks, hot water systems) of *Legionella* are well-documented, but drinking water has turned out to be a major source of infection because it is used for aerosol producers such as drinking fountains, showers, saunas, and sprinklers in and around homes, hospitals, and other building types (CDC 2015; Sabria & Yu 2002). This is because *Legionella* can populate municipal and premise plumbing (e.g. within biofilm), providing a source of bacteria to faucets, shower heads, and other outlets. The problem is likely exacerbated by

other factors such as ageing infrastructure, and water and energy conservation measures that lead to tepid or stagnant water (Melton 2014).

For this reason, Germany now includes *Legionella* in its Drinking Water Ordinance (TrinkwV)(BMJV 2016), and the United States Environmental Protection Agency (USEPA) issued a Legionella Drinking Water Health Advisory (USEPA 2001). The USEPA considered inclusion of *Legionella* in its fourth Unregulated Contaminant Monitoring Rule (UCMR 4) list of drinking water contaminants that require monitoring (USEPA 2015b). In his comments regarding the EPA's ultimate decision not to include *Legionella* in its draft UCMR 4 and instead citing it as only a premise plumbing issue, Randy Ellingboe of the Minnesota Environmental Health Division argued that it is a situation similar to coliform bacteria and lead/copper contamination, whose monitoring are required (Total Coliform and Lead & Copper Rules, respectively). He also pointed out that although the Surface Water Treatment Rule (SWTR) protects against *Legionella*, it “does not establish a treatment technique reduction or removal target” (Ellingboe 2016).

In general, drinking water treatment is effective against *Legionella*, with most cases of contamination arising from bacteria that are living within premise plumbing. However, there are exceptions, and this is due to a number of factors. First, *Legionella* is partially resistant to chlorine (Kuchta *et al.* 1983), so it can survive in water that has reduced chlorine levels resulting from either improper disinfection or other causes (see Flint, Michigan, case below). In fact, it can survive even in ‘normal’ drinking water treatment scenarios (Kuchta *et al.* 1985). Second, amoebae, which can temporarily harbor *Legionella* and release the bacteria at a later time, can withstand drinking water treatment and are found in municipal distribution systems (Thomas & Ashbolt 2011). These factors would explain how premise plumbing is seeded with *Legionella* in the first place.

Case study: Flint, Michigan. The high salt (chloride) concentration and relatively low pH (Torrice 2016) of Flint River water enhanced its corrosiveness and resulted in the lead contamination of Flint's drinking water. It may also have caused the *Legionella* outbreak at the nearby McLaren Regional Medical Center, according to Marc Edwards' research group at Virginia Polytechnic Institute and State University (Edwards *et al.* 2016). Because the water was not treated with a corrosion inhibitor, exposure of the inside surfaces of pipes to the water removed their protective phosphate layer (which had formed over time). This facilitated corrosion of the pipe metal and the release of lead and iron into the drinking water. The iron served as a nutrient for *Legionella*, but also inactivated the chlorine disinfectant [as demonstrated by EPA's monitoring of Flint drinking water (USEPA 2016)]. In addition, switching water sources (from Lake Huron to Flint River) likely caused strong changes in water pressure, which disrupts biofilm, limescale, and sediment that can harbor *Legionella* bacteria, according to the EPA's *Legionella* Human Health Criteria document (USEPA 1999).

Like Flint, many communities possess ageing water infrastructure. As utilities and building owners endeavor to replace old pipes and other water equipment, the disturbance of biofilms and pipes' protective coatings could enhance *Legionella* levels in the water flow.

METHODS FOR *LEGIONELLA* MONITORING

Given the increasing incidence of legionellosis around the world, it is important to have access to a variety of ways to detect *Legionella* in water pipes and equipment. The method used will depend on multiple elements, including degree of urgency, facility type, and amount of data desired or required. Specific details of sample collection will in turn vary by method, but in general, samples are collected in sterile containers (pre-treated with sodium thiosulfate if finished water), from multiple locations or taps. Samples sent to a testing laboratory are shipped overnight on ice packs. For testing on-site, samples are filtered prior to the analysis.

Table 1 cross-references water sample analysis methods with their features, including advantages and disadvantages.

Table 1. Techniques for detecting <i>Legionella</i> in water					
Feature	Culturing	PCR (Standard and qPCR)	IDEXX Legiolert	Legipid: Immunomagnetic Separation Capture Enzyme Immunoassay (IMS-CEIA)	Strip Tests (Dipsticks)
Time to results	7-14 days	8-24 hours	7 days	1 hour	30 minutes
Identification to species-level	Yes ¹	Yes	Yes ¹	No	No
Species Detected	Nearly all	<i>L. pneumophila</i> , many <i>L. spp.</i> ²	Only <i>L. pneumophila</i>	100% <i>L. pneumophila</i> / 90% <i>L. spp.</i>	Only <i>L. pneumophila</i> serogroup 1
Quantification	Yes	Yes (qPCR only)	Yes	Yes	No
Status of detected cell	Viable & culturable (based on growth)	Dead and alive (based on presence of DNA) ³	Viable & culturable (based on growth)	Viable (based on envelope integrity)	Unknown
Detects VBNC (Viable But Not Culturable)	No ⁴	Yes	No ⁴	Yes	Yes
Interpretation of the results	Complex and subjective (pleomorphism, stains, and agglutination)	Simple and objective (does not correlate with cfu)	Simple and objective (corresponds with cfu)	Simple and objective (corresponds with cfu)	Simple and objective (no quantification)
Sample interference	Yes (accompanying organisms)	Yes, but additional steps may help	Yes (accompanying organisms)	Minimal (protocol includes washing steps)	Minimal
False positive rate	Low	Varies with sample quality ³	3.5% as long as pretreatment protocol is used ⁵	12.0% ⁶	Unknown
False negative rate	Can be moderate without additional measures ⁷	Low if certain measures are taken ⁸	Assume similar to Culturing	3.4% ⁶	Does not detect all pathogenic species

Table 1. Techniques for detecting *Legionella* in water

Detection readout	Visual or Mechanical, Counting	Visual or Mechanical	Visual or Mechanical, Colorimetric	Visual or Mechanical, Colorimetric	Visual, Color
Theoretical limit of detection	100 cfu/L	100 GU/L (GU=genomic unit) ⁹	1 cfu/100mL (potable water) ⁵	40 -93 cfu/L ¹⁰	100 cfu/L ¹¹
Simplicity of test	Laborious	Specialized	Simple	Training needed	Simple
High throughput capability	Medium	High	Medium	Medium	Medium
Multiplexing with other analytes	No	No	No	Yes	No
Cost for in-house testing	Capital investment required	Capital investment required	Capital investment required	Relatively inexpensive	Relatively inexpensive
Approximate cost per test	\$150 - 300 ¹²	\$150 - \$500 ¹²	\$50 ¹³	\$28 - \$130 ¹⁴	\$36 - \$100 ¹⁴
Certification	CDC ELITE, others ¹⁵	ISO 12869, AFNOR ¹⁶	No	AOAC ¹⁷	No

Table footnotes: ¹ Additional identification test may be needed; ² qPCR for quantification and identification of many spp., standard PCR plus sequencing for full identification of most spp.; ³ Measures can be taken to reduce detection of dead cells or free DNA; ⁴ Measures can be taken to enhance detection of VBNC; ⁵ According to IDEXX-commissioned report: <https://www.idexx.com/resource-library/water/legiolert-vs-iso-11731-potable-water-technical-summary.pdf>; ⁶ Bedrina et al. 2013--the ‘false positive’ rate included VBNC not detectable by culturing, meaning it is artificially high; ⁷ False negative rates for culturing vary--see text; ⁸ Measures can be taken to reduce sample interference; ⁹ Kirschner et al. 2016; ¹⁰ Colorimetric data are converted to cfu/L using a formula that was generated by correlating to culture results (see Bedrina et al. 2013; Rodríguez Albalat et al. 2014; Díaz-Flores et al. 2015); ¹¹ LOD stated by manufacturers; ¹² Includes shipping of sample to testing laboratory; ¹³ Price is for in-house testing, does not include equipment or other consumables; ¹⁴ Price varies depending on throughput; ¹⁵ See text for others; ¹⁶ For qPCR--see text; ¹⁷ AOAC: Association of Official Agricultural Chemists, a “globally recognized, 501(c)(3), independent, third party, not-for-profit association and voluntary consensus standards developing organization” that “develops analytical methods for a broad spectrum of safety interests” and is “an A2LA accredited Proficiency Testing Provider.”

Culturing. Culturing on agar plates is the original, standard method for *Legionella* testing. A 250-1000 milliliter sample of water is collected, filtered, and concentrated. The concentrate is then spread onto specialized agar plates, and analysis occurs after sufficient time (7-14 days) has allowed the bacteria to grow under controlled conditions. Culturing is sensitive and enables quantification [in colony-forming units (cfu)] of nearly all species of *Legionella*. Last year, the

American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) released its Standard 188-2015, which features culturing as the test method for building water systems (ASHRAE 2015). According to Public Health Ontario (Public Health Ontario, 2014), culturing can be subject to false negatives: 1) *Legionella* can reside within amoebae or protozoa, protecting it from detection by culturing [this can be avoided with heat treatment prior to culturing]. 2) Other microorganisms in a sample can overgrow *Legionella* on the culture plate, obscuring it from detection. 3) Culture methods will not detect Viable But Not Culturable (VBNC) *Legionella*, which are quiescent but can activate, grow, and infect humans (Epalle *et al.* 2015); however, new methods can alleviate this effect (Ducret *et al.* 2014).

Special equipment and extensive training and experience are needed for execution and interpretation of results, but for a cost of \$150-\$300 per sample, culturing as a service is offered by many companies that are certified by the CDC Environmental *Legionella* Isolation Techniques Evaluation (ELITE), International Organization for Standardization (ISO 11731:1998), American Industrial Hygiene Association (AIHA), American Association for Laboratory Accreditation (A2LA), or New York Department of Health (NYDOH) Environmental Laboratory Approval Program (ELAP) programs.

IDEXX recently launched its Legiolert™ test (performed in the laboratory for about \$50 per sample). It is culture-based, with a streamlined workflow and very little hands-on time. Nevertheless, it requires capital lab equipment, takes 7 days to results, only detects *Legionella pneumophila*, and is subject to the other disadvantages of culturing such as sample interference and failure to detect VBNC.

Polymerase chain reaction (PCR). PCR detects and examines DNA from *Legionella*. Up to one liter of water is collected, filtered, and concentrated. The bacterial cells are broken open and the DNA is isolated, then subjected to amplification and analysis (8-24 hours). Quantitative PCR (qPCR) quantifies *Legionella* spp. in the sample, and standard PCR followed by sequencing is used to identify specific species and serotypes cultured from the sample. PCR is extremely sensitive, and has the potential to identify all *Legionella* species, serotypes, and strains. False positives can occur because PCR detects *Legionella* DNA whether or not the bacteria are alive, or even present (Shih & Lin 2006). False negatives result from susceptibility of the PCR amplification step to interfering factors in the sample (Schrader *et al.* 2012). Proper experimental controls will reveal whether interference has occurred, and methods can be employed to overcome some of these challenges, for example, viability PCR (v-PCR) and reverse transcriptase qPCR (RT-qPCR) to detect only live cells (Kirschner 2016; Whiley & Taylor 2016).

Like culturing, PCR uses special equipment and extensive training and experience, but companies will perform this service for \$150-\$500 per sample. Companies offering qPCR may be certified by the Association Française de Normalisation (AFNOR NF T90-471:2010) or ISO/TS 12869:2012.

Rapid antibody-based tests. Antibody-based methods for *Legionella* detection give results in an hour or less after filtration. They utilize antibodies that recognize and bind to specific elements on the bacteria. As with the other methods, a 250-1000 milliliter sample of water is collected, filtered, and concentrated. Detection can then be performed in two different ways.

Strip tests (or dipstick tests) resemble home pregnancy tests, where the filtered water sample is applied to the test strip and a color change indicates presence or absence of *Legionella*. They are easy to use and require no special training for use on-site. Strip tests detect only *Legionella pneumophila* serogroup 1, the most common serogroup of *Legionella pneumophila*, so other pathogenic serogroups and species will not be detected. The cost per test varies between \$28-\$130.

Immunomagnetic separation - capture enzyme immunoassay (IMS-CEIA)¹, a relatively new method for *Legionella* detection (Díaz-Flores et al. 2015), employs magnetic beads coated with antibody to purify *Legionella* from a sample, followed by a color reaction to quantify the amount of *Legionella* present. It recognizes nearly all *Legionella* species, and like PCR and strip tests, detects VBNC bacteria. It has a very low false negative rate, but it is subject to false positives. At least one IMS-CEIA kit [Legipid®, manufactured by Biotica (Castellón, Spain) and distributed in the U.S. by Abraxis, Inc. (Warminster, PA, USA, <http://www.abraxiskits.com/products/legionella/>)] is certified by the Association of Official Agricultural Chemists (AOAC), and with training can be utilized by facility staff on-site, or samples can be sent for analysis, for \$36-\$100 per test.

Monitoring strategy. Monitoring frequency and response to positive test results will be determined by many factors, including type of water (e.g. potable, industrial, cooling tower), structure age, history, potential for human exposure, system maintenance, and prophylactic control measures. Guidelines for monitoring and remediation can be found from resources such as Occupational Safety and Health Administration (OSHA 2016), CDC (CDC 2016a), and ASHRAE (ASHRAE 2015).

As mentioned earlier, the detection method(s) used will depend on multiple factors, including degree of urgency, accuracy, cost, training, and amount of data desired or required. Culturing is the primary method used today, but it is slow, and its propensity for false negatives may cause it to overlook positive samples. PCR is reproducible, very sensitive, and faster than culturing. However, PCR results cannot be expressed in cfu, and it can be subject to false positive and negative results. Antibody-based methods are rapid, relatively inexpensive, can be performed on-site, and require no scientific expertise. Strip tests are very simple, though not quantitative and only detect one *Legionella* serotype; IMS-CEIA is quantitative and accurate, but requires training.

Water and building managers can choose to send samples away for testing (usually the case for culturing, PCR, or IMS-CEIA); or test on-site with either strip tests or IMS-CEIA. A third possibility is to contract a facilities service that handles *Legionella* monitoring, control measures, and remediation.

One monitoring strategy would be to perform regular on-site testing with one of the faster methods (qPCR, strip test, or IMS-CEIA), followed by confirmation via culturing and/or PCR-sequencing. In regions (e.g. New York) where periodic (e.g. 90 days) monitoring by culture is required, one of the faster methods may be used for interim testing. This enables rapid detection of contamination, identification of its source, and timely application of disinfectant, while results are confirmed and analyzed by additional methods. For example, in December 2015, health

authorities used a rapid method to identify and remove potential sources of infection in the early stages of an outbreak in Manzanares, Spain. While the expected mortality rate from *Legionella* infection in Europe is 5-12%, the low mortality observed in this case (1.7% of confirmed cases) suggests that shortening the duration of contamination by promptly identifying and removing suspected sources may have prevented continued exposure, thus mitigating the impact of the outbreak (Rodríguez et al. 2016).

CONCLUSION

The continued increase in reported cases of *Legionella* contamination and infection can be explained by a number of factors. They include more frequent testing and better diagnosis, ageing population, and ongoing industrialization and ageing of infrastructure. This underscores the need for implementation of monitoring strategies employing rapid, reliable methods that enable prompt action by utility and facility employees while samples are sent for confirmation using other methods.

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