

An Overview of *Legionella*
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Background

The first recognized outbreak of Legionnaires' disease occurred in the US at the American Legion Convention in Philadelphia during the summer of 1976. There were several hundred people who were stricken. Thirty four people died from the disease. As a result of the efforts of the US Centers for Disease Control (CDC), this was the first time the bacteria was cultured and identified. Earlier outbreaks of the disease went undiagnosed. Since that time, there have been many identified outbreaks in this country and abroad prompting professional organizations and health departments worldwide to implement guidelines and regulations for diagnosing and reporting the disease, monitoring the organism, and implementing remediation action levels. In 2015 as a result of an outbreak that affected over 100 people, both the New York State Department of Health and the New York City Department of Health and Mental Hygiene passed groundbreaking legislation for cooling towers and healthcare facilities. However there are no health standards for safe exposure to *Legionella* since the infectious dose is unknown in both healthy and immunosuppressed people.

Transmission and Epidemiology

Ubiquitous in all aquatic environments, *Legionella* bacteria are found in groundwater, municipal water as well as fresh, brackish and marine surface waters. The bacteria enter our plumbing systems, whirlpool spas, and cooling towers via these water sources. Unless control measures are conducted properly and routinely, the biofilm, scale, and corrosion that increases

over time in these systems will protect the organism and allow it to multiply.

Contaminated aerosolized water from cooling towers, whirlpool baths, nebulizers, faucets and showerheads become aerosolized. When a susceptible host inhales the contaminated aerosol, legionellosis can occur. Aspiration of contaminated water or melted ice chips can also cause the disease. *Legionella* can cause a very severe form of pneumonia (Legionnaires' disease) often accompanied by serious long term health effects, or it can cause the mild, transitory, flu-like illness called Pontiac Fever. Blood infections, organ infections, native and prosthetic heart valves, prosthetic hip and knee replacements and asymptomatic infections may occur.

Risk factors include age, gender (male), compromised immune systems, and pre-existing medical conditions such as chronic obstructive pulmonary disease, cancer, diabetes, kidney failure, asthma, the use of immunosuppressive drugs, and chemotherapy. Men over 50 years of age who are immunocompromised, heavy smokers and drinkers are at greatest risk. However, there have been cases of the disease in healthy, younger people and women. Premature, immunocompromised, or ventilated neonates are also at risk from hospital acquired infection. Babies born in home birthing pools filled with tap water have also developed Legionnaires' disease resulting in septicemia, organ failure, and death.

While it was previously believed that *Legionella* was not contagious; there were 2 cases that occurred in Portugal that researchers suspect had been transmitted from son to mother based on the temporal occurrence of the two illnesses and the identical molecular fingerprints of isolates obtained from the bacteria of the two patients (Correia et. al. 2016).

Although the disease is under-reported, travel (cruise ships), hotel, and resort related outbreaks are reported each year. These are mostly associated with the use of whirlpool spas and ornamental and recreational water features.

While community-acquired outbreaks involving cooling towers and whirlpool spas receive the most media attention, studies indicate that building potable water sources account for most of the infections. This is particularly true in hospitals and nursing homes where there are large numbers of immunosuppressed or critically ill people. For these reasons, many state health departments have guidelines that recommend routine monitoring for *Legionella* in critical care hospitals and nursing home facilities. In August, 2014 the Veterans Administration (VA) developed a directive that requires all VA locations that have overnight care to implement monitoring of their potable water systems. There are also draft guidelines for the prevention of legionellosis in building water systems that were created by the American Society of Heating, Refrigeration and Air Conditioning Engineers (ASHRAE).

Recently CDC released statistics indicating that the fatality rate of community acquired cases have a fatality rate of about 10% while healthcare acquired cases have a fatality rate of up to 25 %.

Choosing Sampling Methods

Proper methods for collecting and analyzing samples are necessary to ensure defensible results that are based on the correct sample size. Ensuring that a representative number of samples are taken is also critically important before interpreting results. Since the bacteria in drinking water are present in very low levels, a 1000 milliliter (ml) potable water sample is recommended by the US Public Health Centers for Disease Control (CDC). This sample size allows for the bacteria in the water to be concentrated, allowing for a more sensitive detection and quantitation limit. Many professional guidelines recommend semi-annual sampling for potable water sources.

In non-potable water sources such as cooling tower water, a 250 ml sample size is sufficient. Professional guidelines suggest these sources be monitored quarterly.

Sampling should be conducted in a way that maximizes recovery of the organism and mimics the route of exposure. *Legionella* samples should be collected wherever water aerosolization may occur.

Sampling water alone, however, will likely miss the real source of the organism. The actual reservoir for the bacterium is the biofilm or slime that is always found in our plumbing systems, cooling towers, and whirlpool baths.

Biofilm (slime) consists of other bacteria, blue green algae, amoeba, and protozoans. Biofilm protects *Legionella* from direct exposure to ultra-violet (UV) light, desiccation, and the chemicals used to control its growth. *Legionella* bacteria are ingested by the protozoans and amoeba and

will continue to multiply inside these organisms. The infection of these host organisms will cause them to die releasing huge numbers of *Legionella* bacteria into the surrounding environment. This is exactly what happens inside our bodies when our pulmonary macrophages become infected by *Legionella*.

Because biofilm protects the organism and enhances *Legionella* multiplication, incorporating swabs in your sampling protocol is very important. Swab sampling of biofilm found in cooling tower sumps, potable water faucets, showerheads, and whirlpool spa filters is necessary to obtain a true picture of the presence of *Legionella*. Very often, biofilm swab samples demonstrate the presence of *Legionella* undetected by water sampling alone. Taking swab samples of drinking water and ice machines may reveal *Legionella* contamination due to stagnating water.

While collecting air samples for *Legionella* mimics the route of exposure, this sampling method is not recommended for non-research sampling projects. During air sampling, the bacteria likely are killed from the impaction of the bacterial cells on the collection media.

Legionella are unlikely to survive the exposure to sunlight and desiccation for long periods of time. Air transmission of *Legionella* from cooling towers is greater during periods of cloudy, high humidity days and after rainfall (Simmerling et.al., 2017).

When taking samples an N95 or HEPA particulate respirator, face shield or splash goggles, and waterproof gloves should be worn. When taking samples of hot water storage tanks, it is prudent to wear a face shield, a thermal resistant apron, and

thermal, water resistant gloves. Take care not to generate any aerosols when collecting the samples. Only sterile, appropriately preserved bottles and sterile, Dacron (not cotton) swabs obtained from your lab should be used for sampling. Potable water bottles should be preserved with sodium thiosulfate to neutralize halogen biocides in the water sample. These preserved bottles should also be used when collecting water from cooling towers that have been treated with chlorine or bromine compounds.

After collecting a water sample, be sure to leave an air space in the bottle. Since *Legionella* require oxygen for their survival, an air space in the bottle will ensure that aerobic conditions are maintained during shipment to the lab.

Samples should be packed and shipped to minimize the multiplication of non-*Legionella* bacteria. Using an insulated cooler with freezer packs is recommended to avoid temperature extremes during shipping. Samples should be shipped overnight to the lab.

Analytical Methods- Culture Test

Legionella are aerobic, fastidious bacteria; they have very strict requirements for growth. Two of these requirements are the presence of iron and L. cysteine. They are weakly gram-negative and grow slowly compared to other bacteria. *Legionella* are often overgrown by faster growing bacteria. Their growth can be inhibited by some other bacteria, most commonly *P. aeruginosa*, which is commonly found in biofilms. *Legionella* do not grow on standard microbiological media used for aerobic plate counts or on selective agar used for detecting other, non-*Legionella* pathogens.

Currently the most recognized method in the US for identifying and enumerating *Legionella* in clinical and environmental samples is the culture method. This method uses an improved procedure developed by the International Standards Organization (ISO). This method incorporates routine concentration, acid treatment and heat treatment of all potable and non-potable water samples to enhance the recovery of very low levels of *Legionella* in any sample. Buffered charcoal yeast extract agar (BCYE) with antibiotics is the base formulation to which additional supplements are added to control non-*Legionella* bacteria and recover more *Legionella* species.

The samples must be concentrated in order to recover low concentrations of *Legionella*. This is usually done by filtering the entire sample through a sterile membrane filter. The filter is then vortexed in sterile, distilled water. Aliquots are then taken for plating onto 4 different formulations of BCYE agar. Non-potable water often has a large concentration of bacteria that inhibits the growth of *Legionella*. Since *Legionella* are more resistant to acidic pH levels, these samples are pretreated with a buffered acid solution to eliminate the non-*Legionella* bacteria.

Samples containing large amounts of protozoans such as municipal wastewater or wastewater from paper mills require heat pretreatment. Heat pretreatment is needed to kill the protozoans in order to release the *Legionella* so that they may be recovered, grown and quantified.

The BCYE plates are incubated at 35-37 ° F (which is body temperature) rather than

ambient temperature. Since *Legionella* bacteria grow slowly, incubation periods are specified as 7 days to confirm a positive result to no less than 10 days to confirm a negative result. For this reason, culture methods cannot be rushed.

After 72 to 96 hours, the colonies are examined using a dissecting microscope with UV light. *Legionella* colonies appear as convex, circular white colonies having a center that resembles ground glass. The colonies often exhibit a blue-white, green, or red auto fluorescence.

These suspect *Legionella* colonies are streaked onto BCYE plates that do not contain iron and cysteine. If suspect colonies do not grow on these BCYE plates, they are presumptively identified as *Legionella*.

The presumptive colonies are then analyzed using Direct Fluorescence Antibody (DFA) or Latex Agglutination to confirm the identification of species and identify the serotypes. Since *Legionella* in environmental samples grow slowly, a confirmed negative sample result should be provided only after the 10th incubation day.

Due to cross reactivity and the potential for false positive and false negative results, DFA should be used only on pure colonies obtained after incubation. According to CDC, DFA should not be used directly on environmental water samples as some laboratories claim.

While it is estimated that 85% of the outbreaks in the US are caused by *L. pneumophila* serotype 1, there are other serotypes of *L. pneumophila* and even other

Legionella species that can cause the disease. Not all labs employ the same method for isolating and identifying the organism. Ascertain whether your lab uses the method to give you the level of identification and quantitation you need.

The results for the culturable method are expressed as Colony Forming Units per CFU/milliliter (CFU/ml) in the US. Other countries commonly express results as CFU/liter (CFU/L). While it is standard microbiological practice to express results as CFU/ml, this can be confusing to non-microbiologists.

Analytical Methods-Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a genetic test which looks for the deoxyribonucleic acid (DNA) that is specific for *Legionella*. While PCR is not considered the “gold standard” for *Legionella* analysis in the US, it is very useful for quickly determining the presence or absence of *Legionella* in a sample. Since same day results can be obtained, the quick turnaround time can be useful for confirming the presence of *Legionella* during an outbreak when time is critical.

Unlike culture analysis where inter and intra-laboratory variability is high, PCR results are reproducible, accurate, precise, and very sensitive. The detection limit is theoretically a single DNA fragment.

PCR measures the DNA associated with both viable and non-viable *Legionella*. (To date, the culture method only measures viable bacteria which will grow on the selective media.)

The primary disadvantage of PCR is the potential for sample matrix effects. The presence of common divalent cations in the sample such as calcium, magnesium, or

silver, and the divalent form of copper will cause false negative results unless the samples are processed properly. This requires that the lab have a strict Quality Assurance program that includes positive, negative, and sample matrix controls.

Other disadvantages of PCR are it cannot identify individually the 50 species known to cause disease and it cannot identify all serotypes of these species. While most PCR labs can identify *L. pneumophila*, there may be other species or serotypes colonizing your water system or causing the disease that you would like identified. As a result of a new test procedure it is now possible to identify *L. pneumophila* serotype 1 using PCR.

Non-microbiologists often confuse the terms genus, species, serotype and strain. These are independent terms for the identification of organisms and each is used to reach a successively more specific level of identification. (i.e., *Legionella pneumophila*, serotype 1, Philadelphia is the identification of the genus (*Legionella*), species (*pneumophila*), serotype (1) and strain (Philadelphia) that caused the 1976 outbreak in Philadelphia.

The culture method provides quantification and identification of *Legionella* species and serotypes. Currently, the limited number of commercial labs using PCR will only identify to species level. As of this writing, EMSL is the only commercial lab that has a validated PCR method for determining *Legionella* spp., *Legionella pneumophila*, and *Legionella pneumophila* serotype 1 (Lp1) in the same test. Species and serotype identification is not sufficient for determining the actual source of the contamination during an outbreak. During an epidemiological investigation, it is necessary to employ strain identification to

determine if the bacteria in the clinical samples match the bacteria found in the environmental samples.

In the US, Pulsed Field Gel Electrophoresis (PFGE) was the most commonly used method to identify strains within *L. pneumophila* serogroup 1 and other species. However, newer molecular techniques such as Sequence Based Typing (SBT) which looks at seven specific genes within Lp1, as well as Whole Genome Sequencing (WGS) which can discriminate between the roughly 3.4 million genes in the bacterium are used by CDC, some state health departments, and the European Working Group for *Legionella* Infections (EWGLI) for subtyping *L. pneumophila* serogroup 1 and source identification. *The use of PFGE and SBT does not have enough discriminatory power to identify strains of Lp1 or to identify the genetic mutations in Lp1 or other species and serotypes* (LaPierre et.al., 2017). Therefore only WGS can be used for source tracking. As of this writing EMSL is the only commercial testing lab that can provide the raw data of WGS to be used for source tracking.

Intent of the Risk Assessment

The intent of your *Legionella* risk assessment will determine the type of data you need. Proactive monitoring is conducted to determine baseline conditions and concentrations of *Legionella* or to validate the effectiveness of an existing maintenance program in the absence of suspected cases of legionellosis. With this type of monitoring a qualitative, present/absent result or a quantitative result *Legionella* spp. is sufficient. Species and serotype identification is optional. Strain identification or subtyping is not needed.

Reactive monitoring is conducted when a

suspected or a confirmed case of Legionnaires' disease occurs. In this situation, individual species and serotype quantification is necessary. For an epidemiological investigation of an outbreak, only whole genome sequencing (WGS) can determine if the genetic "fingerprints" of the environmental and clinical samples are related.

Whether your risk assessment is proactive or reactive, the results should indicate predominately non-detectable (ND) amounts of the bacteria.

The actual concentration provides useful information concerning the degree of contamination. However it should be understood that the concentrations are relative and are not an absolute number. Bacterial populations are always in flux; bacterial cells are multiplying, dying, or dormant. Since bacteria multiply logarithmically, an order of magnitude difference (10x) in the results between sampling periods is significant. A difference of a few CFUs or a low single digit multiplication of results is not significant. *The goal is to demonstrate a history of non-detectable results over time.*

To recap, the analytical method used determines the type and accuracy of the results. While using BCYE agars to isolate *Legionella* is the recognized "gold standard" worldwide, there are still some labs using other methods. Also, the reagents and methods used for *Legionella* identification are not standardized. This makes comparing lab results from two different labs very difficult. Be sure to identify the isolation method the lab is using as well as the identification methods used. This will ensure you are drawing correct conclusions when comparing results and will also ensure

you obtain the level of detailed information you need.

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