

An Overview of *Legionella* Analyses

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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 for diagnosing and reporting the disease, and monitoring the organism.

Transmission and Epidemiology

Ubiquitous in all aquatic environments, *Legionella* bacteria are found in groundwater as well as fresh 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 builds up 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 becomes airborne. When a susceptible host inhales the contaminated aerosol, legionellosis can occur. Aspiration of the contaminated water can also cause the disease. Legionellosis can cause two types of illness: 1. a severe form of pneumonia (Legionnaires' Disease) often accompanied by serious long term health effects, and 2. a mild flu-like illness called Pontiac Fever. Other infected organs, and asymptomatic infections may also occur.

Historically, risk factors for getting the disease included age, gender, compromised immune systems, and pre-existing medical conditions

such as chronic obstructive pulmonary disease, cancer, and diabetes. Men over 65 years of age who were heavy smokers and drinkers were identified as being at greatest risk. While that is still true, recent research from Neil and Berkelman at Emory University has identified an abrupt increase in the incidence of Legionnaires' Disease in the US in all age groups in the last 20 years. This trend has also been noted internationally by other researchers. They have noted an overall increase in the disease among all people aged 45 to 64. Rates of disease in males still exceed the rates in females.

There also have been cases of the disease in healthy, younger people. Pre-mature, immunocompromised, or ventilated neonates are at risk from hospital acquired infection. In addition cases have been reported in children aged 15-19 years old.

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 potable water. 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 homes. In 2008, the Veteran's Administration promulgated a directive which requires all VA hospitals and rehabilitation centers to implement monitoring for the bacteria in their potable water systems.

Choosing Sampling Methods

Proper methods for collecting and analyzing samples are necessary to ensure defensible results. Since the bacteria in water are present in very low levels, 1000 mL potable water samples are 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 better detection in potable water samples. . Many professional guidelines recommend semi-annual sampling for potable water sources.

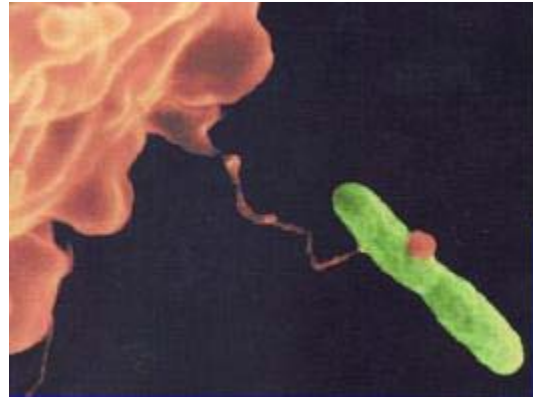
In non-potable water sources such as cooling tower water, a 250 ml sample size is sufficient. Professional guidelines suggest these sources be monitored quarterly.

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 aerosolized water alone, however, will likely miss the real source of the organism. This source is the biofilm or slime that is often found in our plumbing systems, cooling towers, and whirlpool baths.

Biofilm 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. In addition, the *Legionella* bacteria are ingested by the protozoans and amoeba and will continue to multiply inside these organisms. Once these organisms die large numbers of *Legionella* bacteria will be released into the surrounding environment.

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.



A *Legionella* bacterium being ingested by a macrophage

While collecting air samples for *Legionella* mimics the route of exposure, it is generally not recommended for routine monitoring purposes. *Legionella* are unlikely to survive the exposure to UV light and desiccation for long periods of time. In addition, the bacteria may be killed from the impaction of the bacterial cells on the collection media.

When taking samples around cooling towers a high efficiency particulate respirator, safety glasses, and gloves should be worn. Take care not generate any aerosols when collecting potable water samples. Only sterile, appropriately preserved bottles and swabs obtained from your lab should be used. Potable water bottles should be preserved with sodium thiosulfate to neutralize chlorine in the water sample. These preserved bottle should also be used when collecting water from cooling towers that have been treated with chlorine dioxide.

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. Since *Legionella* remain viable at low temperatures, using an insulated cooler with freezer packs is recommended. Samples should be shipped overnight to the lab.

Analytical Methods- Culture is the “Gold Standard” Worldwide

Legionella are aerobic, fastidious bacteria; they have very strict requirements for growth. Two of these requirements are iron and L. cysteine. They are weakly gram negative and grow slowly compared to other bacteria. *Legionella* are often overgrown by faster growing bacteria, inhibited by some bacteria, or will not grow on standard microbiological media. For this reason labs should use methods that are selective for isolating and identifying the organism.

Currently the definitive method worldwide for identifying *Legionella* in clinical and environmental samples is the culture method. This method uses an improved procedure developed by the CDC when it first isolated the organism after the American Legion outbreak in Philadelphia in 1976. The method uses buffered charcoal yeast extract agar (BCYE) as the base formulation.

For potable water, the samples must be concentrated in order to enhance the quantitation limit. This is usually done by filtering the entire 1000 ml through a sterile membrane filter. The filter is then vortexed in 5 mL of sterile, distilled water.

Non-potable water often has a large concentration of bacteria that surpasses or 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.

Equal sample aliquots are then plated onto the BCYE agar containing iron and L. cysteine (BCYE+), BCYE agar with Polymixin B, Cycloheximide, Vancomycin (PCV), PCV with Glycine (GPCV), and PCV without iron and L. cysteine (PCV -). These plates are incubated at 35-37 °F. Because the *Legionella* bacteria from environmental samples may grow slowly, the plates are incubated for 10-14 days.

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 edges of the colonies often exhibit a blue, green, purple or red autofluorescence.

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

The presumptive colonies are then analyzed using Direct Fluorescence Antibody (DFA) technique to confirm the identification of species and identify the serotypes. Since *Legionella* in environmental samples grow slowly, a confirmed non-detected 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. DFA should not be used directly on environmental samples as some laboratories claim.

While 90% 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 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 (CFU) /ml or per volume sampled. Even though some labs prefer to express the results as CFU/volume sampled, it is standard microbiological convention to express results as CFU/ml. CFU/ml is also the concentration used in the OSHA and other international guidelines.

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, it may be very useful for quickly determining the presence or absence of *Legionella* in a sample. Since same day

qualitative results can be obtained, the quick turnaround time can be useful for confirming the presence of *Legionella* during an outbreak when time is critical. PCR may also be useful for rapid assessment of the effectiveness of a treatment program.

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*. (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.

Another disadvantage of PCR is that it is a species specific test. While most PCR labs can identify *L. pneumophila*, there may be other species colonizing your water system or causing the disease that you would like identified.

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 1 is the identification of the bacteria 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. Species and serotype identification is insufficient 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.

Currently in the US, Pulsed Field Gel Electrophoresis (PFGE) is most commonly used to identify strains within *L. pneumophila* serogroup 1. However, a newer molecular technique, Sequence Based Typing, is used by CDC and the European Working Group for *Legionella* Infections (EWGLI) for subtyping *L. pneumophila* serogroup 1. EWGLI has proposed the use of SBT as the standard method for strain identification for travel related outbreaks in the European Union.

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 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 of *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 confirmed case of legionellosis occurs. In this situation, species and serotype quantification and identification is necessary. If the case or outbreak was diagnosed as *L. pneumophila* serotype 1, strain identification will be useful to link clinical isolates to the environmental samples to identify the source.

Whether your risk assessment is proactive or reactive, the results should indicate non-detectable amounts of the bacteria. This is the OSHA recommended performance goal.

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 is significant. A difference of a few CFUs or a low single digit multiplication of results is not significant. To reiterate, 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 very difficult. Be sure to identify the isolation method the lab is using as well as the identification methods used. This will ensure you obtain the information you need.

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