



Advances in Environmental Detection and Clinical Diagnostic Tests for *Legionella* Species

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J Health Allied Sci^{NU} 2022;12:168–174.

Abstract

Keywords

- ▶ extrapulmonary infection
- ▶ iron
- ▶ *Legionella*
- ▶ *Legionella*-contaminated aerosol
- ▶ Legionnaires diseases
- ▶ Pontiac fever
- ▶ water distribution system

Legionella is a fastidious organism that is difficult to culture in the lab but is widely distributed in environmental, domestic, and hospital settings. The clinical manifestations due to *Legionella* infections range from mild fever to fatal pneumonia and multiorgan pathologies. *Legionella* outbreaks though prevalent globally are not reported in developing countries due to difficulties in isolating this organism and the lack of simple diagnostic protocols. Here, we review the literature from across countries to present various methods used to detect *Legionella* from environmental and clinical samples. We compare the sensitivity and the specificity of the conventional culture-based assays with the recent methods and discuss approaches to develop better detection and diagnostic tests. With better cost-effective detection techniques and regular monitoring of the susceptible sites, which may harbor *Legionella* colonies, most of the *Legionella* infections can be prevented. As a result, considerable burden, caused by *Legionella* infections, on the healthcare system, in especially economically weaker countries, can be mitigated.

Legionella: Discovery and General Features

Though reported from a soldier's blood culture in the 1950s, the importance of *Legionella* as a human pathogen was recognized only in 1976 when an unexpected outbreak of fever with pneumonia was reported among participants of the American Legion Conference in Philadelphia, United States of America. This led to the discovery of the causative organism called *Legionella* and new diseases called Legionnaires disease (LD) and Pontiac fever (PF) getting added to the list of bacterial infections.¹ *Legionella* are common in soil and aquatic systems often found associated with protists.² The cells of *Legionella* are thin, pleomorphic, Gram negative, nonspore-forming bacilli with a size ranging from 2 to 20 μm

in length and 0.3 to 0.9 μm in width. Most species are motile by means of a single polar flagellum and have pili. The members of this genera utilize amino acids rather than carbohydrates for energy and require L-cysteine and iron salts for their survival. They can withstand a wide range of temperature from 20 to 45°C.³ *Legionella* are pervasive in all aquatic habitats and known to enter man-made water systems easily.²

Till date, about 60 species of *Legionella* have been documented and of these at least 24 have been found associated with human disease condition. *L. pneumophila*, *L. longbeachae*, *L. anisa*, *L. bozemanii*, and *L. micdadei* are commonly detected agents of Legionellosis. Other pathogens include *L. feeleii*, *L. hackeliae*, *L. sainthelensi*, *L. spiritensis*, *L. erythra*, and

DOI <https://doi.org/10.1055/s-0041-1731863>.
ISSN 2582-4287.

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L. quinlivanii. Environmental isolates such as *L. adalaidensis*, *L. beliardensis*, *L. brunensis*, and *L. busanensis* are some of the nonpathogenic species.⁴

Reservoirs

Water is the primary natural reservoir, although *Legionella* represents a minor component accounting for less than 1% of the residential bacterial population in water.⁵ *Legionella* are found worldwide in freshwater environments such as stagnant lakes, springs, mud streams, and damp soil with relatively low concentration. However, they are rarely found in the marine ecosystem.^{6,7} Ocean waters receiving treated sewage contain *Legionella* species and their numbers do not drop even after the treatment process.⁸ Since *Legionella* are autochthonous in aquatic habitats, it is impossible to prevent their entry to public water systems. Storage tanks, decorative fountains, spa pools,⁹ whirlpools, air conditioning vents, cooling towers in recreational centers and water systems of homes, industries, resorts, healthcare facilities, and cruises^{10,11} have been identified as sources of *Legionella* outbreaks. The disease is mainly transmitted via air from the water source typically contracted by inhaling contaminated aerosols from anthropogenic devices.

The bacteria colonize and transmit from plumbing fittings such as shower heads and hot-water taps.¹² In India, *L. pneumophila* was found in 6.66 to 15.2% of distal outlet waters in patient care areas in tertiary hospitals.^{13,14} Outbreaks of the LD are more common in late summer and early autumn because of stagnation of water which provides an ideal ground for bacterial growth.¹⁵

In wake of the ongoing pandemic crisis due to coronavirus disease 2019, exposure to aerosolized water from recently reopened office and market buildings is considered as an epidemiologic risk factor for *Legionella* exposure.¹⁶ Outbreaks from a dishwasher in a reopened restaurant in Italy, Rome, were reported just after the prolonged lockdown. Stagnating water in the water pipes and dispersing water units such as air conditioning system, and spa pools provide conditions where harmful *Legionella* can proliferate.^{17,18}

Clinical Presentations of Legionella

Legionellosis is the descriptive terminology used to define infections caused by the members of *Legionella* including LD, a severe and presumably fatal form of pneumonia,⁶ and PF, a self-limited flu-like illness. Subclinical infections may even go undetected as being entirely asymptomatic.^{19,20} In exceptional cases, *Legionella* can get transmitted to other organs through the bloodstream and the lymphoid system resulting in extrapulmonary infections.²¹

In all cases, the incubation period is 24 to 48 hours. When an outbreak occurs, more than 90% of the population exposed to the contaminated aerosol contract PF. Recovery from PF occurs within a week, while the more susceptible population may progress to LD.²²

Legionnaires Disease

LD does not have explicit or critical medical features, but presents a wide range of clinical manifestations and symptoms. LD often occurs in elderly patients, immunocompromised patients, smokers, and patients with the history of diabetes and chronic obstructive pulmonary disease. The disease is initially characterized by high fever, fatigue, malaise, and sleeplessness. Additional variable symptoms may include simple cough,²³ cough with blood-streaked sputum and hemoptysis, or pleuritis. Gastrointestinal symptoms such stomach ache, vomiting, and diarrhea are common.²⁴ Some patients also manifest neurologic signs such as neuralgia, disturbed state, lethargy, anxiety, and delusion.²⁵ Radiographic manifestations include alveolar opacities and infected pulmonary tissue reveal bronchopneumonia. Air-space inflammation and thrombosis and alveolar wall necrosis can also occur. Often, antibiotic treatment does not reduce the progression of pneumonia even after 5 days.

Pontiac Fever

PF is seen in a majority of the population exposed to contaminated aerosols. PF occurs in an acute outbreak mode and is not related to pneumonia.²⁶ As with LD, the infection occurs from the inhalation of a *Legionella*-contaminated aerosol but PF appears in healthy individuals as well.²⁷ Clinically, symptoms of PF resemble asthenia, influenza with fever, muscle pain, headache, cough, and pharyngitis.^{26,28} Patients with these symptoms recover without medication in 2 to 5 days.²⁹ Unlike LD where pneumonia is the distinct pathology, the pathogenicity of PF is poorly defined. However, age, gender, and smoking habits are underlying risk factors for LD, and these are not notable risk factors for PF.³⁰

Extrapulmonary Infections

L. pneumophila has been isolated from internal organs including lymph glands and alimentary canal indicating that the bacteria spread into different parts of the body through the respiratory system or via surgical sites.³¹ The heart is the most frequently affected organ.³² The clinical manifestations of extra pulmonary *Legionella* are often abrupt with manifestations of erysipelas, peritoneal inflammation, or kidney infection.³³ *Legionella* occasionally progress through the nervous system and lead to neurological symptoms of encephalitis, cerebrum infection, and chronic fatigue.³⁴

Incidence

The worldwide prevalence of Legionellosis is mysterious. Although *Legionella* is a well-known problem, according to World Health Organization, this pathogen is neglected in developing and underdeveloped countries and hence no or limited reports appear from many regions.³⁵ Some data from longitudinal research has shown that outbreaks in healthcare systems are more likely to occur when more than 30% of peripheral sites in the water source are colonized by the pathogen.³⁶ Despite the availability of various guidelines and standards for preventing the spread of *Legionella* and minimizing risks in the healthcare sector, the recorded incidence

of Legionellosis has risen from 0.42 to 1.62 per 100,000 individuals in the United States from 2000 to 2014.³⁷ In Europe, the incidence rate of LD was 2.2 per 100,000 in 2018. Reports from Hong Kong and China also report an increase in incidence rate from 0.16 per 100,000 in 2005 to 0.91 per 100,000 in 2015.³⁸ A study conducted at a medical care center in India from 2015 to 2020 in patients with confirmed pneumonia found 14 out of 597 patients were positive for *Legionella* infection.³⁹

Diagnostic Methods

Since the discovery of *L. pneumophila*, several techniques have been developed to detect the pathogen. Though first reported by culturing in yolk sacs of embryonated eggs, the first diagnostic technique for specific identification of the causal agent employed immunofluorescence labeling.⁴⁰

Conventional Culture Method

Mueller-Hinton agar accompanied with hemoglobin and IsoVitaleX was the first culture medium used to isolate *Legionella*.⁴¹ The medium used for the isolation has undergone several modifications with improvements, resulting in the medium currently used. This conventional culture technique is traditionally recognized as the gold standard for *Legionella* isolation and detection.

Even though this protocol allows to isolate and quantify legionellae from environmental water, it does have its restrictions. These techniques require extended growth time, they are tedious, retrieval rates are habitually low, and concentration of samples by centrifugation or filtration leads to bacterial cell injury and thus loss.⁴² The most commonly used method for the environmental surveillance of *Legionella* is the standard culture technique, which allows the estimation of the number of bacteria present in the water. Laboratories across the world follow International Organization for Standardization (ISO) 11731:2017 culturing method for *Legionella* enumeration. This method is applicable to all kinds of samples such as natural waters, potable, industrial and waste waters and also include water associated matrices such as biofilms and sediments. The bacterial portion of the sample is concentrated by filtration and treated with acid or heat to selectively isolate *Legionella*. Isolation of *Legionella* from clinical and environmental samples is currently done on buffered charcoal yeast extract agar base (BCYE) containing 0.1% alpha-ketoglutarate (ISO 11731:1998). Two types of selective BCYE agar supplemented with antimicrobial agents are included in the standard processing of the environmental samples. The first is designated as PCV that contains polymyxin B, cycloheximide, and vancomycin antibiotics along with BCYE. The second is GPCV that contains glycine, polymyxin, cycloheximide, and vancomycin to inhibit the growth of environmental flora in the medium. PCV without L-cysteine is used as a negative control medium as *Legionella* fail to grow in the absence of cysteine.⁴⁰ Further strain identification requires additional serological methods.⁴³ Presence of other interfering microorganisms and the ability of *Legionella* to enter into the viable

but not-culturable state in limited nutrient conditions further hinder the recovery of *Legionella* by conventional culture method. The underestimation of *Legionella* could occur due to failure of *Legionella* to grow in harsh sample processing conditions and overestimation (false-positives) occurs due to growth of other genera that have evolved to grow in formulated *Legionella*-specific media.⁴⁴ Alternatively, a co-culture protocol for resuscitation of *Legionella* in the presence of amoeba was developed to promote the intracellular multiplication of *Legionella* and enhance recovery.⁴³ This enrichment takes about 72 to 100 hours for recovery of ~10 bacteria and the bacteria thus recovered are more invasive and virulent.⁴⁵

Molecular Methods

Culture-independent methods of detecting have shown higher number of *Legionella* when compared to the culture technique.⁴⁶ Nucleic acid probe detection methods and polymerase chain reaction (PCR) techniques have been developed or adapted for rapid detection and quantification of *Legionella* DNA in water samples for routine monitoring.⁴⁷ Molecular methods such as PCR are more sensitive than the culture method. Some of the common targets for PCR-based detection of *Legionella* are the 5S rDNA, 16S rDNA, the macrophage infectivity potentiator (*mip*) gene, *rpo B*, and defective organelle trafficking(*dotA*) gene of *L. pneumophila*.^{48,49} A comparative study between PCR and culture-based method showed PCR to accurately detect 6% more samples than culture technique from respiratory samples using the 16S rDNA as target. However, ambiguity rests when testing environmental samples: 5S rDNA had higher level of sensitivity than culture methods, while *mip* and 16S rDNA had same levels of sensitivity.⁵⁰ Not all PCR assays can discriminate between living and nonliving (noninfectious) *Legionella* cells. *Legionella* detection for environmental monitoring is a qualitative test and hence quantification is often not reported.

Quantitative (qPCR) amplifies and quantifies a target DNA thereby providing rapid enumeration of *Legionella* from environmental samples.⁵¹ A review on 28 studies conducted from 2003 to 2013 that concurrently used culture and qPCR to quantify *Legionella* from environmental sources showed 26 of 28 studies reporting better sensitivity using qPCR.⁵² The limitations of qPCR include amplification of dead cells, need for optimizing the sample types every time, and presence of inhibiting environmental compounds.⁵³

Viability quantitative polymerase chain reaction (v-qPCR) is a relatively recent analytical approach applied for detecting live *Legionella* in environmental sample. While v-qPCR yields better or equal sensitivity when compared to the culture method, its sensitivity is lower when compared to qPCR thus establishing that it detects only viable forms.⁵⁴

Loop-mediated isothermal amplification (LAMP) has gained attention for molecular diagnostics due to its higher sensitivity to detect lower nucleic acid concentrations and the ability to perform this test in the absence of any sophisticated equipment. The technique has found applications in the detection and diagnosis of several pathogens. Thus, the

LAMP assay can be considered as a potential diagnostic tool with higher sensitivity and specificity than conventional methods, such as PCR and culture method.⁵⁵ Recent developments on the application of LAMP for detection of *Legionella* are listed in ▶Table 1.

MALDI-TOF: Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has emerged as an advanced, efficient, and low-cost tool for species-level identification using ribosomal protein patterns.⁶³ Owing to slow growth and the lack of specific and rapid diagnostic methods, *Legionella* nonpneumophila species are less commonly identified in clinical diagnosis or environmental sampling. While MALDI-TOF MS is widely used throughout the world, few studies have been published on its use to identify *Legionella* in clinical and environmental samples. A comparative study was conducted between the cultural and *mip*-gene sequencing findings. Additionally, phylogenetic analysis was also conducted to quantify the correlations between isolates. In this experiment, MALDI-TOF MS achieved exact species-level identification for 45.0% of the isolates displaying a high concordance with the *mip*-gene results. In comparison, the remaining 55.0% of isolates showed less consistent, as it was not added in the database. The findings support the use of MALDI-TOF MS in routine monitoring, as it provides more accurate and species level identification, as well as the ability to identify species that

are not yet in the database. No errors were observed in the result.⁶⁴

Immunological Methods

While culture methods are time exhaustive but provide for accurate detection, molecular methods provide for rapid but probable nonspecific detection. Immunological methods provide for specific rapid detection using antibodies that are specifically raised against *Legionella* more specifically against a particular serotype. The direct immunofluorescence assay (DFA) was first used for the discovery of *Legionella* as a pathogen. DFA is now used to detect *Legionella* effectively from sputum, endotracheal suction aspirates, and lung biopsies.⁶⁵ This method has the advantage of delivering a report in 2 to 4 hours, but it is technically challenging and can only be practiced by professional laboratory staff. The urinary antigen test is often used in a clinical setting for rapid diagnosis as *Legionella* gets eliminated via the urine and provides for a noninvasive detection method. Here too, the antibody is specific for a particular serotype, most often *L. pneumophila* serotype 1. Other species of the genera or other serotypes of *L. pneumophila* other than that detected by the antibody would get omitted by these methods and hence the immunological methods though specific and rapid have low sensitivity for the detection of *Legionella* infections.⁶⁶

Table 1 Applications of LAMP in the detection of *Legionella*

Method	Gene target	Outcome	References
LAMP combined with electrochemical transduction system	16S rRNA	Detects 10fg nucleic acid corresponding to only two copies of the bacteria	Olabarria et al 2020 ⁵⁶
LAMP Droplet digital based LAMP	16SrRNA <i>Lep B</i>	10 ³ fg µL ⁻¹ 1,233 droplets with positive target DNA amplification out of 9103 droplets for the <i>lepB</i> gene sequence	Reuter et al 2020 ⁵⁷
LAMP	<i>Mip</i>	1% culture positive 3% PCR positive 7% LAMP positive	Moosavian et al 2019 ⁵⁵
Multiplex isothermal RPA amplification	<i>Mip</i>	10 CFU	Kersting et al 2018 ⁵⁸
LAMP	16S rRNA	31.9%- LAMP for water sample 5.8% culture method 11.1%-LAMP swab sample 1.1%-culture method	Kuroki et al 2017 ⁵⁹
Real time LAMP on-filter direct amplification	16SrRNA, <i>cadA</i>	~1 CFU /100 mL	Samhan et al 2017 ⁶⁰
Real time LAMP	16S rRNA	LAMP 56.07% Culture assay 47.66%	Lu et al 2011 ⁶¹
LAMP	16SrRNA gene to detect wide range of <i>Legionella</i> spp.	detection limit 6 CFU per test	Annaka 2003 ⁶²

Abbreviations: CFU, colony forming unit; LAMP, Loop-mediated isothermal amplification; PCR, polymerase chain reaction; RPA, recombinase polymerase amplification.

The use of polyclonal antibodies or various monoclonal antibodies can provide for a broader spectrum of sensitivity. However, cross reactivity within the antibodies can give false positive results.⁴⁶ This method detects and enumerates *L. pneumophila* within 3 to 4 hours. However, immunological methods and molecular methods detect both viable and nonviable cells.

ELISA: Tilton was the first to develop enzyme-linked immunosorbent assay (ELISA) to identify a soluble antigen in patients urine infected by *L. pneumophila* serogroup 1 (Lpn 1) and several other assays have since been published. For the detection of specific antibodies to serogroup 1 and each of the six serogroups of *L. pneumophila*, ELISA has been found to give many advantages over standard indirect fluorescent antibody and other serological tests in terms of simplicity, rapidity, quantitative measurement, and automation ability.⁶⁷ Cross-reactivity between serogroups or species is typical in commercial ELISA kits that use *L. pneumophila* whole-cell protein as the coating antigen. A study conducted by Sun et al using *L. pneumophila* proteins such as FLA (Flagellin A), MOMP (major outer membrane protein), MIP (macrophage infectivity potentiator), and PILE (type IV pilin) IP, and PILE (Type IV pilin) applied in serological diagnosis of *L. pneumophila* infections compared with *Legionella* ELISA kits. The results of the five purified proteins used in indirect ELISA revealed that IgG had a sensitivity of 90.4% and a specificity of 97.4%. The proteins seemed to be suitable coating antigens for the serological diagnosis of *L. pneumophila*.⁶⁸

Physicochemical Methods to Indicate *Legionella* spp.

The biggest hurdle to detect *Legionella* is its fastidious nature. The conventional culturing technique considered as the benchmark detection can take up to 14 days to give a result. Therefore, rapid detection methods are the need of the hour for disease prevention and management of outbreaks if any.⁶⁹ One approach to develop a low cost, low infrastructure, and rapid detection method is to detect environmental indicators for the pathogen of interest. Among many transition metals, iron, copper, zinc, and magnesium act as cofactors for many biological enzymes. More often than not the microorganisms source these metal ions from their immediate environment. Thus, screening of concentration of essential metal ions allows development of sensitive and specific assay for the detection of sites that favor the growth of microorganism of concern.

Iron is associated with many enzymes linked with respiratory chains. Since legionellae are iron-dependent, it is often expected that their growth will be promoted by the use of iron piping and in water containing high levels of iron. Iron positively correlates with *Legionella*.⁷⁰ We have reported that iron concentration of 300 mg Fe/L in cooling tower water shows a positive correlation with *Legionella* presence and could be used as a strong indicator of *Legionella*.⁷¹

Copper pipes have been found to temporarily restrict colonization by *Legionella* and hence may not be a suitable indicator for this pathogen.⁷²

Zinc has been associated with lower levels of *Legionella*, suggesting that between 100 and 200 ppb may be the optimal amount of zinc for *Legionella* growth.⁷³ Manganese levels below 3 µg/L are correlated with less number of *Legionella*, but a negative association appears to exist above 10 µg/L.⁷⁴ Conflicting results were found with respect to the calcium and *Legionella* interactions, with two experiments showing favorable, one negative, and another noncorrelated results.⁷⁵ Overall, among the inorganic ions that are present in water, iron seems to be the only parameter that consistently appears along with *Legionella* thus being a suitable indicator for the pathogen.

Conclusion

The incidence of *Legionella* infection is underdiagnosed in many parts of the world due to lack of optimal diagnostic tests. To improve the diagnosis protocols, in terms of specificity and sensitivity, newer methods are being developed to understand the epidemiology of LD. Recent advances in the *Legionella* detection include PCR-based methods. However, combinatorial tests based on culture, serological testing, urinary antigen detection, and analytical techniques continue to be the first line of investigation in many medical microbiology laboratories. The better availability and use of improved diagnostic tests will help to better characterize the epidemiology of LD, including the true incidence and geographic variation.

Conflict of Interest

None declared.

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