Lab Diagnosis of Legionnaires' Disease

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ABSTRACT

Legionnaires' disease is a multi-system disease which causes atypical pneumonia due to *Legionella* bacteria, most commonly of the species *Legionella pneumophila*. About one out of every 10 people who get sick from Legionnaires' disease will die. Most common source of infection-contaminated water supply through inhalation of contaminated water droplets (aerosols). A laboratory diagnosis of Legionnaires' disease can be made using a variety of laboratory tests including: culture/isolation of the causative organism, antigen detection in urine, a significant rise in antibody titres or PCR methods. Determination of the monoclonal subtype and molecular sequence typing can support linking between strains from the sampled environment and from patients. The control measures available to reduce the amount of *Legionella* in a water system include structural adaptations to the water system, temperature control, disinfection using chemicals or other oxidizing materials, and use of biocides or installation of filters. JMS 2014; 17(2):50-54

Key words: *Legionella*, diagnosis, antigen, pneumonia, sequencing, culture.

INTRODUCTION

Legionellosis is a collection of infections that emerged in the second half of the 20th century. Includes a pneumonia-type illness called Legionnaires' disease, a mild flu-like illness called Pontiac fever and extra-pulmonary Legionellosis. *Legionella* is an important pathogen in nosocomial pneumonia and also causes community acquired pneumonia resulting in a high rate of hospital admission. *Legionella* was discovered after an outbreak in 1976 among people who went to a Philadelphia convention of the American Legion.^[1] The affected suffered from pneumonia that eventually became known as *Legionnaires' disease (LD)*. Although cause of the outbreak went undetected for many months, eventually Joseph McDade and Charles Shepard (CDC) discovered the etiologic agent "*L.pneumophila*" in 1977.

L.pneumophila is a fastidious, Gram-negative thin rod or coccoid and is uncapsulated motile with one to three polar or subpolar flagellae. It is aerobic and uses amino acids for energy (L-cysteine). It does not form spores and the cell

wall in it has large amounts of branched-chain cellular fatty acids.^[2] Above 70°C (158°F) *Legionella* dies almost instantly, at 60°C (140°F): 90% die in 2 minutes. At 50°C (122°F): 90% die in 80-124 minutes, depending on strain 48 to 50°C (118 to 122°F): can survive but do not multiply. 32 to 42°C (90 to 108°F) is ideal growth range. 25 to 45°C (77 to 113°F) growth range. Below 20°C (68°F) can survive, even below freezing, but are dormant. Chlorination at 0.5ppm residual in hot water kills it. Hyper chlorination (50ppm for 24hrs) takes care of it in biofilms also.^[3] Copper-silver ionization is recognized by WHO for *Legionella* control and prevention. Chlorine dioxide is approved by the U.S. Environmental Protection Agency as a primary disinfectant of potable water since 1945.^[4]

Pathogenicity is enhanced by Type II Secretion System, which favours intracellular growth, virulence and environmental persistence of *Legionella*. It dampens the cytokine secretion from infected macrophages and epithelia and limits the levels of cytokine transcripts in infected macrophages. It uses another DOT/ICM Type IV secretion system (Defective Organelle Trafficking) Or ICM (Intracellular Multiplication) for its pathogenic effects also by enhancing bacterial entry, secreting and translocating multiple bacterial effector proteins into the vacuolar

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membrane and cytosol of the host cell. Inhibition of host cell apoptosis and egress of *L. pneumophila* from host cells is prevented. Heat shock protein 60 also enhances invasion and cytokine expression in macrophages. Macrophage infectivity potentiator (Mip) protein promotes adherence and phagocytosis. Flagella provide motility. Type IV pili enhance entry of *L. pneumophila* into macrophage. Outer membrane protein is responsible with binding and delivery of packaged materials into the eukaryotic cells it also inhibits the fusion of phagosomes with lysosomes. MOMP (Major outer membrane protein) is responsible for adherence and intracellular replication, biofilm development and formation and horizontal gene transfer.

Epidemiology

The clinical spectrum of disease can occur in three forms Legionnaire's disease, Pontiac fever and Extra-pulmonary Legionellosis. Extra pulmonary Legionellosis being the rare but most common site in adults is the heart. In children, extra pulmonary sites are- liver, spleen, brain, and lymph nodes. Other manifestations may include: sinusitis, cellulitis, peritonitis, pyelonephritis, pancreatitis and wound infection.

It is worldwide in distribution and cause 2-15% of all CAP cases requiring hospitalization. About 6,000 cases of LD were reported in the United States in 2013. LD is likely under-diagnosed, this number may underestimate the true incidence. About one out of every 10 people who get sick from Legionnaires' disease will die. More illness is usually found in the summer and early fall, but it can happen any time of year.

A case is defined as a person having clinical symptoms compatible with Legionella plus a confirmatory Laboratory Test: by Urinary Antigen or Culture or validated positive PCR or DFA or Serology (fourfold increase from baseline).

Reservoir

The natural reservoirs are water resources- lakes, rivers, streams, ponds, hot & cold springs etc. Artificial reservoirs are large plumbing systems, intracellular pathogens of free-living protozoa and other aquatic eukaryotes also harbor legionella. Amoebas such as *Acanthamoeba*, *Hartmannella*, *Naegleria* provide the bacteria with an ideal environment for reproduction and development. It has the capability of surviving within biofilms particularly in man-

made water systems. Extracellular growth is often a necessity, and the biofilms permit this growth. Majority, if not all, Legionella are associated with biofilms in showers and faucets in cooling towers (air-conditioning units for large buildings). It is also found in hot tubs that aren't drained after each use, decorative fountains and water features, hot water tanks and heaters, large plumbing systems, humidifiers. Worth mention is that home and car air-conditioning units do not use water to cool the air, so they are not a risk for *Legionella* growth. It also does not occur as commensal flora in man. There is no person to person transmission. Inhalation of contaminated water droplets (aerosols), aspiration- oropharyngeal colonization or drinking contaminated water can cause the disease.^[5]

People at Risk

Most healthy people exposed to *Legionella* do not get sick. However presence of virulent *Legionella* in environment is a risk factor when there is overnight stay outside home (travel/camping), exposure to aerosols of potable water, recent plumbing repairs or maintenance work. CDC categories the disease as: Travel-associated; Healthcare-associated and Community-acquired. An outbreak occur when two or more people are exposed to Legionella and get sick in the same place at about the same time, and is commonly associated with buildings or structures that have complex water systems like hotels and resorts, long-term care facilities, hospitals, cruise ships^[6]

Lab Diagnosis

1. MICROSCOPY

Microscopy is the basic diagnostic aid in the diagnosis and the specimen for staining can be respiratory secretions (sputum, bronchial aspirate or washings), pleural fluid, lung biopsy or autopsy material. On Gram staining direct specimens stain poorly as very small gram-negative bacilli are seen. Sputum Gram stain is insensitive, especially if safranin rather than basic fuchsin counterstain is used, as small, intracellular bacteria are rarely recognized. *L. micdadei* and rarely, *L. pneumophila* may be acid-fast or weakly acid-fast. Silver staining by Dieterle's Silver Impregnation Method can also be used. Gimenez staining is used for tissue & water staining and in amoeba.

Figure 1: Gram stain of *Legionella*

DFA is most sensitive for detecting *Legionella* in clinical specimens; in it Fluorescein-labeled monoclonal or polyclonal antibodies directed against *Legionella* species are used.^[7] For IFA specimen to be used is serum and is incubated with a hyper immune antiserum. Then it is visualized by applying a fluorescently tagged anti-*Legionella* antibody FluoresceinIsothio cyanate-conjugated immunoglobulin (FITC).

2. CULTURE

Culture remains the Gold standard and clinician should request for *Legionella* culture. The specimens for culture can be Sputum, BAL/Tracheal aspirations, pleural fluid, lung biopsy, blood, environmental samples (water). It is strict aerobe 37°C (29-40°C) for 3-10 days (slow growth), pH; 6.9. It does not grow on ordinary media and L-cysteine & iron is required. Grows on Buffered Charcoal Yeast Agar, Tyrosine BYE Agar, Feelay Gorman Agar (FG). BYCE Agar is recommended for *Legionella* culture and is enriched with iron & cysteine, incubated for 3-10 days. Growth on BCYE medium may appear after incubation for 48 h at 36°C (heavy infections). Colonies are , 3 to 4 mm in diameter, Gray-white to bluegreen, glistening, convex, and circular ,colonies exhibit a 'cut glass' appearance on plate microscopy, Flourescent under UV light. Cultures are identified by use of specific antisera in an immunoflourescence test, latex agglutination or by gene sequencing^[8].

Fluorecein-labeled antibody attached to *Legionella* bacilli

Figure 2: Direct Immunoflourescence assay

Figure 3: Colonies of *Legionella*

Other media used is FG-agar which is enrichment medium for *Legionella* species. Under Fluorescence (366 nm) *Legionella bozemannii* growth appears blue-white, *Legionella micdadei* does not impart any colour, *Legionella pneumophila* imparts bright yellow. Safety precautions are to be employed and Bio-safety cabinet 2 is needed with PPE. Decontaminate work surface with either 5% hypochlorite or 5% phenol. Since *Legionella* disease is primarily a pulmonary infection, prevention and containment of aerosols is essential.

3. SEROLOGY

Antibodies usually develop after 8-10 days of illness and then increase in titer, some patients may not produce antibody for some weeks or, rarely, for several months. A single titer of 256 or more is presumptive of infection. A four-fold or greater rise in antibody titer in convalescent serum taken at 2, 4 & 6 weeks is significant.

i. ANTIGEN TEST

Antigen is detectable in urine of most patients between one and three days after the onset of symptoms, but may persist for some weeks or months. Rapid detection of *Legionella* antigen in urine and other body fluids has been accomplished by Enzyme immunoassay (EIA) and Immunochromatography⁹.

-Antigen Test-Immunoassay

i. EIA

Immunoassay for detection of urinary antigen is the method of choice for *L.pneumophila* serogroup-1 as it is convenient and rapid test with excellent specificity and sensitivity for *L. pneumophila* sg 1. (80-85% specificity). Commercial EIA kits are available. Immunochromatographic assays can detect *L. pneumophila* sg-1 antigen in urine within 15min. Does not require laboratory equipment however, concentration of urine improves the sensitivity.

--ELISA for Urinary antigen detection

5. MOLECULAR METHODS

The specimen used is urine, serum and respiratory specimens. It has potential to offer rapid results and increased sensitivity on respiratory and urine samples. Direct detection of *Legionella* nucleic acid by: Conventional and real-time polymerase chain reaction (PCR). Targets for PCR assays: Ribosomal RNA (rRNA) genes or their intergenic spacer regions, Gene coding for heat-shock protein (*dnaJ*), RNA polymerase gene (*rpoB*), macrophage infectivity potentiator (*mip*) gene. BD Probe Tec assay is only available for sputum specimens and detects serotype.^[10]

Prevention

Keeping *Legionella* out of water systems in buildings is key to preventing infection. Routine culture of hospital water supply is recommended^[11]. Engineering and building codes routinely advocated preventive measures- little impact on presence of *Legionella*. Maintaining the temperature of water supplies <25 or >50°C. Chemical treatment (chlorination) of water supplies.^[12] Avoid water supplies from known sources of *Legionella*^[13]. No vaccine is available for Legionellosis. Vaccination studies using heat-

killed or acetone-killed cells have been carried out in guinea pigs. Both vaccines were shown to give moderately high levels of dose-dependent protection. A licensed vaccine for humans is most probably still many years away.

REFERENCES

- Heymann DL, editor. Control of communicable diseases manual. 18th ed. Washington, DC: American Public Health Association; 2004.
- Fry NK, Harrison TG (1998). An evaluation of intergenic rRNA gene sequence length polymorphism analysis for the identification of *Legionella* species. *Journal of Medical Microbiology*, 47:667-678.
- European Centre for Disease Prevention and Control. European Legionnaires' Disease Surveillance Network (ELDSNet): Operating procedures. Stockholm: ECDC; 2012. Available from: <https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/1202-TED-ELDSNet-operating-procedures.pdf>
- Newton LH et al. (1996). Legionnaires' disease surveillance: England and Wales 1995. *Communicable Disease Report Review*, 6:R151R156.
- Watson JM et al. (1994). Piccadilly Circus legionnaires' disease outbreak. *Journal of Public Health Medicine*, 16:341-347.
- Alary M, Joly JR (1991). Risk factors for contamination of domestic hot water systems by legionellae. *Applied and Environmental Microbiology*, 57:2360-2367.
- Yu VL, Beam TR, Lumish RM, Vickers RM, Fleming J, McDermott C, et al. Routine culturing for *Legionella* in the hospital environment may be a good idea: a three-hospital prospective study. *Am J Med Sci* 1987; 294:97-9.
- Dieterle RR (1927). Methods for the demonstration of *Spirochaeta pallida* in single microscopic sections. *Archives of Neurology and Psychiatry*, 18:73-80.
- Benin AL, Benson RF, Besser RE. Trends in Legionnaires' disease 1980-1998: declining mortality and new patterns of diagnosis. *Clin Infect Dis* 2002;35:1039-46.
- Robinson PN et al. (1996). Species-specific detection of *Legionella* using polymerase chain reaction and reverse dot-blotting. *FEMS Microbiology Letters*, 140:111-119.
- Best, M. 1984. "Heat eradication measures for control of hospital-acquired Legionnaires' disease: implementation, education, and cost analysis." *Am. J. Infect. Control* 12:26-30.
- Stralin K. Usefulness of aetiological tests for guiding antibiotic therapy in community-acquired pneumonia. *Int J Antimicrob Agents* 2008; 31:311.
- Sidari FP, Stout JE, VanBriesen JM, Bowman AM, Grubb D, Neuner A, et al. Chlorine dioxide as a disinfection method for *Legionella* control. *J Am Water Works Assoc* 2004;96:111-9