Laboratory diagnostics of Legionnaires' disease in clinical practice

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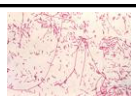
Legionnaires' disease (LD)

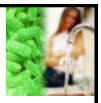
- Nosocomial
- Community acquired
- Travel related

- The source of Legionella infection:
  - potable water systems that become colonized by the microorganism

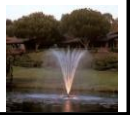
- Caused by organisms belonging to the genus *Legionella*

Legionella = aquatic organisms





- Gram-negative bacterium found in freshwater environments
- Humans are accidental hosts



Parasitic relationships and colonization

- Legionella multiplies within protozoas' host cells and the human macrophage
  - Many amoebae permit its intracellular growth (*Acanthamoeba*, *Hartmannella*, *Echinamoeba*, *Tetrahymena*, *Vahlkampfia*)
  - Amebas provide an ideal environment for reproduction and development

- Biofilm layer commonly associated with water distribution systems = important niche for *Legionella*

- More resistant to adverse environmental conditions.

Legionellosis

- Two different forms of disease in humans:
- Legionnaires' disease
  - incubation period of 2-10 days
  - Multysistem illness that involves lungs (pneumonia), neurological symptoms, diarrhea and high mortality
- Pontiac fever
  - incubation period of 1-2 days
  - Acute, self-limited, influenza-like disease that does not cause pneumonia

Legionella species and serogroups listed in chronological order

based on the date of isolation or identification

Species	No. of serogroups	No. associated with disease	Species	No. of serogroups	No. associated with disease
L. pneumophila	15	15	*L.birminghamensis*	1	1
L. bozemanii	2	2	*L. cincinnatiensis*1	1	1
L. dumoffii	1	1	*L. gormanii*	1	1
L. micdadei	1	1	*L. sainthelensi*	2	2
L. longbeachae	2	2	*L. tucsonensis*	1	1
L. jordanis	1	1	*L. anisa*	1	1
L. wadsworthii	1	1	*L. lansingensis*	1	1
L. hackeliae	2	2	*L. erythra*	2	1*(serogroup 2)
L. feeleii	2	2	*L. parisiensis*	1	1
L. maceachernii	1	1	*L. oakridgensis*	1	1

28 *Legionella* species not associated with disease

Species	Species	Species
L. spiritensis	*L. gratianas*	*L. londoniensis*
L. jamestowniensis	*L. adelaidensi*	*L. taurinensis*
L. santicrucis	*L. fairfieldensis*	*L. lytica*
L. cherrii	*L. shakespearei*	*L. drozanskii*
L. steigerwaltii	*L. waltersii*	*L. rowbothamii*
L. rubrilucens	*L. genomospecies*	*L. fallonii*
L. israelensis	*L. quateirensis*	*L. gresilensis*
L. quinlivanii	*L. worsleiensis*	*L. beliardensis*
L. brunensis	*L. geestiana*	
L. moravica	*L. natarum*	

DIAGNOSIS OF LEGIONELLOSIS

- The disease is clinically and radiographically indistinguishable from other causes of pneumonia
- **DIAGNOSIS REQUIRES SPECIFIC DIAGNOSTIC TESTS!**

THE DIAGNOSIS AND DETECTION OF LD CASES IS IMPORTANT

- Significant in the context of severe community-acquired pneumonias (14-37%)
- Recognition of LD cases allows the source of actual or potential **outbreaks** to be identified and hastens the implementation of appropriate **control measures**

MAJOR METHODS FOR DIAGNOSIS

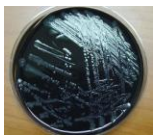
- Actual isolation of the organism on culture media
- Demonstration of the bacterium in tissues or body fluids by using immunofluorescent microscopy
- Detection of antigenuria
- Determination of antibody level
- Molecular methods

SPECIMENS FOR CULTIVATION

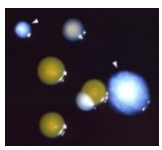
- Isolation from:
  - respiratory secretions (sputum or bronchoalveolar lavage)
  - bronchial aspirates
  - blood
  - lung tissue
  - lung biopsy specimens
- Sputum containing few polymorphonuclear leukocytes should not be rejected !

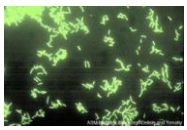
ISOLATION AND IDENTIFICATION

- Culture is the gold standard
- Requires up to 10 days (3-10 days)
- Specificity 100%; sensitivity 25-75%



Growth on buffered charcoal yeast extract (BCYE)

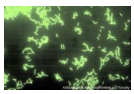




- Evaluation of morphology of colonies
- Requirement for amino acid L-cysteine
- Species are identified using specific antisera

DIRECT FLUORESCENT ANTIBODY STAINING

- Rapid method
- Monoclonal fluorescein-conjugated antibody against *L.pneumophila*
- Polyclonal antisera for different serogroups and non-pneumophila Legionella
- Technically demanding and unreliable
- Very poor sensitivity
- Cross reactivity with Gram-negative bacteria (Campylobacter)



MOLECULAR METHODS

- Detection of DNA
  - 5S rDNA, 16S rDNA, *mip (macrophage infectivity potentiator)*, 23S-5S spacer
- Polymerase chain reaction (PCR) and real-time PCR
- Specimens: BAL, pharyngeal,nasopharyngeal swabs, peripheral blood mononuclear cells, urine, serum
- Sensitivity 11-100%; specificity <99%
- Doesn't distinguish between living and dead cells
  - but Legionella is not considered to be a part of normal human flora → presence or absence will suffice
    - quantification is not necessary
- PCR is generally not used as a routine analysis
  → hampered by the fact that *L.pneumophila* pneumonia often is **unproductive (dry cough)** especially in acute phase and in milder cases

DETECTION OF ANTIGENURIA

- Urinary antigen detection by enzyme immunassay or immunochromatographic membrane assay (ICA,POC)
- Mainstay of diagnosis of *L.pneumophila* sg1
- Antigen detected = component of the lipopolysaccharide portion of the cell wall that is heat stable and resistant to enzymatic cleavage
- Appear within a few days of illness
- Usually remains detectable for 10-15 days, although it may be positive for several weeks or for almost a year

Urinary antigen detection *L.pneumophila* serogroup 1

- Specificity >99.9%; sensitivity 56-99% for LP sg1
- Sensitivity appeared to be associated with clinical severity of the disease:
  - 40-53% positive in mild LD
  - 88-100% positive in severe LD
- Sensitivity in:
  - travel associated LD → 94%
  - community-acquired LD → 76-87%
  - nosocomial LD → 44-46%

SEROLOGIC DIAGNOSIS

- Seroconversion
- Increase in antibody titers of at least fourfold in the convalescent period
  - Indirect immunofluorescence (IFA)
  - Enzyme immunoassays (EIA)
  - Microagglutination test (MAT)

SEROLOGICAL INVESTIGATION

- 80% of diagnostic titers were seen within 4 weeks after onset of disease
- Possible **late seroconversion** after 2 months or more
- Ab still detectable 48 months after disease onset in 33% of patients
  - → **single high or standing titers** detected in sera from patients with pneumonia may be result of **past** infection with *Legionella* spp.
- Low titers of Ab → indicate **previous** exposure
- **Subclinical seroconversion** is known to occur sporadically or during outbreaks

SEROLOGICAL INVESTIGATION – NEGATIVE RESULTS

- **Negative result does not rule out the possibility of infection with legionella!**
  - Serum specimens taken too early during the course of infection may not yet have significant antibody titres
    - **4 - 8 weeks** may be needed to detect an antibody response and antibody levels can fall to undetectable levels within a month of infection
  - Some culture positive cases of legionella do not develop antibody to legionella
- Early **antibiotic therapy** may suppress antibody response and some individuals may not develop antibodies above detectable limits

SEROLOGICAL INVESTIGATION – POSITIVE RESULTS

- A number of apparently **healthy individuals** may carry antibodies to legionella
- **Cross-reactivity** with different serogroups is not unusual
- Positive results may be due to **cross reactivity** with antibody generated as a result of **non-legionella infection**
  - *P.aeruginosa*,
  - several *Rickettsia* species,
  - *Coxiella burnetii*
  - enteric gram-negative rods,
  - *Bacteroides* species,
  - *Haemophilus* species,
  - *Citrobacter freundii*
  - *Campylobacter jejuni*
- The use of haemolytic, lipaemic, bacterially contaminated or heat inactivated specimens should be avoided - erroneous results may occur

Sensitivity of IFA, MAT and EIA in patients with LD

Type of assay	Antigen preparation	Gold standard	Sensitivity
IFA	Whole cell	Epidemic criteria	46%
IFA	Formalized yolk sac antigen	DFA or culture	60%
MAT	Formalin-killed suspension	DFA or culture	63%
EIA	EDTA	IFA	IgM 58% IgG 41%
EIA	LPS	Culture or MAT	IgM 75% IgG 70%

Eur J Clin Microbiol Infect Dis 2004;23:871

COMPARISON OF DIFFERENT TESTS' SENSITIVITY AND FALSE-POSITIVE / CROSS-REACTION

		Sensitivity %	False positive rate %
In-house IFA	Gold standard		4.0
Focus IFA	Sg.1 Sg.2-6+8 pool Combined sg.1 and 2-6+8 pool	72.8 25.9 81.5	12.0 4.0 16.0
SERION EIA LP sg 1-7	IgM IgG Combined IgM/IgG	60.0 53.4 76.5	4.0 2.8 5.6
Zeus EIA LP sg 1-6	IgM/IgG/IgA	68.8	29.0
Vircell EIA LP sg 1-6	IgM+IgG	62.5	2.7

Eur J Clin Microbiol Infect Dis 2008;27:149

Estimates of the Positive and Negative Predictive Values

- **Positive Predictive Value (PPV)** is the chance that a positive result is a true, rather than a false, positive.
- **Negative Predictive Value (NPV)** is the chance that a negative result is truly negative and not a false negative.

- Legionella infection is estimated to be responsible for between 2% and 5% of community-acquired pneumonia requiring admission to hospital in the UK.
- For such low prevalence infections the PPV is very dependant on the specificity of the assay being used while in contrast the NPV is very high almost irrespective of test specificity.

Positive predictive values (PPV) and negative predictive values (NPV) of any positive result assuming a prevalence of *L.pneumophila* sg 1 infection of 2% or 5%

RSIL Evaluation Report LP antibody assays, CPHL Colindale, UK, 1999.

			PPV		NPV	
Assay	Sensitivity	Specificity	prevalence		prevalence	
			2%	5%	2%	5%
EIAs						
Sigma 1-6	77.9	98.2	46.9	69.5	99.6	98.9
Zeus 1-6	87.7	98.2	49.9	71.9	99.7	99.4
IFAT Sgp 1						
Zeus	84.4	99.1	65.7	83.2	99.7	99.2
MRL (Heat)	69.7	93.9	18.9	37.6	99.4	98.4
MRL (Formalin)	91.0	98.2	50.8	72.7	99.8	99.5
IFAT Sgp 1-6						
Zeus	85.2	96.5	33.2	56.2	99.7	99.2
Gull	95.1	89.5	15.6	32.3	99.9	99.7

TG Harrison, HPA centre for Infections, UK

CONFIRMED CASE - EWGLI:

an acute lower respiratory infection with focal signs of pneumonia on clinical examination and/or radiological evidence of pneumonia and one or more of the following:

- Isolation of any Legionella organism from respiratory secretion, lung tissue or blood
- A fourfold or greater rise in specific serum antibody titer to *L.pneumophila* sg1
- The detection of specific legionella antigen in urine using validated reagents and methods

PRESUMPTIVE CASE - EWGLI:

an acute lower respiratory infection with focal signs of pneumonia on clinical examination and/or radiological evidence of pneumonia and one or more of the following:

- A fourfold or greater rise in specific serum antibody titer to *L.pneumophila* other serogroups or other *Legionella* species
- A **single high titer*** in specific serum antibody to *L.pneumophila* sg1 or other serogroups or other Legionella species
- The detection of specific Legionella antigen in respiratory secretion or direct fluorescent antibody (DFA) staining of the organism in respiratory secretion or lung tissue using evaluated monoclonal reagents
- The detection of Legionella specific DNA by polymerase chain reaction (PCR)

A single high serological titer*:

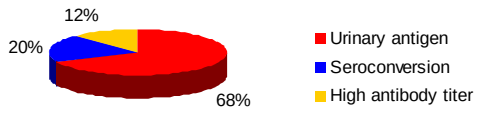
- As different serological testing methods are used, and as an internationally accepted validation exercise has not been carried out, **no specific serological test** or titer level can be specified.
- It is suggested that the single high titer result considered to **indicate** recent Legionella infection, in the presence of compatible **symptoms**, be set at a **sufficiently high** level to be specific for Legionella infection (i.e. to produce a low level of false positives).

Legionella case definition in the 5-years period at the University Hospital for Infectious Diseases, Zagreb

EWGLI criteria	Microbiological diagnosis	2004.	2005.	2006.	2007.	2008.	Total %
Confirmed cases	Urinary Ag detection	10	14	16	20	14	74 **35.2%**
	Ab. seroconversion	7	9	8	4	9	37 **17.6%**
Presumptive cases	4x rise in Ab. titre*	0	3	0	0	4	7 **3.3%**
	Single high titer	9	10	11	6	3	39 **18.6%**
?	Positive IgG	16	14	11	5	7	53 **25.2%**
Total		42	50	46	35	37	210

*four-fold rise in antibody titer to LP sg1 = confirmed diagnosis
four-fold rise in antibody titer to non-LP sg1 = presumptive diagnosis

Diagnostic criteria for acute LD in patients from the University Hospital for Infectious Diseases



EXPERT COMMENTARY

"Not much has changed over the last decade, in relation to prevention of Legionella in the hospital and in the community."

Anna S. Levine

Expert Rev Anti Infect Ther. 2009;7:57-68

Expert commentary - Anna S. Levine

Expert Rev Anti Infect Ther. 2009;7:57-68

- *Legionella* infection is underdiagnosed due to the specific tests needed not always being available for routine use.
- The introduction of molecular methods may improve the diagnosis of *Legionella* other than *L. pneumophila* serogroup 1, but will probably not be available to all hospitals in the near future.
- The best test remains the urine antigen-detection test because it is fast and easy to perform, and has a low cost, although it is limited to *L. pneumophila* serogroup 1.

CONCLUSION

- None of the available diagnostic tools can fulfill all expectation in Legionnaires' disease (LD) diagnostics.
- LD can be diagnosed more promptly and accurately only by using a combination of different methods at the same time.
- Urinary antigen is the mainstay of diagnosis of *L.pneumophila* sg1.
- Serology based on paired sera still remains an important tool in the diagnosis of legionellosis, and it is used to confirm a presumptive diagnosis of legionellosis by PCR or other non-confirmatory methods.


Oh... E... mens... It is a real pleasure !