EPA
United States
Environmental Protection
Agency

Widespread Molecular Detection of *Legionella pneumophila* Serogroup 1 in Cold Water Taps across the United State

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Why is *Legionella* important to U.S. EPA

The mission of EPA is to protect human health and the environment.

EPA United States Environmental Protection Agency
Office of Water

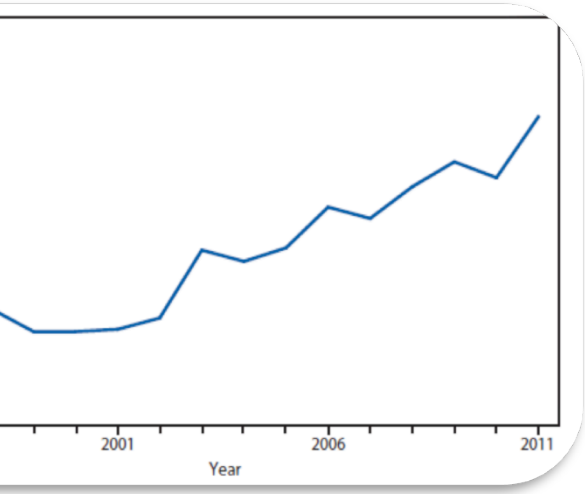
Legionella pneumophila is on the Office of Water's Contaminant Candidate List (CCL)

Legionella pneumophila is a microorganism of the natural environment found in soil and water. However, it also causes Legionellosis / Legionnaire's Disease, a disease that affects the respiratory system.

Legionellosis : Climbing Incidence of Disease in U.S.

CDC Notifiable Disease Report 2000-2011
Reporting ONLY Confirmed Cases

SIS.
y year — United States, 1996–2011



2005 National Notifiable Diseases Surveillance Systems (NNDSS) Case Definition for Legionellosis.

Laboratory Criteria for Diagnosis Confirmed:

1. Culture isolation of any *Legionella* organism from the respiratory specimen.
2. Detection of *L. pneumophila* Sg1 antigen in urine.
3. Seroconversion: fourfold or greater rise in specific serum antibody titer.

Year	Number Cases	Age-adju Inciden 100,00
2000	1127	0.40
2001	1168	0.41
2002	1321	0.45
2003	2232	0.74
2004	2093	0.70
2005	2301	0.75
2006	2834	0.91
2007	2716	0.86
2008	3181	0.99
2009	3522	1.08
2010	3346	1.01
2011	4202	1.10
2012*	3688	1.06
2013*	4548	1.18

*** Provisional cases**

<u>ns of the Surveillance Data:</u>

reporting of the disease burden
are reflective of Urine Antigen Test
s (only detects *L. pneumophila* Sg1).

onellosis Hospitalization: Financial Burden

et al 2012:
care costs of selected disease primarily or partially transmitted by water.
y Infection, **140**, p2003-2013

2006-2007 est

Medicaid:	No information
Medicare:	$26,741
Commercial	$38,363
Uninsured:	No information
Total cost (avg)	$33,366
# of Hospitalization/year:	8,000-18,000 Ma avg(13,000)

l Hospitalization Cost: $433,758,0

idespread Molecular Detection
Legionella pneumophila
erogroup 1 in Cold Water Taps
ross the United States

Environmental Science & Technology

Article

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Widespread Molecular Detection of *Legionella pneumophila* Serogroup 1 in Cold Water Taps across the United States

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Supporting Information

ABSTRACT: In the United States, 6,000 cases of legionellosis were reported to the Centers for Disease Control and Prevention in 2009–2010. Of these reports, it is estimated that 84% are caused by the microorganism *Legionella pneumophila* Serogroup (Sg) 1. *Legionella* spp. have been isolated and recovered from a variety of natural freshwater environments. Human exposure to *L. pneumophila* Sg1 may occur from aerosolization and subsequent inhalation of household and facility water. In this study, two primer/probe sets (one able to detect *L. pneumophila* and the other *L. pneumophila* Sg1) were determined to be highly sensitive and selective for their respective targets. Over 272 water samples, collected in 2009 and 2010 from 50 public and private water taps across the United States, were analyzed using the two qPCR assays to evaluate the incidence of *L. pneumophila* Sg1. Nearly half of the taps showed the presence of *L. pneumophila* Sg1 in one sampling event, and 16% of taps were positive in more than one sampling event. This study is the first United States survey to document the occurrence and colonization of *L. pneumophila* Sg1 in cold water delivered from point of use taps.

INTRODUCTION

Legionella pneumophila is an environmental microorganism capable of causing a range of adverse health effects from severe Legionnaires' disease to moderate Pontiac fever influenza-like symptoms in humans. In 2009–2010, CDC's National Notifiable Disease Surveillance System (NNDSS) reported 6,000 cases of legionellosis.[1] Only 2.6% (179/6,900) of these cases were outbreak events associated with drinking water, other noncommunal waters, or recreational waters.[2] The remaining 97.4% cases of legionellosis were characterized as sporadic (e.g., not associated with an outbreak).

According to the CDC's outbreak reports, exposure to *Legionella* is through potable water when other noncommunal uses such as environmental fountains and cooling towers are isolated[3,4] with drinking water. Independent of exposure route, legionellosis is primarily acquired from aerosolized water droplets contaminated with *Legionella* microorganisms.

Water contamination by *Legionella* is commonly monitored by many countries utilizing conventional culture methods based on International Organization for Standardization (ISO) 11731.[5] The Netherlands Normalisatie-Instituut (NEN) 6265[6] or The Association Française de Normalisation (AFNOR) NF T90-431[7] methods. However, as in the case with all methods, there are limitations when culturing *Legionella*. Optimal cultivation requires amino acid supplementation, attention to pH sensitivity, and prolonged incubation periods (up to ten days). With a generation time of 4 to 6 h, overgrowth of cultures with other eukaryotes is likely to occur.[8,9] Another factor limiting the culture-based detection of *Legionella* is the physiological state of the cells. For instance, viable but nonculturable (VBNC) *Legionella* cells and cells present within amoeba will not be detected by conventional culture methods.[10,11]

The use of qPCR for the purpose of surveillance has some merit because qPCR detects and amplifies a specific gene target known to be exclusive to a specific genus/species/serogroup. The qPCR technique, with the use of a standard curve, is far more efficient and efficient at quantifying the presence of a specific microorganism than the traditional culture approach. To date, several qPCR assays for *Legionella* have been developed: a genus-specific assay (targeting the 16S rRNA gene from *Legionella* spp.),[12] a species-specific assay (targeting the *mip* gene, coding the macrophage infectivity potentiator of

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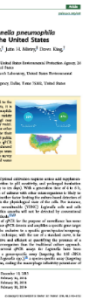


Molecular Detection of *L. pneumophila* Sg 1 in Cold Water Taps across the U.S.: Study Design

2 Year Study: 2009-2010

eographically Diverse Locality

40 Sites

32 Public Buildings

8 Homes

1 Tap/Site

12 Sites

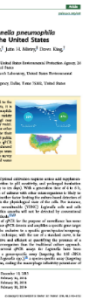
2 Taps/Site

28 Sites

Monitoring Sum

2009-2010

- **40 Sites**
- **68 Taps were mo**
- **4 Sampling even**
- **272 Samples ge**



Molecular Detection of *L. pneumophila* Sg 1 in Cold Water Taps across the U.S.: Sample Collection Proc

- Volunteers

- Willing to participate in four sampling events

- Collect over three liters from the cold tap after a 15 sec

- Package bottles with provided ice packs

- Return within 24hr of collection

Method: Sample Analysis Process and Detection of *L. pneumophila* Sg1

C1

Step 1

Water 3L

Step 2

Membrane Filtration

Polycarbonate 0.4 µm

Step 3

Bead Beating

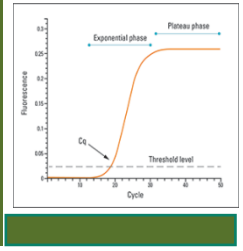
Step 4

DNA Extraction

Step 5

qPCR

Step 6



qPCR Results/ Criteria

C2

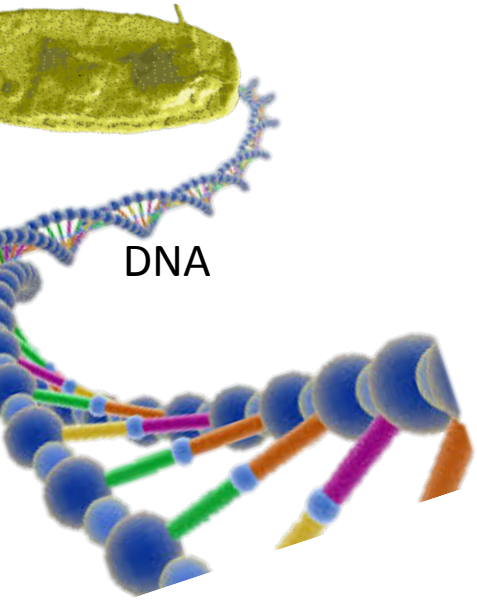
Controls:

C1: Method Blank 100 mL of DNase/RNase Free Water

C2: Non Template Control (TNC) (qPCR chemistry compo

Detection of *L. pneumophila* Sg1
Method: Step 5 DNA Targets for Identity/qPCR

***eumophila* Cell**



Target: 1

pecies-Level
16S RNA
3 copies/cell

DNA Target: 2

Serogroup-Level
LPS gene
~ 1 copies/cell

Merault, 2011

Quantitative PCR

Components in a qPCR Reaction

DNA
Extracted from Water
(DNA Template)

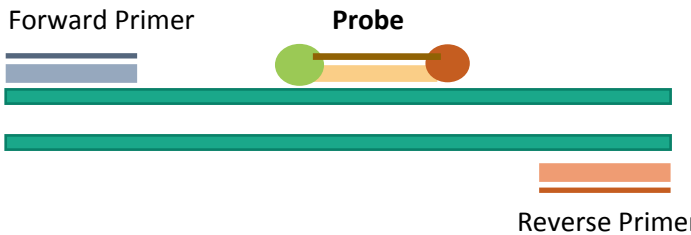
Free Base Pairs
dNTPs

DNA
Polymerase

Buffer
w MgCl

Primers &

PCR Reaction



Products

Fluorophore

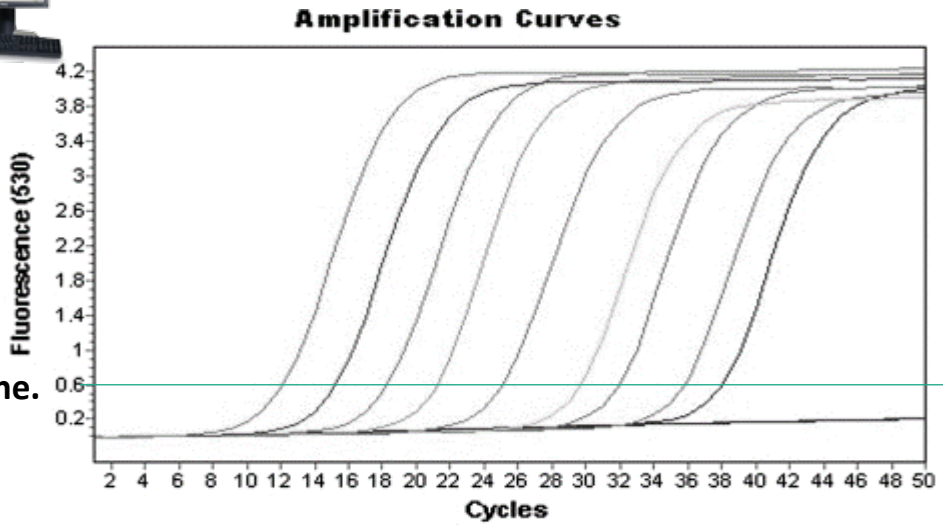
DNA Product

Detection of *L. pneumophila* Sg1
Method qPCR: Step 5/Standard Curve

R

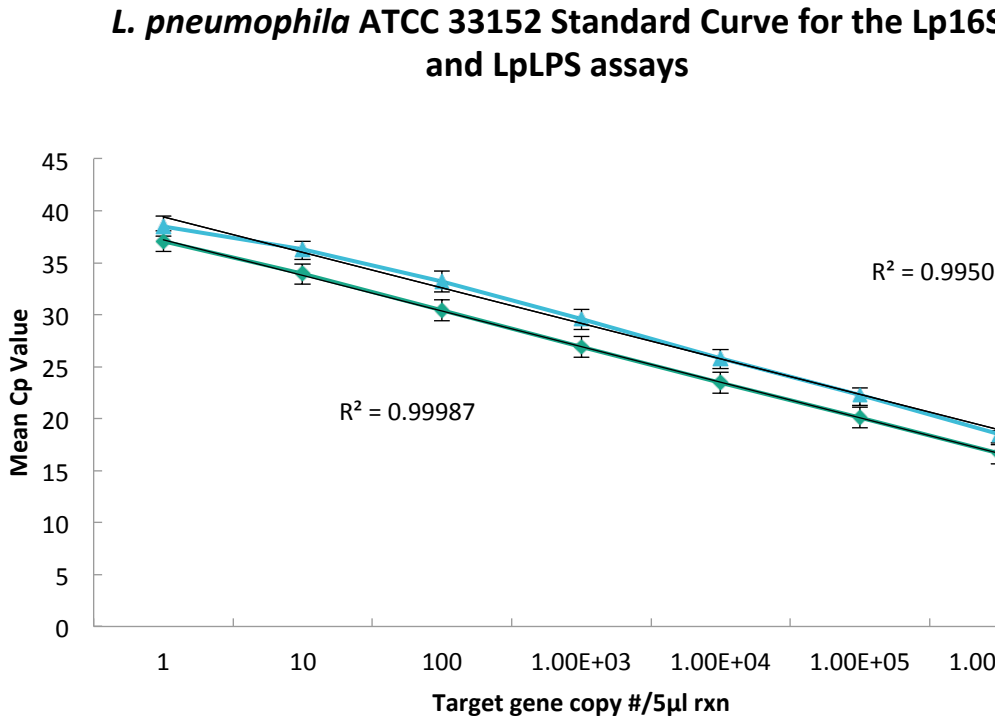
DNA Extracted From ATCC 33152

tion Series



Line.

Standard Curve



Method: Detection of *L. pneumophila* Sg1: Step 6/Reporting Criteria

Assay 1: Lp 16S Assay

- ***Three*** qPCR Reactions were done per sample
  (Each reaction equates to 100 mL of the Original Volume collected)

A Sample was considered L. pneumophila **(+) POSITIVE** if:

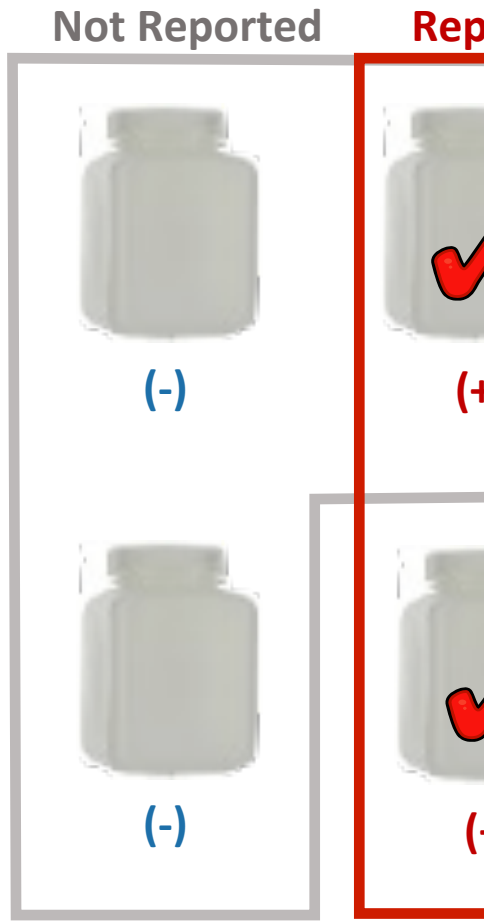
- Cq of < 40 was observed for 2 out of the 3 qPCR reactions.

Assay 2: LpLPS Assay

- ***Two*** qPCR Reactions were done per sample
  (Each reaction equates to 100 mL of the Original Volume collected)

A Sample was considered L. pneumophila Sg1 **(+) POSITIVE** if:

- Cq of < 40 was observed for both qPCR reactions.



BOTH assays need to be posit to be reported

Performance Measures:

Parshionikar, S. (2008) Method Validation of U.S. Environmental Protection Agency Microbiological Methods of Analysis. FEM Document Number 2009-01.

QPCR

- 2 PCR Products (Intact DNA)
- NTC Blank
- Three PCR hybridization need to take place
- ✓Increasing Fluorophor Detection (Logarithmic Curve)
- ✓ DNA Products
  - 100 basepairs (Lp 16S Assay)
  - 75 basepairs (Lp LPS Assay)
- Inhibition Control (s false negatives)

Limit of Detection (LD): 1 genomic target/reaction
Limit of Quantification (LQ): 10 genomic targets/reaction

Method

- Method Blank

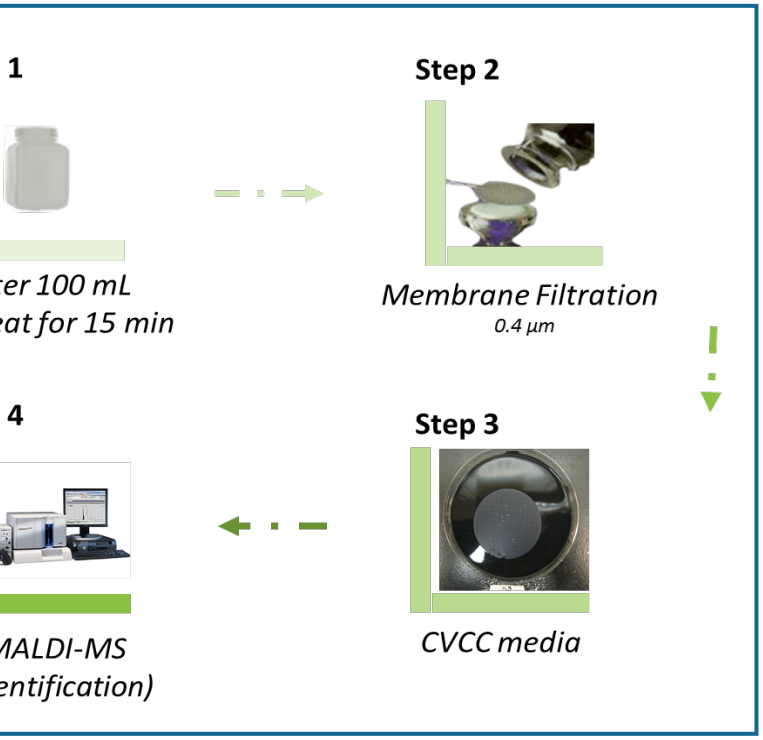
Sensitivity of Method at 100 CFU/L (spiked)
Lp16 S Assay 91% samples
Lp LPS Assay 83% samples

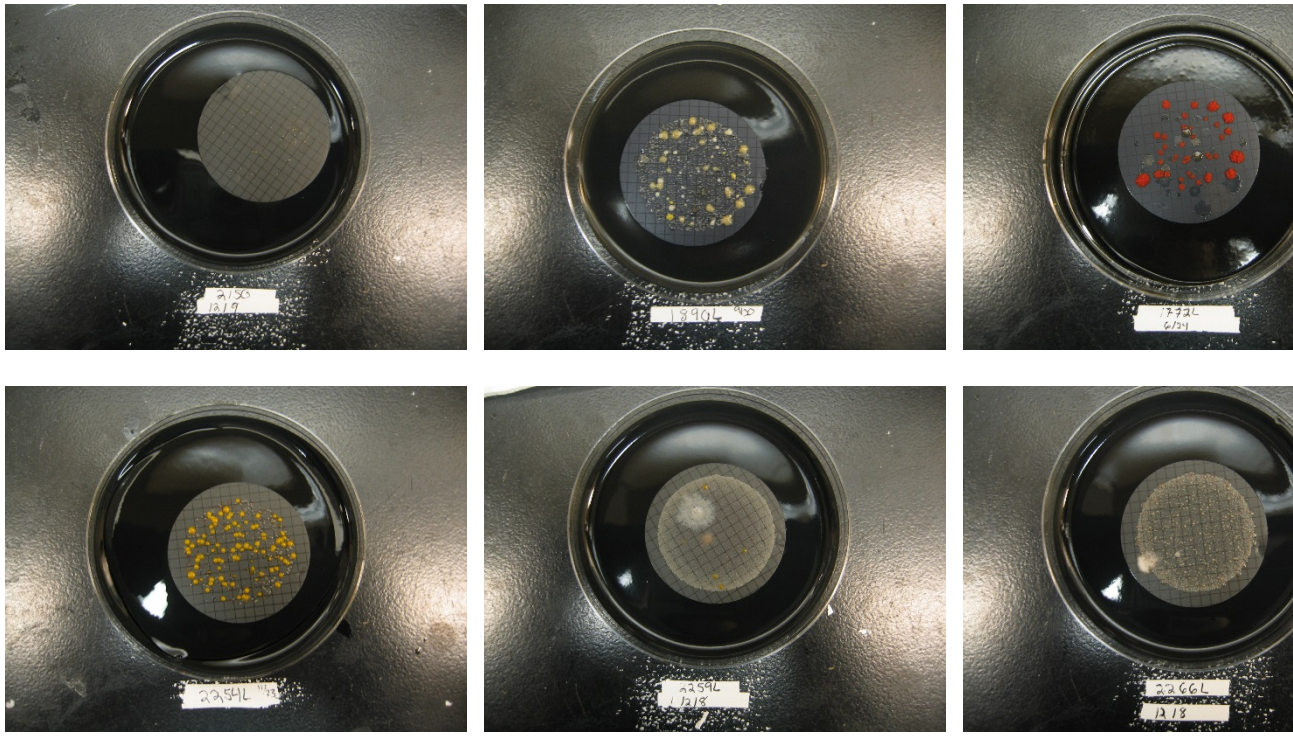
Sensitivity of Method at 1,000 CFU/L (spiked)
Lp 16S Assay 100% samples
Lp LPS Assay 100% samples

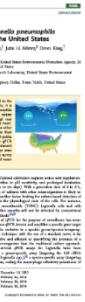
Reporting Crite

- Both Assays must qPCR positive.
- 400 mL of the 500 mL of the original water tested must meet all of the performance measures.

Molecular Detection of *L. pneumophila* Sg 1 in Cold Water Taps across the U.S.: Culture







Molecular Detection of *L. pneumophila* Sg 1 in Cold Water Taps across the U.S.: Results

Monitoring Summary

- **40 Sites**
- **68 Taps**
- **4 Sampling events/tap**
- **272 Samples generated**

Results of Lp16S & LpLPS Assays

272 Samples
- 3 Samples Lost *
269 Samples

* The qPCR Reaction was Completely inhibi
products of the PCR were detected nor pr

	Lp16S / LpLPS (+ / +)	Lp16S / LpLPS (+; - / -)	
	Positive	Negative	Total
Samples	53 (20%)	216 (80%)	269
Taps	32 (47%)	36 (53%)	68

Persistence Results

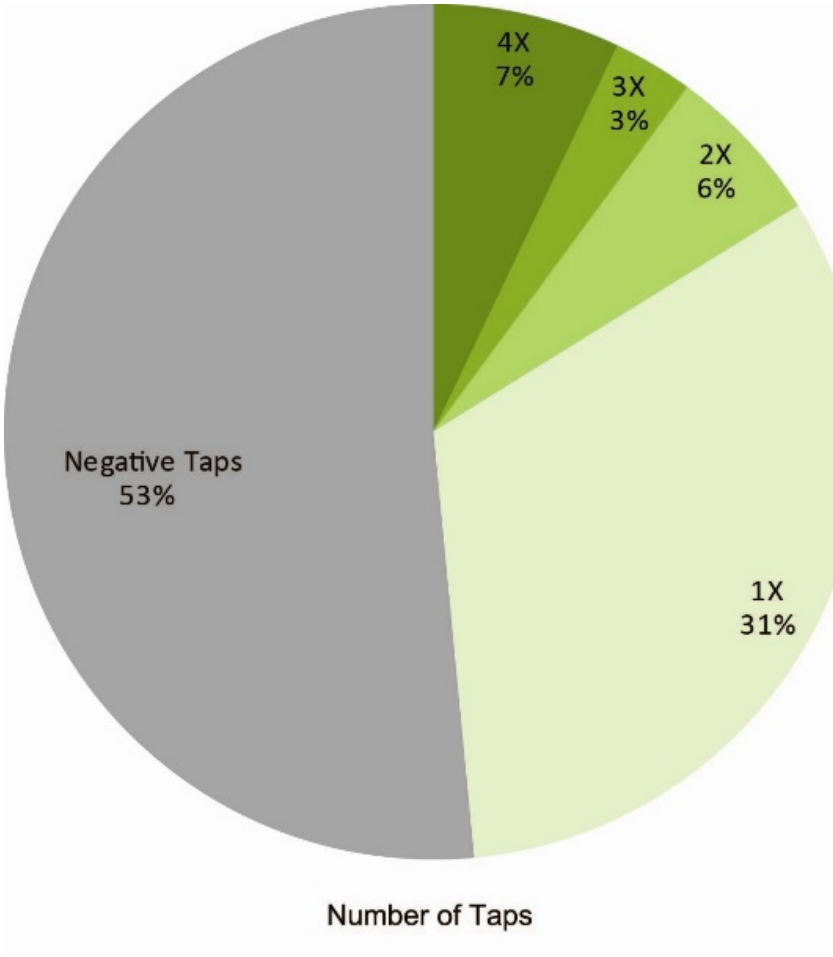
Persistence of *L. pneumophila* Sg1 at Tap

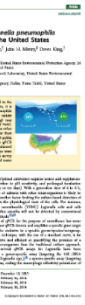
4 Sampling Events

31% (21/68) of the detections were positive for only one of the four sampling events/tap

16% (11/68) of the taps were positive for more than one sampling event.

7% (5/68) of the taps were consistently positive.





Geographic Results

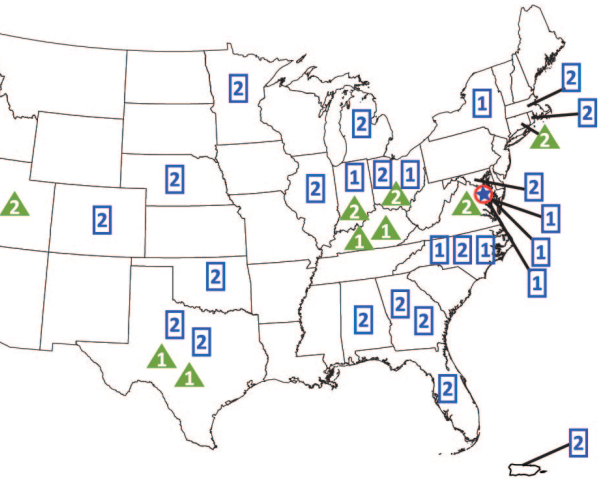
Geographic Occurrence and Persistence of *L. pneumophila* Sg1

Occurrence

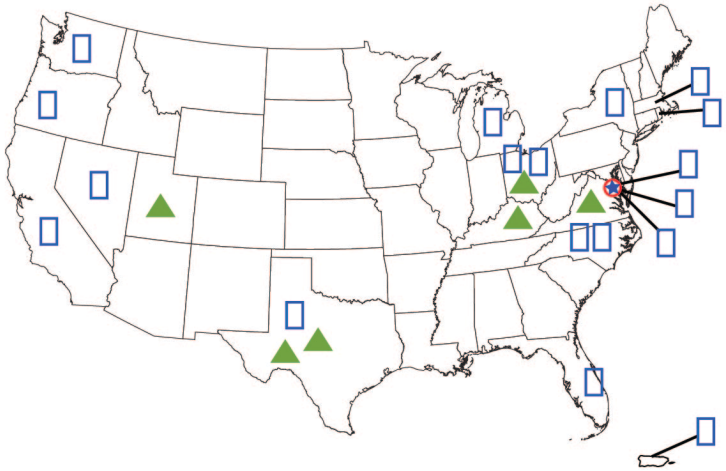
Any site that had a positive sampling event for *L. pneumophila* Sg1

Persistence

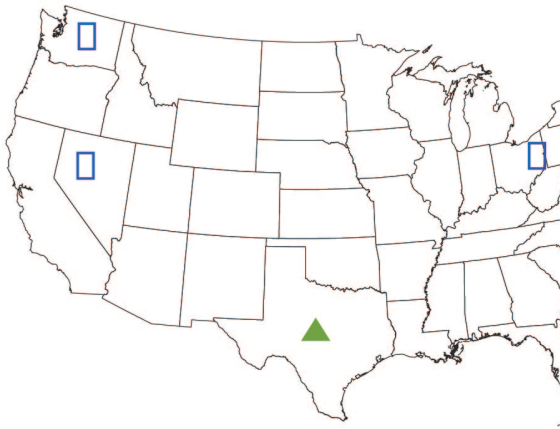
Sites positive for *L. pneumophila* Sg at more than one sampling event



Total of 40 sites in study
68 Taps



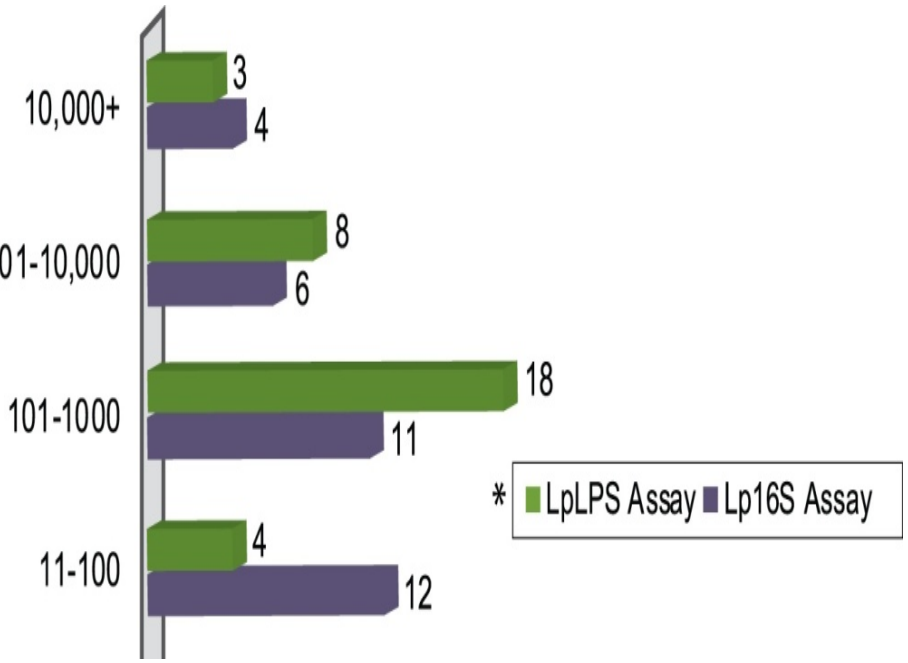
24 Sites
32 Taps



7 Sites
11 Taps

Concentration Results

Distribution of Concentrations of Genomic Target/L Detected at Tap

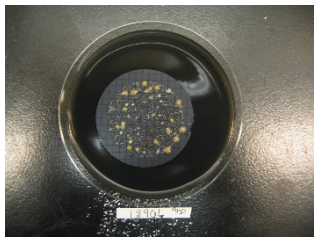


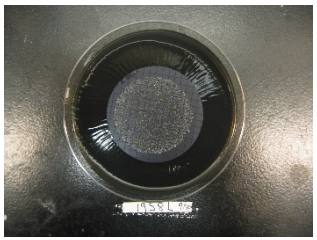
Number of Taps

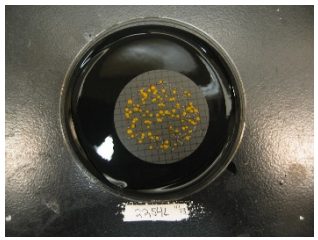
Important Concentration Values

Minimum:	**40 genomic targets/L**
Average:	**1,970 genomic targets/L**
Median:	**62 genomic targets/L**
Maximum:	**365,000 genomic targets/L**

Values based on Lp LPS assay





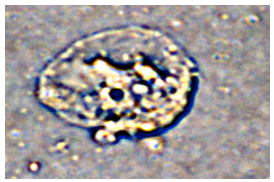


Concentration: What is Being Measured?

Physiological States of *Legionella*

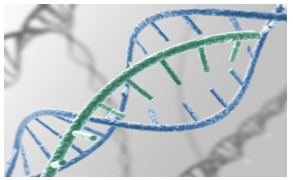
Viable

Amoeba Encysted



Viable but not Culturable (VBNC)

Dead



Culture

Detection, Quantitative (CFU/mL)

Amoeba co-culture

qPCR

Detection, Quantitative (genomic target/mL)

- Due to the extraction technique developed, qPCR will detect *L. pneumophila* Sg1 in these physiological states.

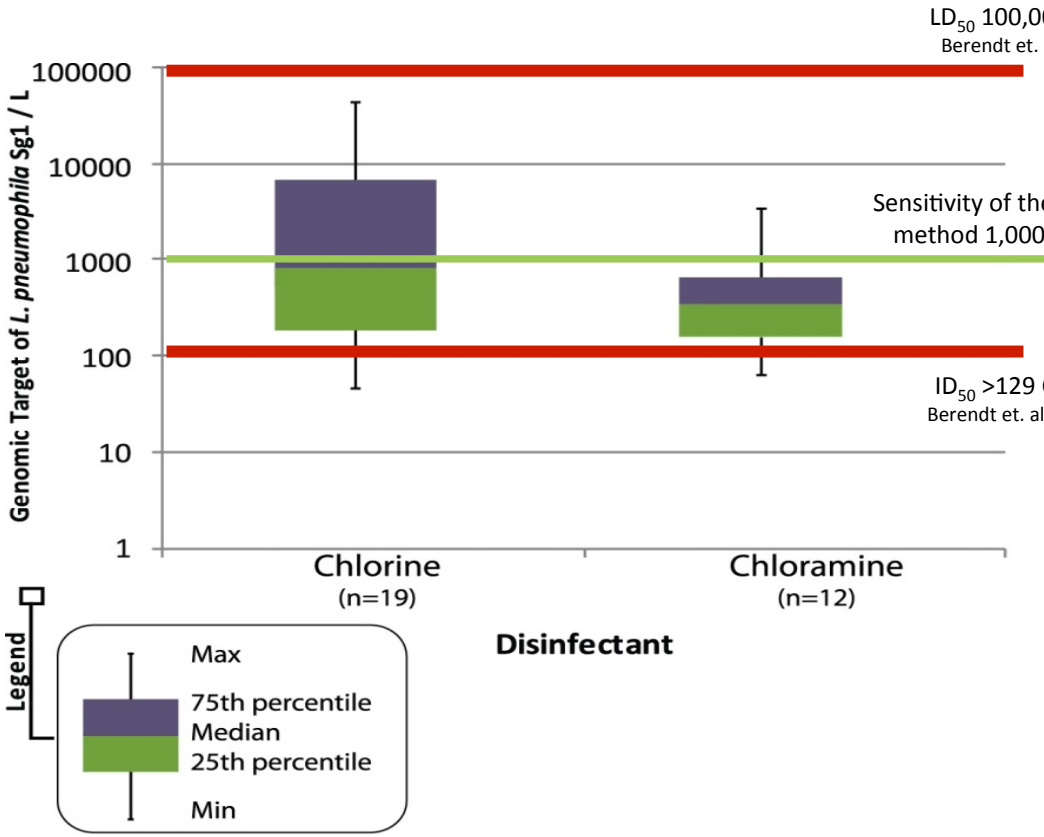
Disinfectant Results

The Role of the Water Disinfectant (chlorine & monochloramine)

43 Taps **chlorinated** water

23 Taps **monochloroaminated** water

- 43% (19/43) of the taps that received **chlorinated** water were positive for *L. pneumophila* Sg1.
- 52% (12/23) of the taps that received **monochloramine** treated water were positive for *L. pneumophila* Sg1.



Molecular Detection of *L. pneumophila* Sg 1 in Cold Water Taps across the U.S: Health Risk (One approach)

Infection Dose ID_{50} Lethal Dose LD_{50}

Guinea pigs exposed *to L pneumophila* by aerosol route.
ID values determined by the on set of a fever
LD values determined by death

Percentage of taps posit
***L. pneumophila* Sg**

Lp LPS Data Avg concentration at

,000 CFU

,000 CFU ← **LD_{50}** **1.4×10^5 CFU** (Berendt, 1980) — 3% of positive taps were a 10^5 CFU in 1L of water.

000 CFU ← **LD_{50}** **10^4 CFU** (Baskerville, 1984) — 9% of positive taps were a 10^4 CFU in 1L of water.

000 CFU

00 CFU ← **ID_{50}** **<129 CFU** (Berendt, 1980) — 88% of positive taps were 129 CFU in 1L of water.

0 CFU ← **ID_{50}** **<20 cells** (Huebner et al, 1984) — 100% of positive taps were above 20 CFU in 1L of wat

0 CFU

Conclusion

- *L. pneumophila* Sg1 was detected in samples from the cold water line at facilities and/or households.
- Exposures can be episodic or more chronic based on the persistence results.
- No significant difference between monochloramine and chlorinated disinfected water, in either the frequency of detection or average concentration *of L. pneumophila* Sg1.
- Assuming that guinea pigs data are applicable to humans,
  - All positive taps monitored in this study had the potential to cause infection.
  - One to three taps had concentrations that could pose a significant risk to human health.

Thank Y