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Detection of *Legionella* spp., *L. pneumophila* & *L. pneumophila* serogroup 1 in drinking waters

In 2nd International Congress on Bacteriology and Infectious Diseases, November 17-19, 2014, Chicago, USA

Jingrang Lu, Ph.D.

- Biohazard Assessment Research Branch
- National Exposure Research Laboratory
- U.S. Environmental Protection Agency

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Legionella: waterborne pathogens

- *Legionella:* Gram-negative bacteria with >50 species, 70 serogroups identified and >24 associated with human infection
- Ubiquitous, but higher counts in water systems with temps of 25-42 ºC
- Environmental factors: temperature, stagnant water/biofilm/sediment, TOC, metals, biofilm and disinfectants, etc.
- Causing Legionellosis: mainly *L. pneumophila*:
  - ✓Legionnaires' disease (LD): severe pneumonia
  - ✓ Pontiac fever: a mild flu like illness

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Legionellosis

- Inhalation of *Legionella*-contaminated aerosols is the only reported cause of Legionellosis
- *L. pneumophila:* > 90% clinical cases, serogroup (sg)1: 84% (Yu et al., 2002); USA: > 30,000 cases/y
- Recently the incidence of observed Legionellosis has significant increase in the United States (Craun, et al 2012) and across Europe (GIDEON 2012)
- 2009-2010 *Legionella* spp. were responsible for 58% USA drinking water-related outbreaks (CDC 2013)
- High fatality rate (28%: ranged from 14-46%)

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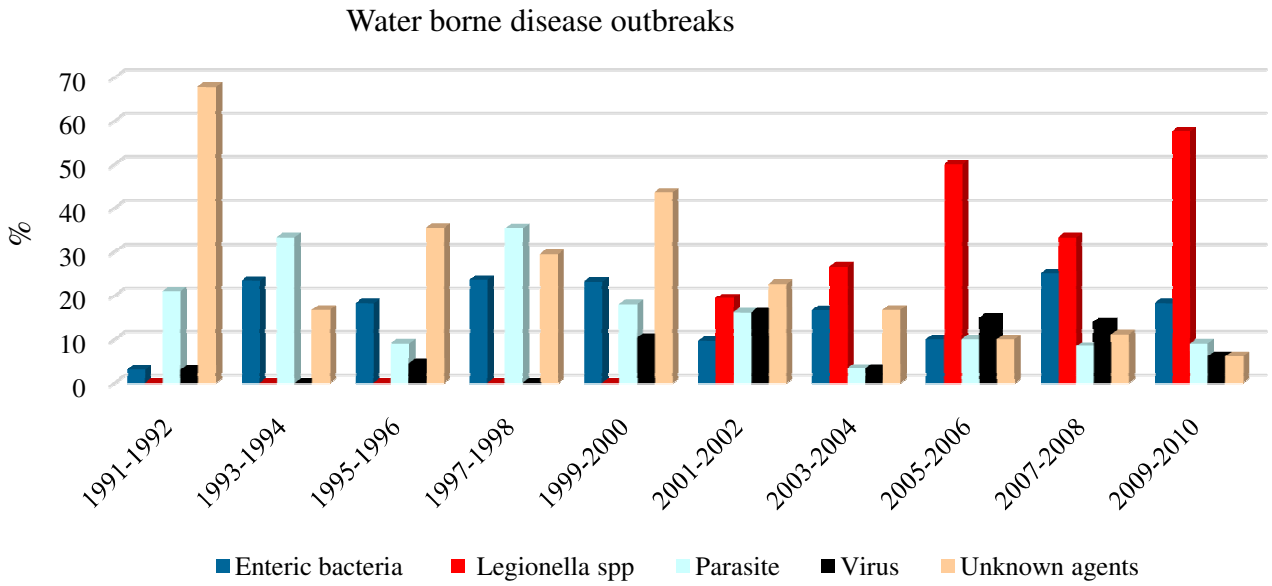
Outbreaks of Legionelloses associated with drinking water from 1991 to 2010 (CDC, MMWR 1998-2011)



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Current problems

Lack reliable detection and control methods for *Legionella* in drinking water distribution systems (DS)

Current detection/diagnostic standard procedures are non-specific, time-consuming and costly

It is hard to collect adequate and accurate data to make risk assessment (Whiley 2014) and to prevent or decrease incidences of LD

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Methods used for *Legionella* detection

Culture, DNA-based and RNA-based methods

1. Culture method: previous "gold" standard

- pros*:* viable estimates
- cons: time-consuming (>48 hr) and no-detection of viable but not culturable (VBNC) stage.

Most *Legionella* presumed to be present in drinking water in the VBNC stage due to low nutrient and chlorinated conditions and intracellular infection of protozoa (Chang et al. 2007, Alleron et al. 2008).

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Detection methods: continue

2. DNA-based methods: PCR/qPCR

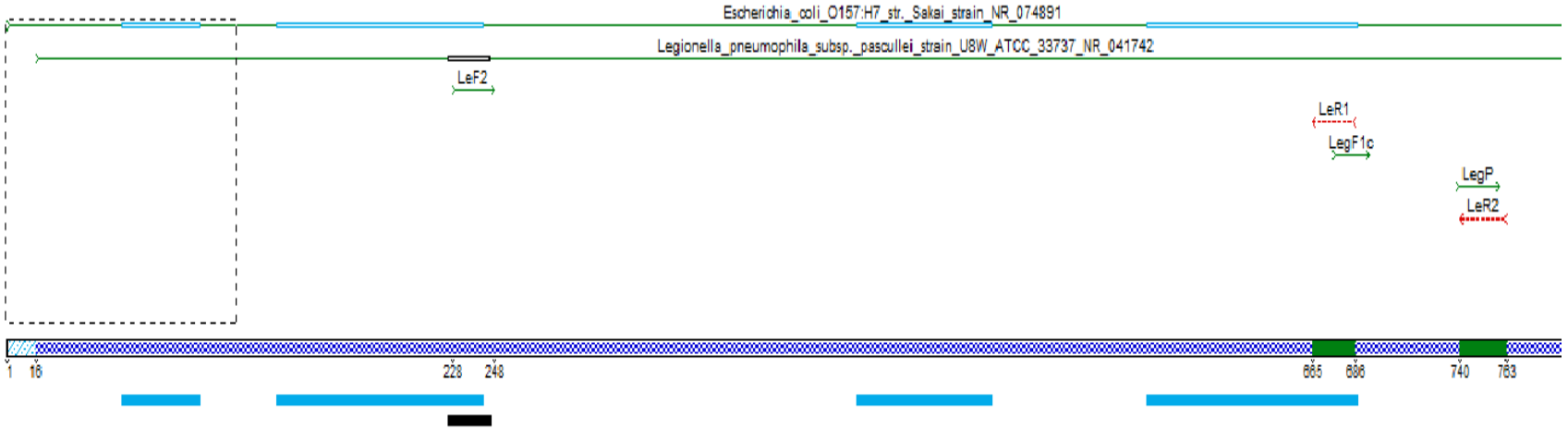
- DNA based standard detection procedures (Ratcliff et al., 1998; Templeton et al., 2003).
  - ✓ 16S rRNA gene qPCR assay for *Legionella* spp.
  - ✓ *mipA* gene assay for *L. pneumophila*
  - ✓ 16S rRNA gene PCR assay, clone-sequencing for species identification

Pros: time saving, specificity & sensitivity

Cons: no differentiation between live and dead cells, so quantity could be overestimated.

- However, modifications are needed for previously published assays, due to their poor specificity and lack of enough coverage for newly added sequences.

DNA-based method: continue PCR/qPCR assays *Legionella*16S rRNA gene



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DNA-based method: continue Test and validation for specificity and sensitivity

- Specificity test DNA panels: Various bacterial strains (21 genera covering 42 species), including various *Legionella* spp. and *L. pneumophila* strains.

- Sensitivity and detection limit:
  - spiked Lp1 into 4 matrices: molecular water, tap water, storage tank sediment and biofilm

DNA-based method: continue Validation

- Validation using isolates from cooling towers, hospital water, and CDC validation samples

Two approaches for validation studies:

1. All assays were tested against CDC reference strains, cooling towers and tap water samples.
2. Positive PCR products amplified from environmental samples of storage tank sediments, hot/cold and distribution drinking water and shower water were validated through clone-sequencing process as the final step.

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3. Test for RNA based assays

- Assays were designed from those previously high-upregulated, especially virulence associated genes under stressor CuO nanoparticles using microarray method (Lu et al., 2013)

assay	assay	assay	assay
Leg16S	p65/66	secA	enhC
rpsL	lvhB3	sidF	rtxA
fusA	lvhB6	sodC	sdhA
ftsy	lvhB5	sseJ	bdhA
gyr A	lvhB4	surA	patD
csrA	lvhB8	pilE3	traD
lphA	lvhB11	pla	magA
rpos	lvhD4	degP	gacA
ccmf	lvr A	mipA	ccmF
flaA	lvr B	icmX	csmY
fliA	lvr C	icmQ	surA
tatB	lvr D	dotA	let A-S
tolB	lvr E	dotC	CAS-2
tolC	lspD	dotD	lolA
gacA	lspG	ceg29	lph A
piE3	aroB	cegC1	
lirR	pla	traV	

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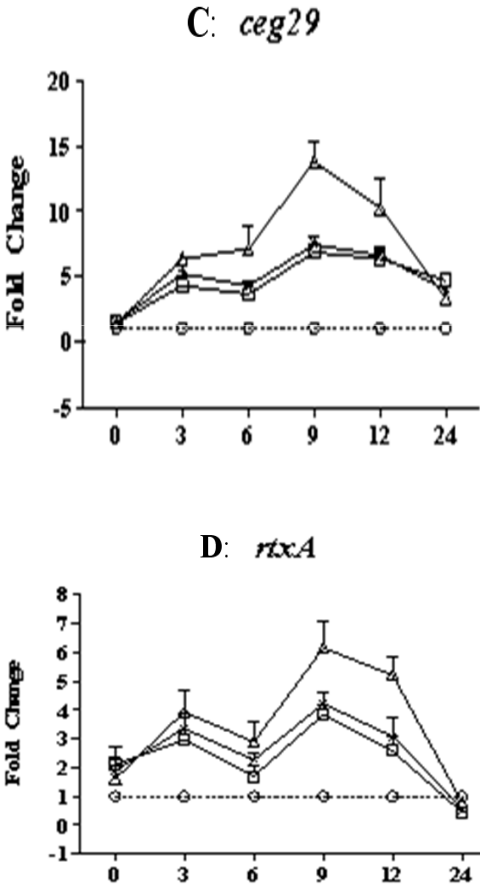
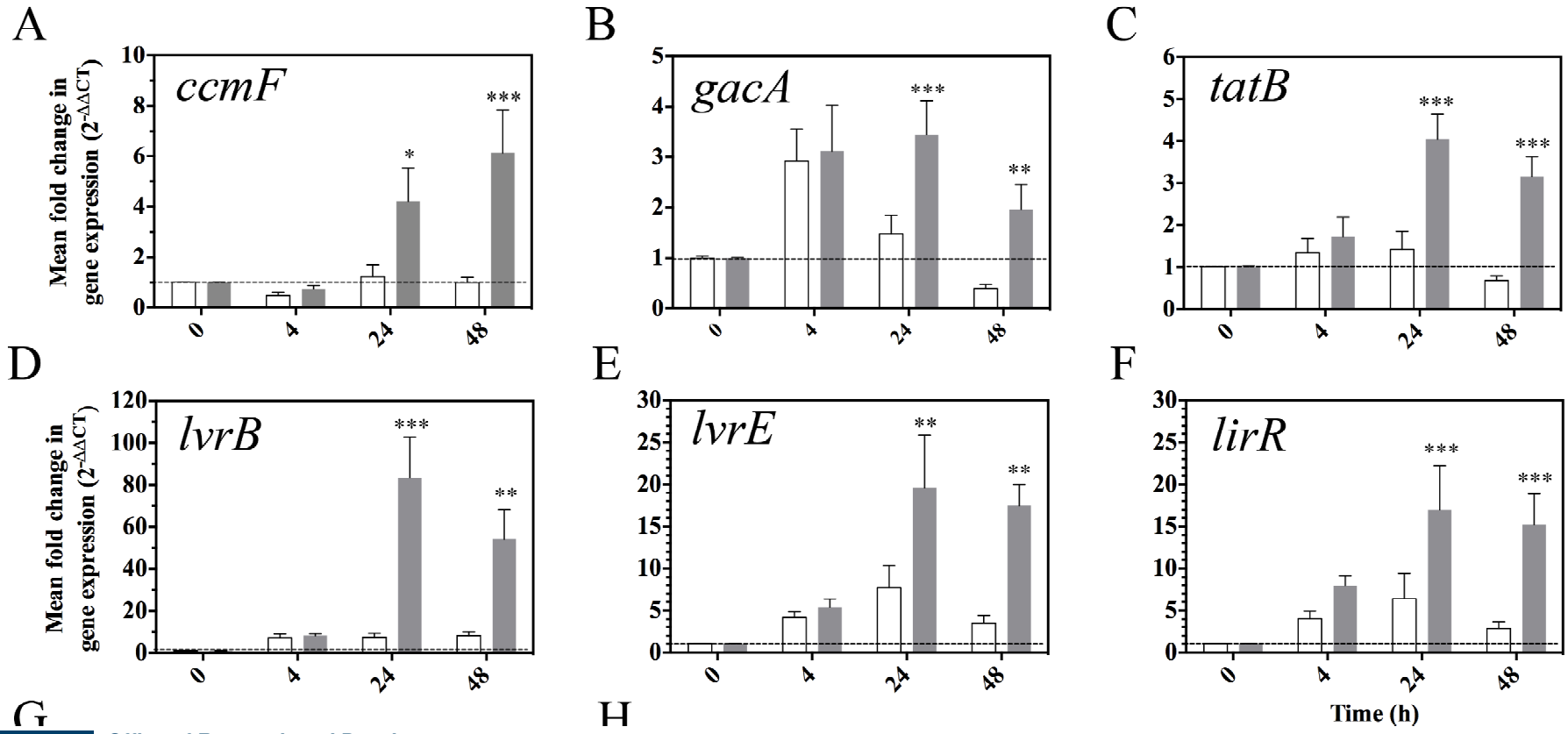
RNA-based method: continue
For example: assays *ceg29* and *rtxA*

- Some assays (*rtxA, ceg29* and *sidF*, et al.) have been used in both DNA- and RNA-based detection and showed very high specificity and sensitivity for Lp1 (Lu et al. 2013)



RNA-based method: continue
Application of the developed assays

- The assays also have been used in the evaluation of greywater on Lp.



- (Buse and Lu et al. 2014)

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Detection limits of RNA based qPCR in tap water

- RT-QPCR has been indicated as a viable detection method for *Legionella* to overcome the under-/overestimated in current methods.
- However, high limit of detection for environmental sample is a hurdle for using in all environmental samples

Primer	Detection Limit
RTX A	6.2x10^4
MIP sigma	6.2x10^4
SID F	6.2x10^4
Ceg C1	6.2x10^6
Ceg 29	6.2x10^5
dot A	no detection
lir	6.2x10^5
Pla	6.2x10^5
sdhA F2R2	6.2x10^5
Lvr D	6.2x10^5
Lvr E	6.2x10^5
csr A	6.2x10^5
fusA	6.2x10^5
gyrA	6.2x10^5
ftsY	6.2x10^5
icmX	6.2x10^7
RPSL	6.2x10^4

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Legionella in drinking water

1. In drinking water distribution system
2. In sediment
3. In biofilm
4. In tap water
5. In shower water

Examined: Quantity, Species & Variation

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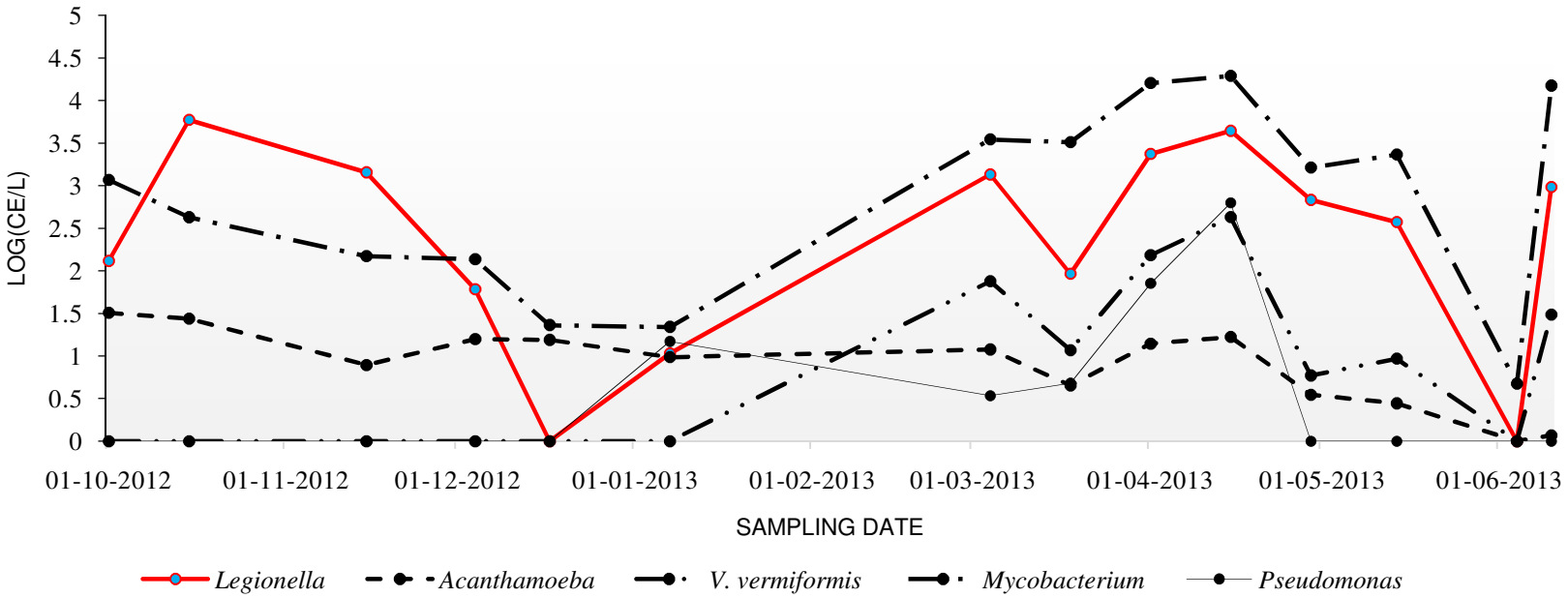
1. In a chlorinated distribution system

Frequency of detection (FOD) & density of *Legionella* spp. & *L. pneumophila* (Lp) in various drinking water systems

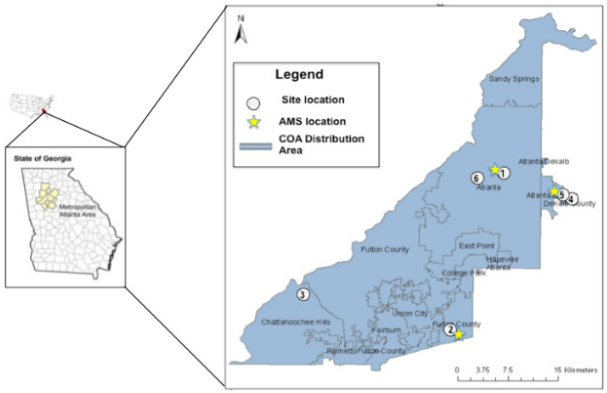
Distribution system	Method	*Legionella* FOD (%)	*Legionella* (CE/L)	Lp FOD (%)	Lp density (CE/L)	Reference
ATL DS (n=60), chlorination	qPCR	57	435	5	70	Lu et al. 2014
Virginia DS, chlorination	qPCR	69		20		Wang et al. 2013
Hot water system	qPCR	48		45		Bargellini et al. 2011

Seasonal variations

- Quantity of *Legionella* spp. varied seasonally and peaked in April (our study)
- In the study by While et al. (2014): peaks of seasonal distribution occurred in summer
- Seasonal variations of *Legionella* spp. were also found in a natural water (Tung et al. 2013), peaks occurred during summer, and coincidently, cases of Legionellosis were also found in that period.



Distribution

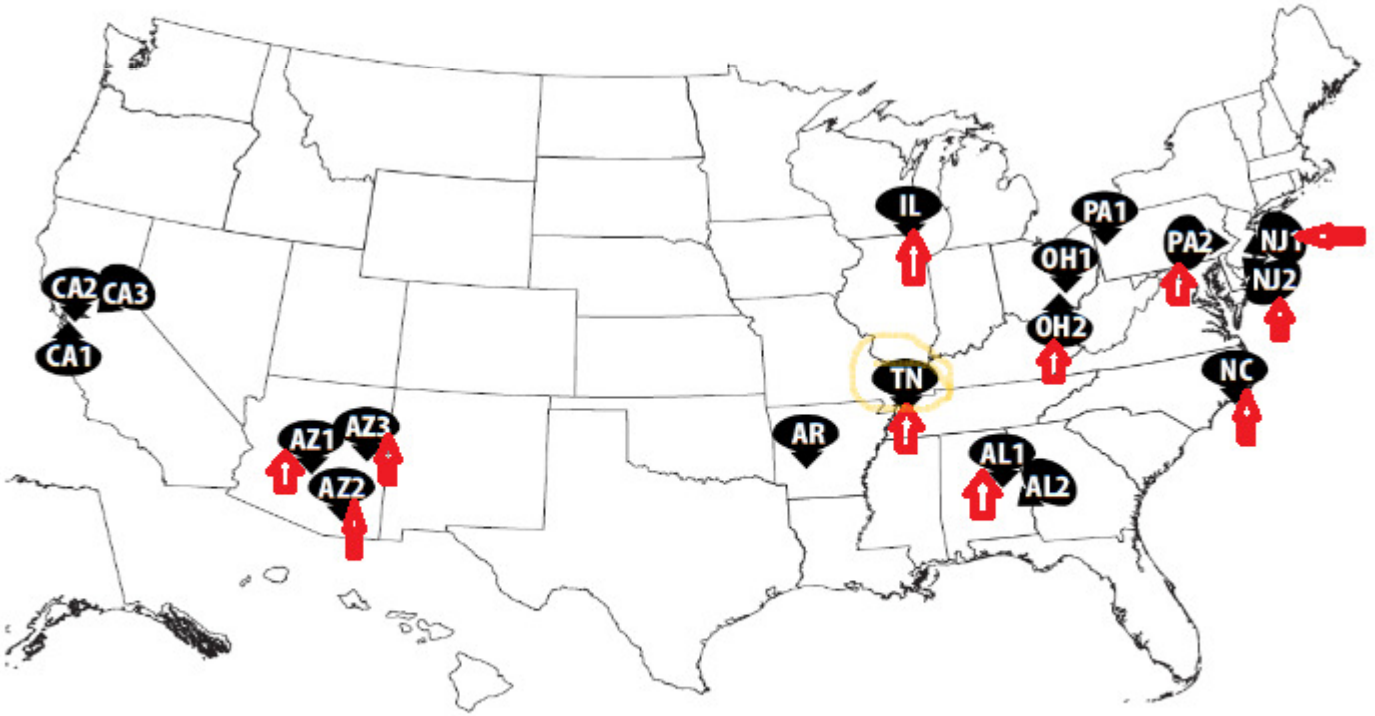


- *Legionella* might come from treatment plant and multiple sites within drinking water distribution system

Site	1 (entry site)	2	3	4	5	6	Mean
FOD (%)	60	83	31	93	50	21	57
Mean (StDev) (CE/L)	9 (11)	1257 (2347)	149 (303)	1111 (1263)	75 (141)	8 (18)	435
Max (CE/L)	27	5913	949	4400	395	57	1957
Ratio of density to entry site		140	17	123	8	1	48

Legionella in drinking water distribution system reservoir sediments

- *Legionella* spp. (66.7 %; 5.2 ±5.9 x 10^3 CE/g,
- Diverse *Legionella* spp.: 115 OTUs of 336 examined, including: *L. pneumophila (33%)*, *L. pneumophila* sg1 (28%) and *L. anisa*



*red arrow indicates sites of detection

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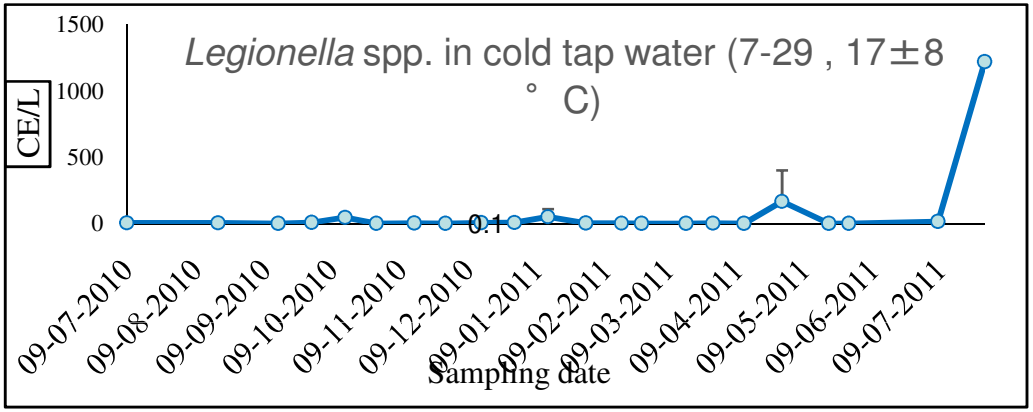
In drinking water biofilms

- *Legionella spp*. have been detected in various drinking water biofilm (Flemming et al. 2002, Keevil 2002, Lehtola et al. 2007, Gião et al. 2009, Moritz et al. 2010)
- Lp colonization's on pipe materials of copper and PVC were detected, more persistent on Cu (Lu et al. 2014)

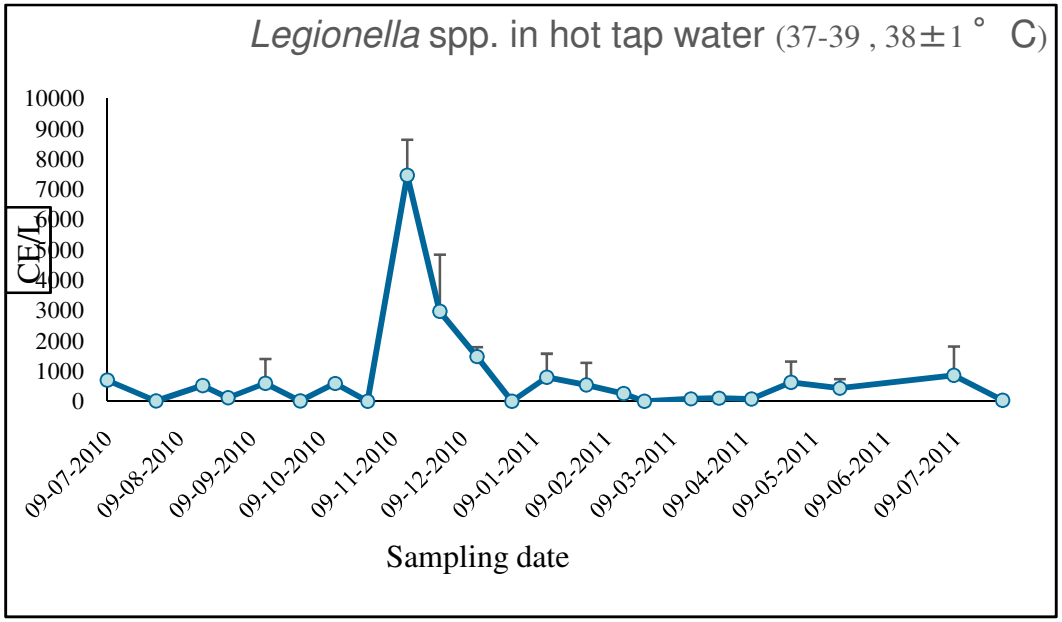
	PVC-control	PVC-Lp	PVC-Lp/Ap	Cu-control	Cu-Lp	Cu-Lp/Ap	P (T-test)
Target	Mean (StDev)	Mean (StDev)	Mean (StDev)	Mean (StDev)	Mean (StDev)	Mean (StDev)	PVC vs Cu
L. pneumophila							
sidF	BDL	BDL	45 (18)	BDL	235 (58)	147 (32)	< 0.0001
dotA	BDL	BDL	55 (32)	BDL	233 (137)	207 (221)	< 0.0001
rtxA	BDL	BDL	12 (5)	BDL	108 (4)	98 (7)	< 0.0001

3. In tap water (July 2010 – July 2011, Cincinnati, OH)

- Frequency of detection (FOD): 68%
- Density: 64 ± 248 CE/L
- *Lp1, L. anisa, donaldsonii, L. dronzanskii*

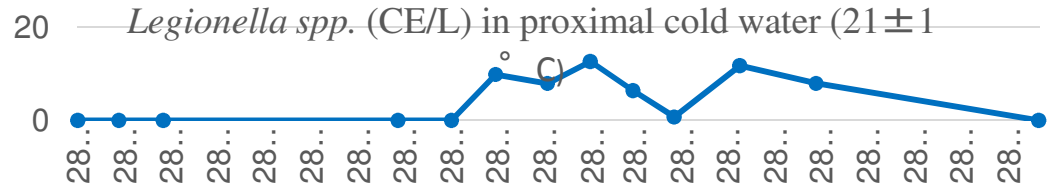


- FOD: 100%
- Density: 763±1596 CE/L
- Peaks: 1.5~7.5 × 10^3 CE/L, at Nov. 15-Dec. 15
- *Lp1, L. anisa, donaldsonii, L. dronzanskii*

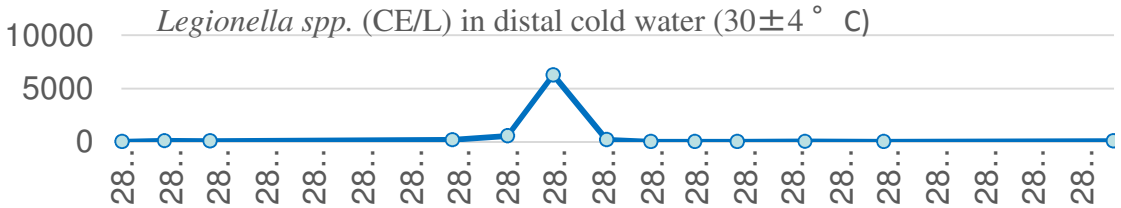


4. In shower water (March 2012 – June 2013)

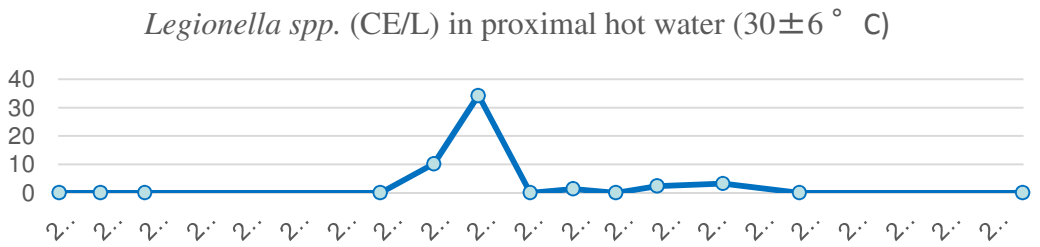
- FOD: 37%
- Density: 4±5 CE/L



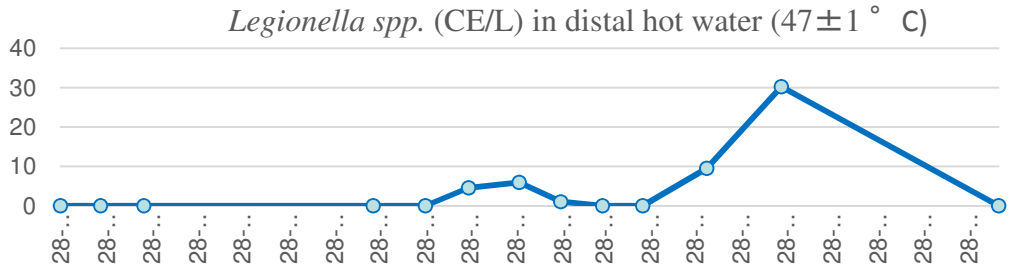
- FOD: 100%
- Density: 30±4 CE/L
- *Lp*1, *L. anisa*, *L. geestiana*, *L. massiliensis*, *L. tunisiensis*



- FOD: 46%
- Density: 4±10 CE/L
- *Lp*1

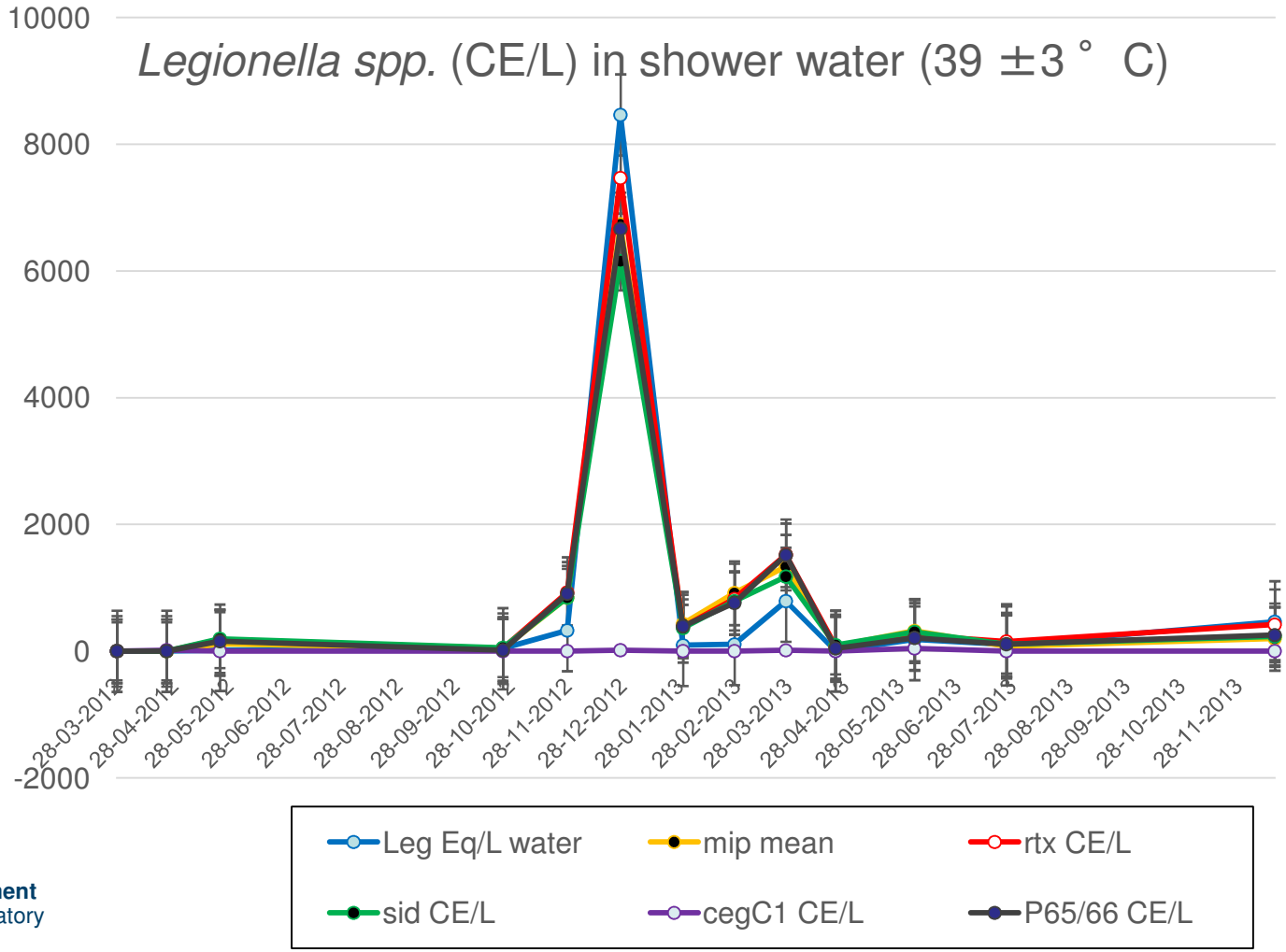


- FOD: 38%
- Density: 4±9 CE/L
- *Lp*1



4. In shower water: continue

- FOD: 85%
- Density: 815 ± 2310 CE/L
- Peaks: *Lp1, L. anisa, L. donaldsonii,* L. *geestiana*
- Lp peak: mipA.
- Also Lp1 peak: rtxA (more sensitive), sidF and P65/66

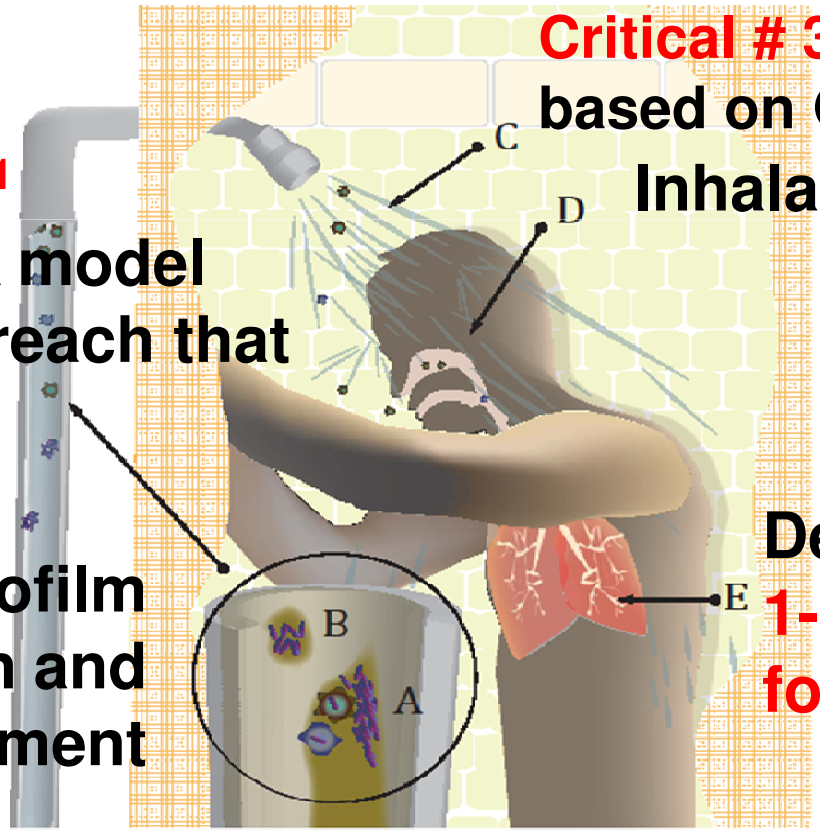


QMRA for critical *Legionella* densities in shower water

Aerosolization
Critical # 35 – 3,500 CFU m^{-3}
based on QMRA model

Inhalation

Critical # in DW
10^6 – 10^8 CFU L^{-1}
based on QMRA model
Needs hosts to reach that



Biofilm colonization and detachment

Deposition
1-1,000 CFU in lung for potential illness

Schoen & Ashbolt (2011) Water Res 45: 5826-5836

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Current approach in RT-qPCR detection

- A protocol for Lp/Lp1 in drinking water developed included:

✓ qPCR for screening samples

✓ PCR-clone-sequencing for ID, if necessary

✓ RT-qPCR for viable detection, when densities reach to > 10^4 CE/L (Implement corrective action level, http://www.ewgli.org/), or even to the QMRA critical level 10^6 – 10^8 CFU L^{-1} (Shoen et al. 2011)

- Further, a RT-qPCR panel test may be applied using the developed panel assays.

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Summary of *Legionella* in drinking water

- The detection of *Legionella* were completed mainly using the DNA-based qPCR method in distribution systems. The newly developed assays proved to be specific by clone-sequencing and sensitive.
- *Legionella* occurred in all the places investigated, but generally at low levels (< 10^3 CE/L) and rarely > 10^4 CE/L. Special attention should be paid to monitor the possible seasonal peak.
- The contamination could be from outside (initial source) or inside (secondary source: biofilm, sediments, etc.).
- *Legionella*, especially the potential pathogens: Lp and *L. anisa*, tended to occur in tap water and shower water when temperatures with 29-39 ºC and reach high densities (~ 10^4 CE/L), which might be of health risk.
- RT-qPCR method should be used to estimate viable number in DS, when high quantities of Legionella occur, in order to provide more accurate densities for QMRA of *Legionella*

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Thanks to

This research was supported by EPA ORD's Safe and Sustainable Water Resources Programs.

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Jay Garland, ORD, US EPA
Nick Ashbolt, University of Alberta, Edmonton, CAN
Jorge Santo Domingo, ORD, US EPA
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