A PCR Assay Detects *Legionella pneumophila* Harboring Mobile Element ICE-βox in a Variety of Water Sources

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Introduction

The most common waterborne disease in the United States is the pneumonia Legionnaire's disease,[1] with a case fatality rate as high as 80%.[3] *Legionella pneumophila* is readily detected in the built environment,[2] and epidemiologic surveys suggest that 63% to 84% of US hospital water systems are colonized with *Legionellae*.[4] In a study of 209 Paris hospital water systems, chlorination treatment correlated with increased prevalence of *Legionellae*.[5] In summer 2015, contaminated cooling towers in Bronx, New York, sickened 128 and killed 12. Even after disinfection, 15 of 35 towers still tested positive for this pathogen.[13] Thus *L. pneumophila* persistence in the built environment is a public health concern.

L. pneumophila acquires traits via horizontal gene transfer. Integrative conjugative elements (ICEs) are mobile genetic elements that are either integrated into bacterial chromosomes or excised as episomes.[6] Excision requires direct repeat nucleotide sequences called attachment (*att*) sites. Recipient bacteria must harbor the *att* site to maintain a newly acquired ICE (Figure 1). The *att* sites align during recombination and facilitate mobility. This process leaves 1 *att* site on the chromosome and 1 on the ICE episome, allowing for reintegration after excision events. Thus those bacteria that do not carry an *att* site cannot integrate an incoming ICE into the bacterial genome.

ICE-βox of *L. pneumophila* strain Philadelphia-1 confers resistance to ß-lactam antibiotics, oxidative stress encountered within macrophages, and bleach.[7] As chlorine disinfectants are the primary method of eradicating waterborne pathogens, the discovery of a mobile element with these fitness advantages is

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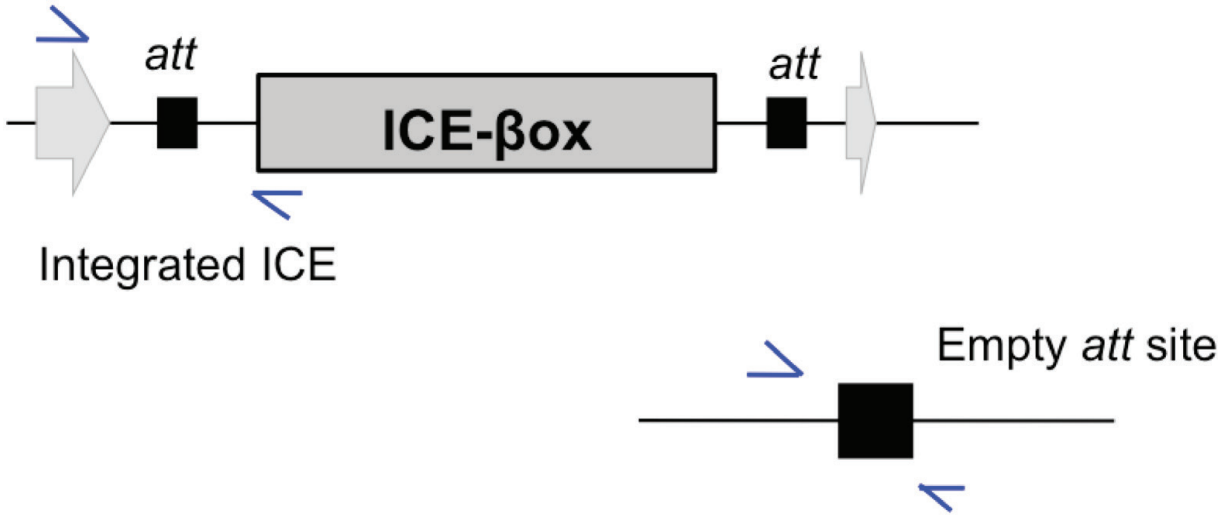

FIGURE 1. Primer locations for multiplex PCR. A block diagram of the genomic location of ICE-βox is shown. The 65 kb element is flanked by two attachment (*att*) sites (black boxes). Primers (blue) to detect integrated ICE-βox are specifically designed to amplify over the *att* site using one primer that lies within the ICE-βox coding region and one that lies in the chromosome opposite the *att* site (gray arrow). Chromosomal primers oriented toward the *att* site detect the empty site.

concerning. Given the prevalence of *L. pneumophila* isolated from chlorinated environments, it is possible these disinfectants select for resistant isolates harboring ICE-βox. In this way, water treatment may actually select for more fit strains of the pathogen it is designed to eradicate.[8,9]

To probe the relationship between disinfection and oxidative stress resistance, we designed a multiplex polymerase chain reaction (PCR) screen to assess ICE-βox presence in clinical and environmental *L. pneumophila* isolates. This screen is specifically designed to detect either the presence of integrated ICE-βox or the *att* site necessary to acquire the element in 1 reaction. In this study, we aimed to determine the prevalence of ICE-βox in *L. pneumophila* clinical and environmental isolates using this assay.

Methods

Bacterial strains

One hundred eighty-three *L. pneumophila* isolates representing serogroups 1 through 17 were selected from the United States Centers for Disease Control and Prevention (CDC) *Legionella* reference collection to determine the prevalence of ICE-βox and the *att* site. Included were typing strains representing several serogroups, clinical isolates, built environment isolates linked to particular infection cases (Table 2, column 4), and 1 natural water isolate with no known exposure to disinfectants. The positive control strain for this assay was *L. pneumophila* strain Philadelphia-1 derivative Lp02, known to contain ICE-βox, and the negative control strain was the derivative JR32, known to lack the element.[7]

DNA extraction

Genomic DNA was isolated at the CDC using the InviMag Bacterial DNA kit/KFmL (Invitek, Hayward, California) on the KingFisher mL (Thermo Fisher Scientific, Philadelphia, Pennsylvania), MagNA Pure Compact (Roche, Basel, Switzerland), or EZ1 advanced XL (Qiagen, Hilden, Germany) platforms according to manufacturer's guidelines.

Real-Time Multiplex PCR

Real-Time Multiplex PCR was performed on 1 ng/ul DNA using Quanta PerfeCTa Multiplex qPCR SuperMix (Quanta Biosciences, Gaithersburg, Maryland)

on the ABI7500 Standard platform (Applied Biosciences). Specific primers were designed manually to amplify the integrated form of ICE-βox, the empty *att* site, or a pan-genome control as described previously[7] (Table 1, Figure 1). Primers were verified using genomic DNA isolated from *L. pneumophila* strain Lp02 as a positive control and strain JR32 as a negative control.[7]

Results

To pilot our surveillance strategy, we screened 183 clinical and built environment *L. pneumophila* strains isolated from outbreaks sent to the CDC using primers specifically designed to amplify the integrated ICE-βox or the empty *att* site. This screening assay proved to be sequence-specific, as the PCR products were of the expected size and did not detect ICE-βox or the *att* site in negative control strains. Of the 183 isolates, 57 (31.1%) contained integrated ICE-βox, and the remaining 126 (68.9%) carried its *att* site (Table 2). One hundred thirteen were serogroup 1 strains, the most common serogroup associated with infection. Of these, 24/84 (28.5%) of clinical isolates and 22/29 (75.8%) of built environment strains carried ICE-βox, and the remainder contained *att* (Table 2).

Although knowledge of the water treatment protocols used in the environments represented is limited, of the 3 outbreak locations where data exist, chlorine disinfectant concentrations ranged from 0.2 to 0.7 ppm Cl_2 (Table 2). In the 1 natural water isolate tested in this initial study, *att*, but not ICE-βox, was detected (Table 2). Compared to serogroup 1 strains, ICE-βox was less prevalent for both clinical (8.2%) and environmental (29.4%) nonserogroup 1 strains.

Conclusion

This pilot study demonstrates that a multiplex PCR assay can detect both integrated ICE-βox and its

TABLE 1. Primers Used in This Study

Primer Name	Sequence (5' → 3')
Philadelphia-F	CGGAATAGACCAGACCCAAATGGCGCG
Paris-F	AGCCGGAATAGACCGATTAAAAATG
Lens-F	TTGGGGAAGAGCCTTTTAAATGG
Lorraine-F	AATAATGTGGGGGTTTACTAAATGGC
HL-F	ATGCAAATTAAATCAACAAAGTGGC
Alcoy-F	AATTGGGAAAGAGCCATTAAATGGC
Sg12-F	GATTTTAAAAGGATTAAATGGCG
Integrated-R	GATTTGATGCATCGTAAGTTGTTGATT
Empty-R	ATAAAATGTTCATCCACACCCCAT
Integrated-ABY-P	ABY-TGTTTTCTATTATTGAGTATCAG-MGBNFQ
Empty-TX-RED-P	TX615-CGCTCGTAGCTCAGCTGGATAGAGTACTT-BHQ2
Pan-Leg-F	GGCGACCTGGCT TC
Pan-Leg-R1	GGTCATCGTTTGCATTTATATTTA
Pan-Leg FAM P	FAM-ACGTGGGTTGCAA-MGBNFQ

TABLE 2. ICE-βox Screening Results

Isolate Source	Contains ICE-βox	Contains *att* Site Only	Location Examples
Clinical	24/84 (28.6%)	60/84 (71.4%)	Lung, sputum, bronchial wash
Built environment	22/29 (75.8%)	7/29 (24.1%)	Cooling tower, faucet, fountain
Cl_2 exposure	2/3 (66.6%)	1/3 (33.3%)	Source treated with 0.2 to 0.7 ppm Cl_2
Natural environment	0/1 (0%)	1/1 (100%)	Soil and outdoor shower in Thailand
Non-Sg1 clinical	4/49 (8.2%)	45/49 (91.8%)	Lung, sputum, bronchial wash
Non-Sg1 environmental	5/17 (29.4%)	12/17 (70.6%)	Showerhewad, tap water
Total	57/183 (31.1%)	126/183 (68.9%)	

Multiplex PCR for integrated ICE-βox (second column) or the *att* site (third column) on DNA isolated from *L. pneumophila* outbreak isolates. The first 4 rows represent screening results from serogroup 1 strains; the last 2 rows cover nonserogroup 1 strains.

att site in a range of *L. pneumophila* serotypes and isolates. Since all 183 strains tested contained the ICE-βox *att* site, it appears that the capacity to accept and stably integrate ICE-βox is widespread in *L. pneumophila*, regardless of serotype.

ICE-βox was more prevalent in built environment samples than in clinical isolates (75.8% vs 28.5%, respectively, $P < 0.001$). The apparent increased frequency of ICE-βox in environmental isolates is consistent with the hypothesis that disinfectant-treated water selects for strains that carry ICE-βox. The 0.2 to 0.7 ppm Cl_2 exposure levels reported for 3 of the environmental isolates are much lower than the 2 ppm chlorine level ICE-βox confers protection to.[7] To continue to assess whether water treatment impacts ICE-βox prevalence, we are next keen to compare ICE-βox prevalence in larger sets of isolates subjected to known disinfectant treatments with natural water *L. pneumophila* isolates with no known exposure to chlorinated chemicals. With this multiplex PCR assay in hand, we are poised to determine the potential for the spread of disinfectant- and antibiotic-resistant *L. pneumophila* in the built environment.

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