**Environmental *Legionella* spp. Collected in Urban Test Sites of South East Queensland, Australia, are Virulent to Human Macrophages *in vitro***

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**Abstract**

Legionellae are frequent contaminants of potable water supplies, resulting in sporadic infections and occasional outbreaks. Isolates of *Legionella* were collected from urban test sites within South East Queensland and evaluated for their virulence potential *in vitro*. Two strains (from the species *L. londiniensis* and *L. quinlivanii*) were demonstrated to have the ability to infect human macrophages, while a strain from the species *L. anisa* did not maintain an infection over the same time course. This suggests that the spectrum of urban environmentally associated *Legionella* with potential to cause human disease might be greater than currently considered.

**Keywords:** *Legionella*; environmental; pathogenicity

**Introduction**

*Legionella pneumophila* is the causative agent of Legionnaires disease, a potentially fatal pneumonia that commonly infects those with an underlying illness or weakened immune system. This species evades host defences by invading and surviving within human macrophages [[8](#_ENREF_8)]. Legionellae are ubiquitous and widely distributed in the environment. They are found naturally in fresh water where they survive as parasites of free-living amoeba or form biofilms [[19](#_ENREF_19)]. Those found in soil habitats (particularly *L. longbeachae*) can cause infection via inhalation of dust from contaminated soils [[18](#_ENREF_18)]. Legionellae are also widely distributed in man-made facilities, including air-conditioning ducts and cooling towers, warm water plumbing systems, humidifiers and whirlpools, with the ability to survive in moist environments for long periods of time, withstanding temperatures of 0 - 68°C and a pH range of 5.0 - 8.5 [[5](#_ENREF_5)]. Legionellae-infected amoebae are frequent contaminants of potable water supplies, especially heated reservoirs, thus posing a significant public health concern.

Legionellae are considered opportunistic pathogens. The major mechanism of human infection appears to be direct transmission from the environment by inhalation of the bacterium in aerosolised, contaminated water or soil particles, possibly within their amoebal hosts [[6](#_ENREF_6)]. Person-to-person spread has not been documented the literature. These bacteria are capable of infecting and multiplying within a variety of mammalian and amoebal cell lines [[6](#_ENREF_6)]. Key to the pathogenicity of this organism is its ability to manipulate host cell processes, permitting the establishment of an intracellular replication niche [[16](#_ENREF_16)].

Since its discovery, more than 60 species of *Legionella* have been recognised, and at least 24 of these have been associated with human disease [[13](#_ENREF_13)], whether the remaining species can cause disease is currently unknown. Diseases caused by this opportunistic pathogen include the pneumonic form, legionnaires’ disease, and the flu-like form, Pontiac fever [[15](#_ENREF_15)]. The conditions under which an individual develops either legionnaires’ disease or Pontiac fever are not fully understood but may depend on the health status of the individual, the degree of exposure to the organism, and/or the strain-specific virulence [[4](#_ENREF_4)]. *Legionella* is a major, yet largely unrecognised, cause of community and nosocomially acquired pneumonia. There has been an average of 397 notifications of Legionellosis annually in Australia over the last 5 years (National Notifiable Diseases Surveillance System, 2015). The disease has a case fatality rate as high as 40-80% in untreated immunocompromised patients and 5 -10% for persons able to develop an immune response (World Health Organisation, 2014). Most pathogenic and environmental studies to date have focused on *L. pneumophila,* particularly serogroup 1, as it is responsible for more than 90% of cases of Legionnaires’ disease [[13](#_ENREF_13)]. Overall, non-pneumophila species account for around only 10% of cases of Legionellosis globally [[11](#_ENREF_11)], with the exception of *L. longbeachae* in Australia and New Zealand. Many *Legionella* species appear to be less virulent than *L. pneumophila*, as they cause disease almost exclusively in highly immunosuppressed persons [[3](#_ENREF_3)]. However, it is not really known if this reported lack of cases is actually a consequence of infrequent testing clinically for these non-pneumophila isolates, or a consequence of these isolates being less virulent in humans. Human macrophage infection models *in vitro* are commonly used to assess virulence of bacterial pathogens including *Legionella* [[13](#_ENREF_13)].

This study aimed to investigate the potential for human infections associated with *Legionella* isolates commonly found during routine sampling and surveillance for *L. pneumophila* conducted in accordance with mandated testing protocols. Environmental isolates of *Legionella* were collected from urban test sites within South East Queensland to characterise the growth and virulence properties of these isolates compared to *L. pneumophila* 130b. A total of 34 *Legionella* isolates were collected and included *L. quinlivanii*, *L. anisa*, *L. pneumophila*, *L. londiniensis* and *L. longbeachae* (as determined by 16s rRNA PCR and sequencing). Isolates considered to be environmental *Legionella* (species not commonly associated with clinical cases) cultured from urban test sites during routine environmental monitoring were demonstrated by this study to have virulence capacity, at least in laboratory models.

**Results and Discussion**

*Collection of environmental* Legionella *isolates*

Environmental *Legionella* isolates were collected by EML Consulting Services, Brisbane, Australia and were collected during 2010 to 2011 from urban test sites within South East Queensland. Sites included commercial and industrial cooling towers and potable waters (including shower heads in nursing homes) in which water samples were taken. These isolates were used to characterise the growth and virulence properties of environmental isolates compared to *L. pneumophila* 130b (AA100) ATCC® BAA-74™type strain. Environmental *Legionella* isolates were identified to species level using 16S rRNA PCR and SANGER sequencing as previously described [[9](#_ENREF_9)]. The PCR assay utilised primers to amplify a 654-bp fragment of the 16s rRNA gene of *Legionella* species. Sequences were compared with 16s rRNA gene sequencing available in the GenBank nucleotide library using BLASTn software. A total of 34 *Legionella* isolates were collected and found to belong to five different species; *L. quinlivanii* (50%), *L. anisa* (20.6%), *L. pneumophila* (17.6%), *L. londiniensis* (8.8%) and *L. longbeachae* (2.9%). Table 1 shows the selection of *Legionella* isolates from these environmental samples that were then used to investigate virulence potential in this study. *L. quinlivanii* was the most common *Legionella* spp. isolated in this study, but clinical cases of pneumonia attributed to this species have not been reported, although antibody titres in a small selection of patients have been reported that might indicate clinical causality [[2](#_ENREF_2)]. *L. anisa* has been implicated in cases of Legionnaire’s disease [[20](#_ENREF_20)], but is mostly associated with cases of Pontiac fever [[7](#_ENREF_7)]. *L. londiniensis* has been implicated in clinical disease, and is often isolated from the environment [[17](#_ENREF_17)]. *L. longbeachae* causes approximately 30% of cases of pneumonia in Australia and New Zealand [[21](#_ENREF_21)] and is commonly associated with compost and potting soil [[18](#_ENREF_18)].

*Growth Curves*

Growth curves were performed in Buffered Yeast Extract (BYE). The distinct broth growth phases of exponential and stationary phase are proposed to correlate (or at least share many attributes) with the replicative and transmissive forms observed in phagocyte cultures and reported to be characteristic of the biphasic lifecycle of *L. pneumophila* [[10](#_ENREF_10)] (Fig. 1). *L. pneumophila* 130b had a doubling time (measured during exponential phase) of 2.86h, *L. longbeachae* E35 1.71h, *L. anisa* E2 2.40h, *L. quinlivanii* E4 2.72h and *L. londiniensis* 1.57h. *L. pneumophila* 130b had a considerably shorter lag phase than did the environmental isolates, however by 30 hours (h) all had reached stationary phase.

*Infectivity in human macrophages*

Central to the pathogenesis of *Legionella* is its ability to survive and multiply within human macrophages. Intracellular replication of the isolateswas analysed in the human acute monocytic leukaemia cell line, THP-1 (Fig. 2A) and infection was performed at an MOI of 1:5 as previously described [[14](#_ENREF_14)]. Survival and distribution of legionellae in the environment are assumed to be associated with their multiplication in amoebae, whereas the ability to multiply in macrophages is usually regarded to correspond to pathogenicity [[12](#_ENREF_12)]. *L. pneumophila* 130b was shown to invade efficiently and multiply consistently over a 48h time period (Fig. 2A). *L. longbeachae* E35, *L. quinlivanii* E4 and *L. londiniensis* E26 also displayed this ability, albeit with significantly lower total CFU/ml. *L. anisa* was able to survive intracellularly to 24h but failed to continue multiplying to 48h (Fig. 2A); this may correlate with this isolate commonly being associated with Pontiac fever and not pneumonia [[7](#_ENREF_7)].

Cytopathogenicity assays were conducted to determine the viability of the THP-1 monolayers after infection with *Legionella* spp. Cytopathogenicity is a trait known to directly correlate with the ability of a particular species to cause disease in a human host [[1](#_ENREF_1)]. At several time-points after infection, the monolayers were treated for 4h with 10% Alamar Blue dye, a cell viability indicator that allows analysis of cell proliferation and cytotoxicity. Viability of the monolayers was determined by measuring the absorbance of Alamar-Blue-treated monolayers using a microplate reader and expressed as percentage cell death compared with uninfected cells (Fig. 2B).

*L. pneumophila* 130b had a more detrimental impact on the viability of human macrophages than did the environmental *Legionella* isolates, however, by 72 h PI *L. londiniensis* E26 lysed 85% of the monolayer and *L. longbeachae* E35 had lysed 60%. *L. anisa* E2 was again shown to be able to invade THP-1 macrophages but are unable to establish an infection. *L. quinlivanii* E4 maintained a low level of cytopathogenicity until 72 h PI whereby no infection remained. Alli *et al* has previously demonstrated that the majority of *L. pneumophila* serogroup 1 isolates tested in the study were able to kill 50% or more of U937 macrophages, while remaining serogroup 1 and non-pneumophila strains were able to kill 49% or less after 72h infection [[1](#_ENREF_1)]. Results obtained from this study demonstrate a higher level of cytopathogenicity for environmental *Legionella* isolates than formally reported.

It is acknowledged that only one isolate of each *Legionella* species collected in this study was examined, however, the heterogeneity and the potential pathogenicity that this bacterium displays cannot be underestimated and the species itself is unlikely to be major determinant of virulence.

Very little is known about these opportunistic pathogens commonly found in the urban environment and the risks they pose to the community. The variations observed here between *Legionella* isolates can potentially be attributed to the varied environmental niches of this organism and, we suspect the different host cell reservoirs (e.g. different protozoan hosts) that exist within these niches. We suspect that the different host environments may well translate into significant differences in phagosome formation in the human macrophage and could be what determines the ability of *Legionella* to cause disease (or different disease severity) in humans.

However, the most important finding from this study is that isolates of ‘environmental’ *Legionella* commonly found in the urban environment have the capacity to infect and replicate within human macrophages and may represent a greater cause of community acquired pneumoniae and greater public health risk than currently recognised.

**Conflict of Interest**

The authors declare that there are no conflicts of interest.

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**Fig. 1.** Determination of growth phase of *Legionella* spp. Isolates from overnight cultures were diluted to an OD660 of 0.2 in broth, and then growth of the cultures was monitored spectrophotometrically (OD660) over 50 h. Shown are mean absorbance with error bars indicating the standard error of the mean from triplicate experimental replicates for each isolate. The isolates are represented by different symbols indicated to the right in the figure legend.

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**Fig. 2. A** Infection and replication within human THP-1 macrophages by *Legionella* spp. Differentiated human acute monocytic THP-1 cells were infected with *Legionella* isolates shown above at an MOI of 1:5, and then at various time points, the CFU in infected monolayers was determined by serial dilution and plating (CFU/ml on y axis on log scale). Shown are mean CFU/ml with error bars indicating the standard error of the mean obtained from triplicate infected wells. The x axis shows the different time points, the different isolates are indicated by different grey scale shading as indicated in the figure legend to the right. Asterisks indicate statistical differences in CFU/ml from *L. pneumophila* 130b (p ≤ 0.05 using one-way ANOVA) **B** Cytopathogenicity of *Legionella* spp. to differentaited human acute monocytic THP-1 macrophages. Cytopathogenicity was performed as previously described [[1](#_ENREF_1)]. Experiments were performed in triplicate and expressed as percentage cell death compared with uninfected cells by using the formula [1-(mean absorbance of treated cells/mean absorbance of untreated cells)]\*100 (y axis). The x axis indicates the time points that the cytotoxicity was measured, and the different isolaes are indicated by different shades of grey as indicated to the right.

**Table 1**

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| --- | --- | --- | --- |
| **Strain name** | **Isolate Identification** | **Source** | **Clinical association for this species** |
| *L. pneumophila* 130b | *L. pneumophila* 130b | American Type Culture Collection (ATCC) | Clinical isolate from USA, most frequent in clinical cases |
| *L. Longbeachae*  | E35 | Environmental isolate (EML Laboratories) | Frequent clinical cases |
| *L. anisa*  | E2 | Environmental isolate (EML Laboratories) | Infrequent clinical cases |
| *L. quinlivanii*  | E4 | Environmental isolate (EML Laboratories) | Infrequent clinical cases |
| *L. londiniensis*  | E26 | Environmental isolate (EML Laboratories) | Infrequent clinical cases |