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Screening-Level Assays for Potentially Human-Infectious Environmental *Legionella* spp.

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In spite of the fact that various *Legionella* species are isolated from nonclinical water settings, there is no standard method to determine whether environmental legionellae may be infectious to humans. Here we provide a screening-level approach based on an *in vivo* murine (A/J mouse) model and three *in vitro* proliferation assays using *Acanthamoeba polyphaga*, and THP-1 human and J774 murine macrophage cell lines to identify potentially human-infectious legionellae. As an initial demonstration the infectivity potential of three clinical (*Legionella pneumophila*, *L. longbeacheae*, and *L. micdadei*) and three environmental (*L. dumoffii*, *L. maceachernii*, and *L. sainthelensi*) legionellae were evaluated. A/J mice were intranasally infected and by 6 h post infection (p.i.), there were significant bacterial titers in the lungs. *L. pneumophila*, *L. dumoffii*, and *L. micdadei* densities were higher than *L. longbeacheae*, *L. maceacherni*, and *L. sainthelensi* at 24 h p.i. However, only *L. pneumophila* and *L. micdadei* persisted in the lungs after 48 h, indicating that the other isolates were rapidly cleared. Results from the *in vitro* assays showed that only *L. pneumophila* significantly multiplied within *A. polyphaga*, THP-1 and J774 cells after 72 h, but lysis of any of the *in vitro* hosts also flagged the strains for potential concern (e.g. L. *dumoffii* and *L. micdadei*). The results demonstrate the value of using multiple approaches to assess the potential level of pathogenicity of *Legionella* strains isolated from different environmental matrices.

***Keywords*:** *Legionella* spp., *Acanthamoeba polyphaga*, biofilms, virulence, macrophages

Legionnaire's disease is a severe type of pneumonia caused primarily by *Legionella pneumophila* serogroup 1, although other strains have been implicated as aetiologic agents (Fields *et al*., 2002; Diederen, 2008). *L. pneumophila* is one of the most important pathogens associated with drinking water exposures in the USA (Craun *et al.*, 2010), but it is less clear as to the human significance of the other fifty species of *Legionella* that naturally inhabit freshwater and soil environments (Fields *et al.*, 2002; Pagnier *et al.*, 2009). Exposure to disease-causing strains has long been attributed to the inhalation of aerosols from cooling towers, heaters, hot tubs, pools, and hot and cold water distribution systems (Bornstein *et al.*, 1986; Breiman *et al.*, 1990). Within such environments *Legionella* spp. are biofilm community members where their interaction with various protozoa (and possibly other metazoa) is considered critical for their environmental survival, amplification, and delivery of infectious strains via aerosols (Borella *et al.*, 2005). Indeed various amoebal species can support intracellular growth of *Legionella* spp. providing a niche for replication and protection from harsh water treatment conditions such as residual (chlorine) disinfectants in treated drinking water systems (Kilvington and Price, 1990; Declerck, 2009; Declerck *et al.*, 2009). Specifically, *L. pneumophila* has been shown to be physiologically distinct, more invasive, and pathogenic after passage through various amoebae compared to cells cultured from artificial media. This may be due to the fact that alveolar macrophages and amoebae have similar phagocytic and microbicidal mechanisms that pathogenic legionellae have evolved to avoid (Lau and Ashbolt, 2009). It is because of those similarities amoebae share with mammalian macrophages and their close relationship with *Legionella* in the aquatic environment that underscores the need to elucidate the role biofilm-amoebae play in *Legionella* pathogenesis.

Acanthamoeba spp. are commonly used to study the correlation between *Legionella* virulence and intra-amoebal growth. Cirillo *et al.* (1999) reported that amoeba-grown *L. pneumophila* is more virulent in susceptible A/J mice resulting in increased rates of replication in the lung. Similarly, amoeba-grown *L. pneumophila* cells were at least 10-fold more invasive and displayed increased replication in human macrophage-like THP-1 cells compared to their agar grown counterparts (Cirillo *et al.*, 1994). THP-1 cells and J774, a murine macrophage-like cell line, are commonly used as primary macrophage cell substitutes, with *L. pneumophila* replication in J774 cells shown to resemble those levels observed in human primary monocyte-derived macrophages (Yan and Cirillo, 2004). Both cells lines have been used to identify *Legionella* virulence genes by evaluating the gene mutants' invasiveness and replication further indicating their value as a model for *Legionella* virulence (Bandyopadhyay and Steinman, 2000; Naylor and Cianciotto, 2004; Ohnishi *et al*., 2004). However, most studies do not couple different approaches to screen *Legionella* strains for their pathogenicity, which would enhance the ability to accurately assess potentially human-infectious, environmental isolates.

Across the drinking water industry there is increasing regulatory interest in culturing environmental legionellae from

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drinking water distribution systems. For example, in the Netherlands, a detection of more than 100 CFU of legionellae per 100 ml of drinking water triggers management action. Although targeting a broad group to trigger action is appropriate, detection of total legionella should not necessarily be equated to health risk. Based on the broad genetic diversity of legionellae, it would be expected that many environmental isolates are not human pathogens (Harrison *et al.*, 2009). Furthermore, as we learn more about the true diversity of bacteria in drinking water biofilms under different disinfectant regimes through molecular and culture-based approaches (e.g. Roeder *et al.*, 2010), a question arises as to how different chlorine residuals impact/control legionellosis through changing drinking water microbial ecology. This is further complicated by the fact that amoebae-infectious but non-culturable legionellae are likely to be generated in disinfected drinking water (Gião *et al.*, 2009) and be outcompeted by non-pathogenic legionellae. Each are examples of why it is important to be able to screen for potentially human-infectious environmental legionellae.

In this study, we use multiple methods, specifically intranasally inoculated A/J mice and growth of the same strains in *A. polyphaga*, J774, and THP-1 cells, to evaluate their use in screening for potentially human-infectious environmental legionellae derived from drinking water systems.

Materials and Methods

Animals and bacterial inoculation

A/J mice were purchased from The Jackson Laboratory and housed in specific pathogen-free (SPF) conditions within the animal care facility at the US EPA, AWBERC building (Cincinnati, USA) until the day of sacrifice. All animals were cared for and all experimental procedures were conducted in accordance with federally approved guidelines developed by the Institutional Animal Care and Usage Committee at the US EPA (National Research Council, 1996). For intranasal inoculation, mice were intraperitoneally injected with a 0.1 ml saline solution consisting of 10 μg ketamine (anesthetic) and 30 μg xylazine (analgesic) and a 10 μl inoculum of 10^7-10^8 bacterial cells was delivered into each nostril followed by a 10 μl flush with sterile PBS in each nostril.

Bacterial preparation

The strains used in this study (Table 1) were grown overnight as pure cultures at 37°C in buffered yeast extract (BYE) broth [10 g N-(2-Acetamido)-2-aminoethanesulfonic acid, 10 g yeast extract, 0.4 g L-cysteine and 0.135 g ferric nitrate per L]. Bacterial concentration was determined by measuring the amount of absorbance at 600 nm and compared to a predetermined standard curve of colony forming units (CFU). Bacteria were then diluted to the desired concentration in either phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI), or Dulbecco's Modified Eagle's Medium (DMEM) medium. To determine *Legionella* densities (as measured by CFU), a small aliquot of the bacterial suspension was serially diluted and plated on buffered charcoal yeast extract (BCYE) agar plates (BD Diagnostics, USA), incubated for 48 h at 37°C and diagnostic *Legionella* colonies counted. Serial dilutions were performed with sterile water to release intracellular bacteria for the amoebae and macrophage cell line assays described below.

Bacterial densities in whole lung homogenates

A/J mice were intranasally inoculated with each of the tested *Legionella* isolates (Table 1) and analyzed 3, 6, 12, 24, and 48 h post infection (p.i.). Mice were euthanized by CO_2 inhalation. Excised whole lungs were homogenized using a tissue homogenizer (Biospec Products, USA) in 1 ml of PBS. *Legionella* densities were estimated as CFU as described above.

A. polyphaga proliferation assay

A. polyphaga (ATCC 30461, USA) was grown in ATCC 712 medium as monolayers at 30°C. Cells were harvested on the day of the experiment and seeded into 24-well plates at a density of 10^5 per well and incubated for 3 h. Cells were washed twice with 1 ml of Page's amoeba saline (PAS: 2.5 mM NaCl, 1 mM KH_2PO_4, 0.5 mM Na_2HPO_4, 40 μM $CaCl_2{\cdot}6H_2O$, and 20 μM $MgSO_4{\cdot}7H_2O$). PAS-diluted *Legionella* cells were added to wells at a bacterium to amoeba cell ratio of 25:1. Plates were centrifuged at 250×g for 3 min and incubated at 30°C. At each time point, cells were resuspended and an aliquot was taken for bacterial enumeration as described above.

Growth of *Legionella* strains in human and murine macrophage-like cell lines

The ability of the *Legionella* test strains to proliferate in the presence of two different mammalian cells lines was assessed. THP-1 cells (ATCC TIB-202™) were maintained in RPMI-1640 modified medium (ATCC, USA) containing 10% fetal bovine serum (FBS) and 100 U each of penicillin and streptomycin per ml in a 5% CO_2 at 37°C environment. For each experiment, THP-1 cells were washed with complete medium, counted and seeded into 24-well plates at a density of 10^5 cells per well and pretreated with 16 nM phorbol-12-myristate-13-acetate (PMA) for 24 h in 5% CO_2 at 37°C, to induce maturation of the monocytes into macrophage-like adherent cells. The monolayer was washed three times with penicillin/streptomycin free RPMI-1640 modified medium -10% FBS. Bacteria were then added at a bacterium to amoeba ratio of 25:1, spun onto the monolayers at 250×g for 3 min, and incubated in 5% CO_2 at 37°C. At each time point, the cells were resuspended in the wells and an aliquot was taken for bacterial enumeration.

Table 1. *Legionella* spp. strains used in this study

Name	Description	Source
L. pneumophila, Lp02	Clinical isolate, derivative of Philadelphia-1 strain	Dr Michele Swanson
L. longbeacheae	Clinical isolate, human lung	ATCC 33462
L. micdadei	Clinical isolate, human blood	ATCC 33218
L. dumoffii	Enviromental isolate, cooling tower, New York	ATCC 33279
L. maceachernii	Enviromental isolate, water in home evaporator cooler, Phoenix, AZ	ATCC 35300
L. sainthelensi	Enviromental isolate, spring water, Mt. St Helens, WA	ATCC 35248

J774A.1 cells (ATCC TIB-67^{TM}) were maintained in ATCC formulated DMEM containing 10% FBS and 100 U of penicillin and streptomycin per ml in 5% CO_2 at 37°C. For each experiment, J774 cells were washed with complete medium, counted and seeded into 24-well plates at a density of 10^5 cells per well for approximately 15 h before each experiment. The monolayer was washed three times with penicillin/streptomycin free DMEM-10% FBS. Bacteria were then added at a bacterium to amoeba ratio of 25:1 and processed as described above.

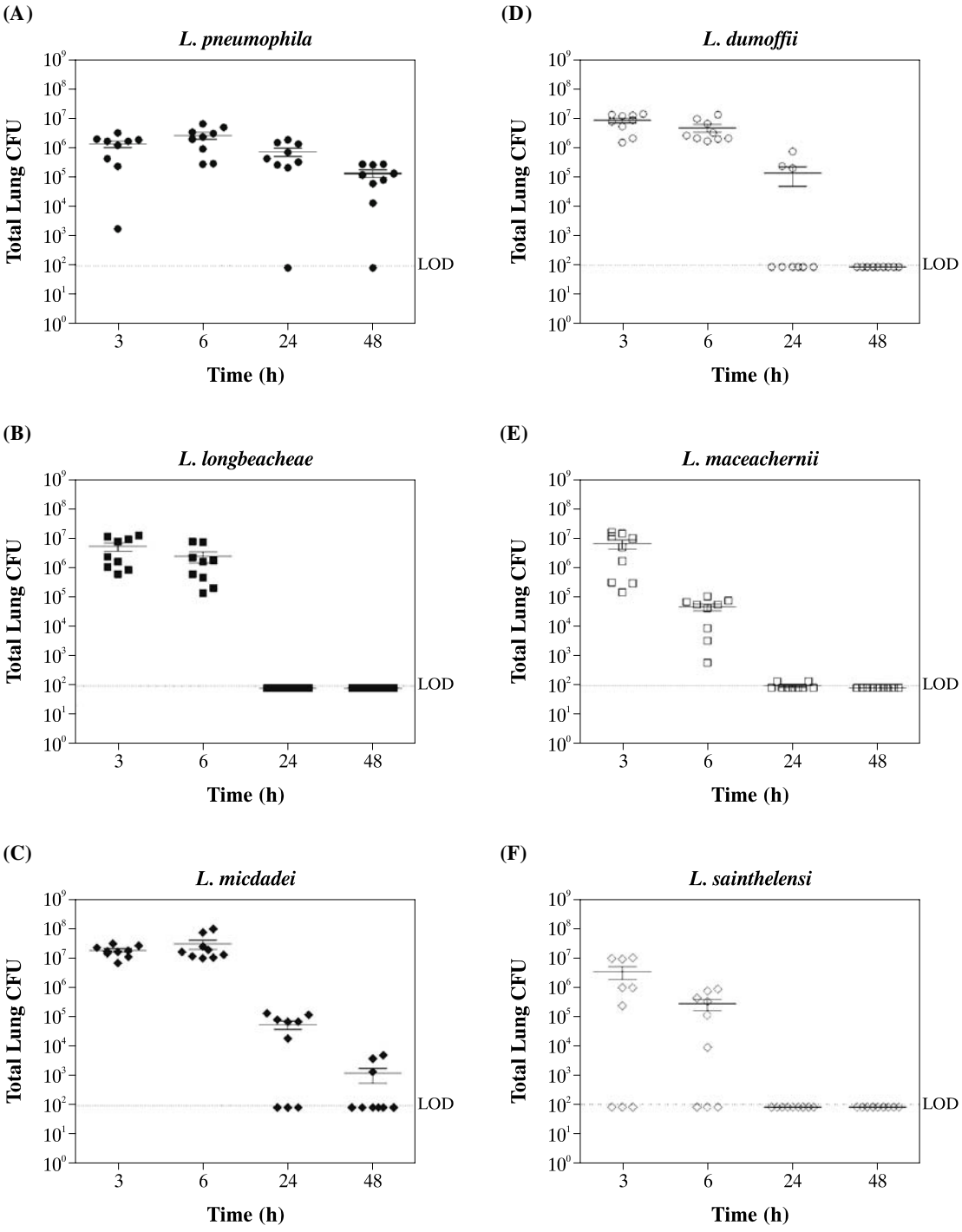

Fig. 1. Total lung CFU of intranasally infected mice. A/J mice were intranasally infected with *L. pneumophila*, Lp02 (A), *L. longbeacheae* (B), *L. micdadei* (C), *L. dumoffii* (D), *L. maceachernii* (E), and *L. sainthelensi* (F). Filled symbols and open symbols represent clinical and environmental isolates, respectively. Each point represents one animal. Data were generated from three independent experiments with 3 animals per group. No bacterial growth was observed in the lungs of control animals. LOD, limit of detection.

Statistical analysis

Statistical significance was determined using the unpaired, two-tailed Student t-test, ANOVA for multiple group comparisons using the Student-Newman-Keuls post-test, and Fisher's exact test. Where appropriate, the unpaired, nonparametric Mann-Whitney test was utilized. Calculations were performed using InStat 3 (GraphPad Software, USA).

Results

Kinetic clearance of *Legionella* spp. from the lungs of A/J mice

Six *Legionella* strains were intranasally inoculated into A/J mice (10^7-10^8 CFU) and their clearance rate was determined at 3, 6, 24, and 48 h post infection (p.i.). At 3 and 6 h p.i., all isolates displayed high bacterial titers, between 10^5-10^7 CFU, in the lung (Figs. 1A-F). However, by 6 h p.i., only *L. maceachernii* and *L. sainthelensi* CFU levels decreased by 1-2 logs, 10^7 to 10^5 and 5×10^6 to 5×10^5 total lung CFU, respectively (Figs. 1E and F). At 24 h p.i., only *L. pneumophila*, *L. dumoffii*, and *L. micdadei* infected mice displayed bacterial titers in the lung, 10^6, 10^5, 10^5 CFU, respectively, while *L. longbeacheae*, *L. maceachernii*, and *L. sainthelensi* bacterial numbers were below the limit of detection (<100 CFU). Interestingly, only *L. pneumophila* and *L. micdadei* inoculated mice contained legionellae cells in the lung at 48 h (Figs. 1A and C; 10^5 and 10^3 CFU, respectively) suggesting that there may be a mechanism resisting or affecting the host's rapid clearance of these strains. Based on this mouse assay, the delayed clearance of *L. pneumophila* and *L. micdadei* implies that these strains are potentially human-infectious compared to *L. longbeacheae*, *L. maceachernii*, and *L. sainthelensi*, which were rapidly cleared by 24 h p.i., whereas *L. dumoffii* appears intermediate between these two groups.

Legionella spp. proliferation in the presence of *A. polyphaga*

Growth rates of the *Legionella* strains in association with *A. polyphaga* were examined for further potential screening of virulent legionellae. The six *Legionella* isolates were incubated in the presence and absence of *A. polyphaga* cells for 0, 24, 48, and 72 h and results are presented as a ratio of the bacterial CFU in the presence of amoebae (Tn) divided by the legionellae CFU in bacteria only wells (Tc) at each time point. PAS was used as the assay buffer since *A. polyphaga* and legionellae will not grow in this medium. The *A. polyphaga* cells remain in the trophozite (active feeding) form for up to 72 h after which they start to encyst. Therefore, substantial amoebae encystation had not occurred all time points examined in this experiment. *L. pneumophila* cell ratios increases slightly at 24 h from 1.6 to 2.4, but substantially rose at 48 and 72 h, being 321 and 108 respectively (Fig. 2, $p<0.001$). In contrast, the other two clinical isolates, *L. longbeacheae* and *L. micdadei*, display no significant increase in their growth ratio at 48 or 72 h. *L. micdadei* did display some growth by 72 h (ratio of 1.1 at 0 h versus 2.8 at 72 h); however, while growth

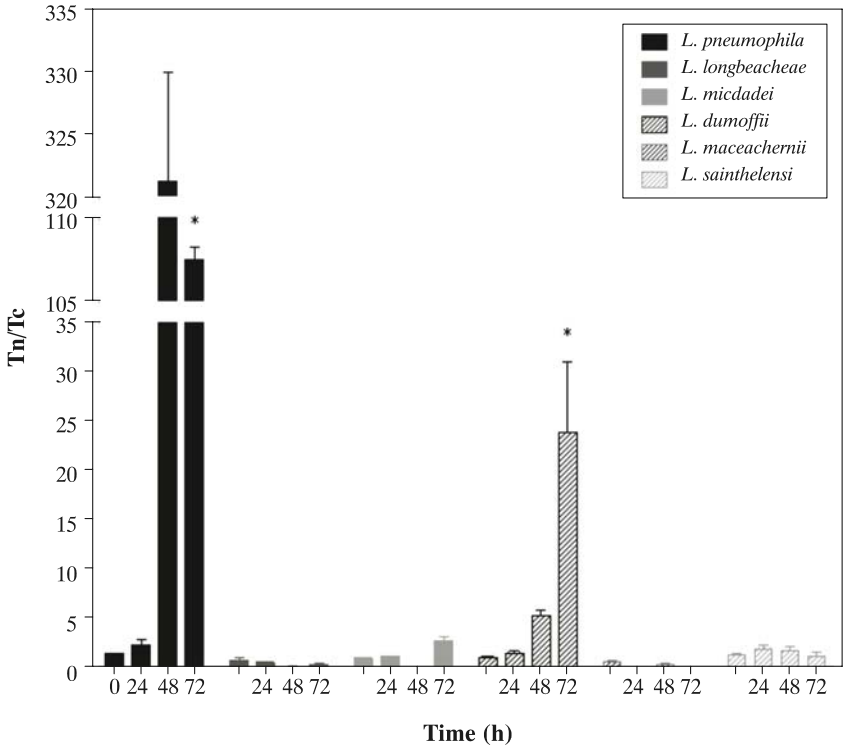

Fig. 2. Growth of bacterial strains in the presence of *A. polyphaga*. Data are presented as a ratio of bacterial CFU levels at each time point (Tn) divided by bacterial CFU levels in control wells (Tc). Fill bars and hatched bars represent clinical and environmental isolates, respectively. Data are representative of two independent experiments. *, $p<0.001$.

was not significant, amoebae were lysed (as with *L. pneumophila* and *L. dumoffii*, data not shown), implying pathogenicity. *L. dumoffii* followed similar trends to *L. pneumophila*, with minimal growth at 24 h (ratios from 1.2 to 1.6), but by 48 and 72 h, ratios significantly increased to 5.4 and 24 respectively (Fig. 2, $p<0.001$). In contrast, the growth ratio of *L. maceachernii* decreased from one to <1 suggesting a decrease in bacterial numbers, while the growth ratio of *L. sainthelensi* remained constant throughout the course of 72 h (ratio of 1.4 at 0 h versus 1.3 at 72 h) suggesting negligible net growth in the presence of *A. polyphaga* cells. Similar to the mouse assay, based on this amoebae assay, the increase in bacterial numbers/lysis of amoebae by *L. pneumophila*, *L. dumoffii*, and *L. micdadei* implies that these strains are potentially human

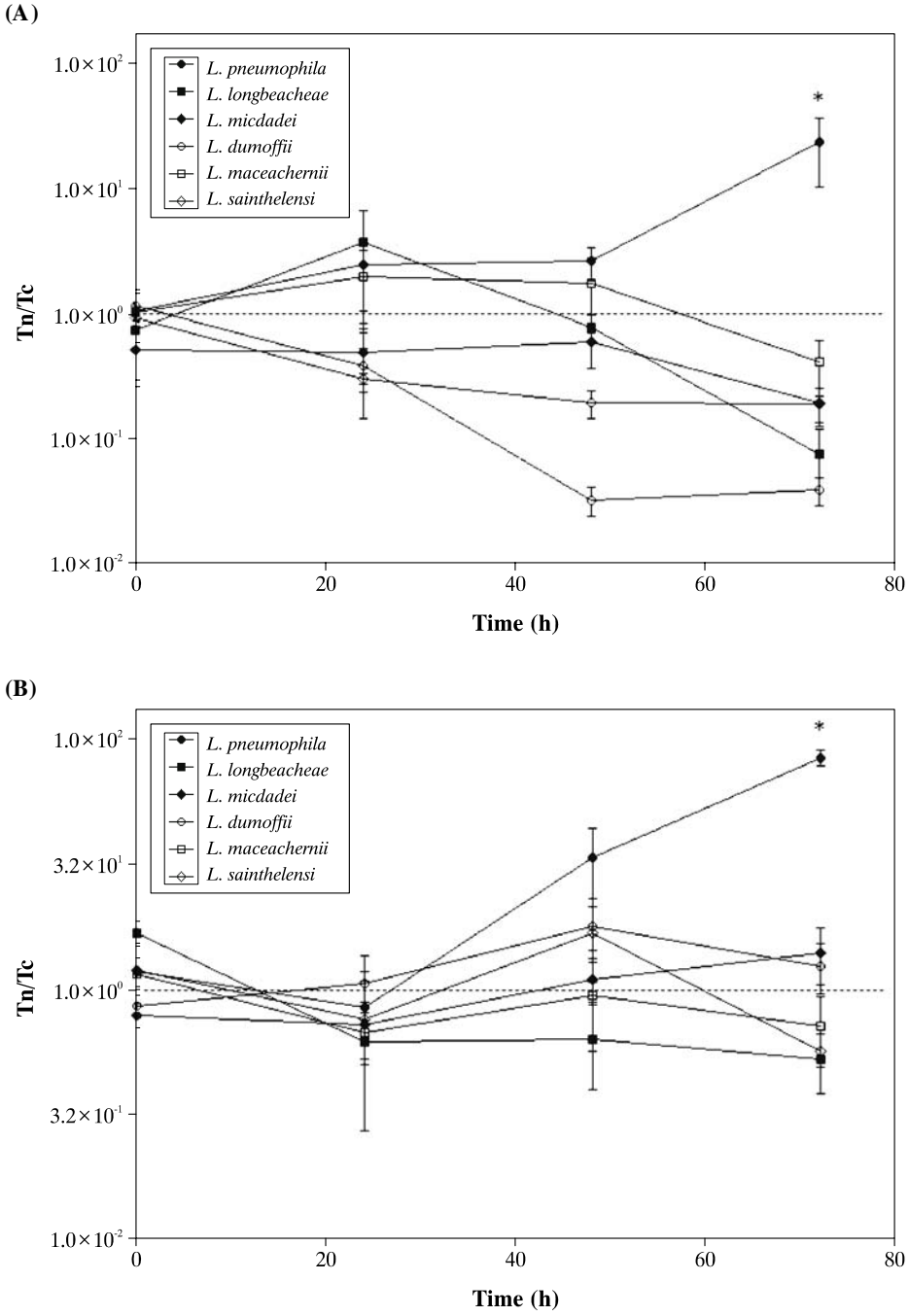

Fig. 3. Bacterial proliferation in the presence of J774 and THP-1 cells. J774 (A) and THP-1 (B) cells were infected with the four bacterial isolates. Data are presented as a ratio of bacterial CFU levels at each time point (Tn) divided by bacterial CFU levels in control wells (Tc). Filled symbols and open symbols represent clinical and environmental isolates, respectively. Data are representative of two-three independent experiments. *, $p<0.001$.

infectious compared to the strains of *L. longbeacheae*, *L. maceachernii*, and *L. sainthelensi* examined.

Infectivity of *Legionella* spp. in mouse and human macrophages

Since three of the *Legionella* isolates were able to increase their densities in the presence of amoebae, the ability of each isolate to utilize both human and murine macrophages to proliferate was further examined through infection of mouse (J774) and human (THP-1) macrophages. As for growth with and without amoebae hosts, the ratio of the bacterial CFU in the presence of macrophages (Tn) was divided by legionellae CFU in bacteria only wells (Tc) at each time point. Incubation of J774 macrophages with the isolates demonstrated no significant net growth of cultivable legionellae except for *L. pneumophila*, whose growth ratio increased significantly from 48 to 72 h, 2.7 and 24, respectively (Fig. 3A, $p<0.001$). *L. longbeacheae*, *L. micdadei*, *L. dumoffii*, *L. maceachernii*, and *L. sainthelensi* counts in J774 cells decreased over time indicating death, eradication and/or non-cultivability of the bacteria from the culture (Fig. 3A). Similar results were observed for *L. longbeacheae*, *L. maceachernii*, and *L. sainthelensi* with THP-1 cells, where bacterial densities dropped over 72 h to below their respective control well levels (Fig. 3B; growth ratios of 1 at 0 h to 0.08 at 72 h, 1 to 0.4, and 1 to 0.2, respectively). In contrast, the growth ratio of *L. pneumophila* steadily increased from 1.3 at 0 h to 8.5 at 75 h (Fig. 3B, $p<0.001$). Interestingly, to a lesser extent, the growth ratios of *L. micdadei* and *L. dumoffii* appeared to increase from 0 to 72 h although not significantly, from 1 to 1.4 and 0.9 to 1.3, respectively (Fig. 3B). Among the strains evaluated by THP-1 cells, only *L. pneumophila* had the ability to proliferate in the presence of both murine and human macrophage-ike cells, which is consistent with findings of previous studies (Yan and Cirillo, 2004; Sahr *et al.*, 2009).

Overall, the results imply that the *L. longbeacheae*, *L. micdadei*, *L. dumoffii*, *L. maceachernii*, and *L. sainthelensi* strains evaluated in this study may not be as virulent as the *L. pneumophila* Lp02 reference strain. However, because of the delayed clearance of *L. dumoffii* and *L. micdadei* from mouse lungs and their ability to lyse and infect amoebae, these latter two strains may be of particular interest for future studies and at this screening-level evaluation should be considered potentially human-infectious along with the *L. pneumophila* strain.

Discussion

Several studies have used mouse models, cell lines, various amoebae or virulence genes to identify pathogenic *L. pneumophila* (Naylor and Cianciotto, 2004; Newton *et al.*, 2008). In this study, we combined these approaches to determine if delayed clearance in mice and prolific intracellular replication within three host cell types would be an adequate approach to screen for potentially human-pathogenic environmental isolates. The *L. pneumophila* strain used in this study was able to proliferate within amoebae and both murine and human macrophage-like cells and exhibited delayed clearance from mouse lungs compared to the other legionellae isolates studied. Interestingly, *L. dumoffii*, and to a lesser extent, *L. micdadei*, was able to proliferate within *A. polyphaga* and THP-1 cells although their growth in those assays was not as robust as that of *L. pneumophila*. Furthermore, *L. dumoffii* and *L. micdadei* also displayed delayed clearance from the lungs of A/J mice. These results suggest that the *L. dumoffii* strain, originally isolated from a cooling tower, and *L. micdadei*, originally isolated from human blood, possess some traits commonly associated with *L. pneumophila* virulence in mice and human cell-line models.

For the *in vitro* cell culture experiments, control wells (i.e., without eukaryotic cells) displayed a decline of viable *Legionella* cells (Fig. 3). Intracellular replication of *Legionella* spp. within phagosomes is a prerequisite for Legionnaires' disease as demonstrated in various studies (Roy, 2002; Weber *et al.*, 2009). Therefore, the ability of *Legionella* spp. to infect and replicate within macrophages is important in determining potential human virulence. O'Connell *et al.* (1996) reported that two out of fourteen *Legionella* species not associated with human disease exhibited an infective dose similar to that of a virulent *L. pneumophila* strain in U937 cells, a human macrophage-like cell line, hence suggesting that numerous other *Legionella* spp. may replicate within macrophages and thus be pathogenic. Others have explored the virulence traits of *Legionella* spp., but apart from *L. pneumophila*, such traits are not well defined in non-*L. pneumophila* strains. For example, Alli *et al.* (2003) demonstrated that several virulence phenotypes, including intracellular replication within macrophages, presence of the dot/icm loci, and cytopathogenicity, commonly exhibited by *L. pneumophila* were largely absent in 24 out of the 27 clinical and environmental legionellae strains tested, which represented 16 different species. Because various *Legionella* species such as *L. anisa*, *L. dumoffii*, and *L. feeleii* are commonly detected in engineered water systems (Muder and Yu, 2002; Yu *et al.*, 2002; Doleans *et al.*, 2004) and because *L. pneumophila* serogroup 1 may account for 90% of clinical cases (Doleans *et al.*, 2004; Cazalet *et al.*, 2008), it is hypothesized that the high percentage of *L. pneumophila* serogroup 1 strains in human disease is not due to the predominance in the environment, but rather is connected with higher virulence of these strains in humans.

Certain biofilm-amoebae conditions may further select/up-regulate for human-infectious strains, independently of free-living environmental growth or growth of non-pathogenic legionellae (Lau and Ashbolt, 2009). In fact, coculture of both human and environmental samples with amoebae has been demonstrated as an efficient means to detect and isolate amoeba-resisting bacteria (ARB) such as various species of *Legionella*. Pagnier *et al*. (2008) utilized the coculture method to recover ARB from different human environments and isolated over 200 strains, which included several human pathogens. Applying the same coculture method, Thomas *et al.* (2008) reported the isolation of ARB, including mycobacteria, legionellae, and *Chlamydia*-like organisms, at various stages of a river-fed drinking water plant. Because free-living ameobae are ubiquitous in the environment and can harbor ARB, investigating the ecology and interactions of these microorganisms in various human environments is important. This is further underscored by the suggestion that a number of ARB utilize their ameoba-resisting capacity within macrophages to become potential human pathogens. In this study, the fact that *A. poly-*

phaga was permissive to growth of *L. pneumophila* Lp02 and to some extent the strains of *L. dumoffii* and *L. micdadei* examined, it implies that amoebae may play a possible role in propagating and selecting for opportunistic pathogens in drinking water systems. Brieland *et al*. (1997) showed that *L. pneumophila* virulence for amoebae was required for maximal intrapulmonary bacterial growth in A/J mice coinoculated with *Hartmannella vermiformis*, further highlighting the role amoebae may play in *Legionella* pathogenesis.

This study indicates that the tools for screening possibly human-infectious legionellae isolated from environmental samples should include assessment of clearance from A/J mouse lung tissue as well as levels of proliferation within *Acanthamoeba polyphaga*, J774 and THP-1 cells *in vitro*. In addition, the results described here support the notion that *L. pneumophila* serogroup 1 strains may be unique compared to other *Legionella* species in regards to their ability to persist in the presence of phagocytic cells. Moreover, the ability of *L. pneumophila* to cause disease may be a result of their species/strain-specific interactions with amoebae within biofilms since not all amoebae are equally permissive to *L. pneumophila*. Specifically, when comparing *A. castellanii*, *H. vermiformis*, *Willaertia magna* strain c2c and Z503, the latter strain was highly resistant to *L. pneumophila* Paris, but not to the Lens or Philadelphia strains (Dey *et al.*, 2009). In addition, a selective mode of *L. pneumophila* uptake by *A. castellanii* and *Naegleria lovaniensis* was shown where the latter took up 20 times less *L. pneumophila* bacteria compared to *A. castellanii* (Declerck *et al.*, 2005). Because *L. pneumophila* may co-inhabitant with other bacteria in biofilm communities, the effect of non-legionella bacteria on *L. pneumophila* uptake and intracellular replication was also examined in the previous study. *L. pneumophila* uptake by both *A. castellanii* and *N. lovaniensis* was not affected by other bacteria. Rather, *L. pneumophila* intracellular replication within *N. lovaniensis* was enhanced by *Pseudomonas aeruginosa* (Declerck *et al.*, 2005). Therefore, there is much to be understood as to the diversity of amoebae, other potential metazoan hosts and the role other bacteria play in environments that support legionellae growth within biofilms (Kuiper *et al*., 2006; Valster *et al.*, 2009). This is especially the case for water distribution systems considering the importance of nosocomial diseases in which drinking water biofilm bacteria are implicated aetiological agents (Yoder *et al.*, 2008).

Future studies include determining the virulence of drinking water *Legionella* isolates after amoebae passage and during co-inoculation into mice. Similar studies have been previously performed by intratracheally infecting A/J mice with a mixture of *L. pneumophila* and *H. vermiformis* (Brieland *et al.*, 1996) and *L. pneumophila* infected *H. vermiformis* (Brieland *et al*., 1997). Co-inoculation of mice with *H. vermiformis* enhanced intrapulmonary growth of *L. pneumophila* while *L. pneumophila* infected *H. vermiformis* were more pathogenic than when infection with *L. pneumophila* alone. However, these studies used intratracheal (surgical) exposure that does not accurately model the inhalation pathway of *Legionella* in contaminated water aerosols. In addition, only *H. vermiformis* and *L. pneumophila* strain AA100 was examined; thus, it would be interesting to test the *in vivo* virulence of other *L. pneumophila* strains and non-pneumophila species both alone and within various free-living amoebae. Such research will aid in our understanding of the role amoebae play in selecting for and releasing potential pathogens within drinking waters and to determine if virulence selection/up-regulation is a *L. pneumophila* serogroup 1-specific phenomena.

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