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Counting *Legionella* Cells within Single Amoeba Host Cells

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Here we present the first attempt to quantify *Legionella pneumophila* cell numbers within individual amoeba hosts that may be released into engineered water systems. The maximum numbers of culturable *L. pneumophila* cells grown within *Acanthamoeba polyphaga* and *Naegleria fowleri* were 1,348 (mean, 329) and 385 (mean, 44) CFU trophozoite^{-1}, respectively.

Legionellosis is a major cause of health burdens associated with drinking water, with *Legionella pneumophila* accounting for some 29% of all drinking water outbreaks since being reported in the United States (7). Direct exposure to aerosols containing *Legionella* spp. that are generated from cooling towers, humidifiers, and shower heads is implicated as the vehicle for bacterial transmission (2, 5). While *L. pneumophila* may be packaged and expelled in vesicles of freshwater ciliates (4), free-living protozoa within the genera *Acanthamoeba* and *Naegleria* are often cited as potential hosts and ecological reservoirs for *L. pneumophila* in drinking water systems (9). Therefore, understanding *Legionella*-amoeba interactions is considered fundamental to aid in developing control strategies and in supporting quantitative microbial risk assessment models for pathogenic legionellae in drinking water (10). Furthermore, if human-infecting legionellae are largely from amoeba hosts (9), it is critical to understand how many legionellae may be released per host cell within drinking water biofilms in order to enable back calculations of host densities that may result in significant pathogen release events (10). There appear to be no previous data reported on cell counts of intracellular *L. pneumophila* within single amoeba host cells. Hence, here we present the first attempt to provide intracellular pathogen ranges in *Acanthamoeba* and *Naegleria* spp. that may colonize drinking water biofilms.

L. pneumophila strain Bloomington 2 (B2; ATCC 33155), *Acanthamoeba polyphaga* (ATCC 30461), and *Naegleria fowleri* (ATCC 30894) were grown as previously described (6). Previous data showed that *A. polyphaga* and *N. fowleri* were more susceptible to *L. pneumophila* B2 at 32 and 30°C, respectively, within 48 h than were other environmental and clinical isolates, which had maximal bacterial growth between 48 and 72 h postinfection (6). Initial tests were performed to determine the Triton-X concentrations necessary to lyse amoebae with minimal effect on *L. pneumophila* cultivability (Table 1). The preferred protocol consisted of flow cytometric cell sorting (BD FACSAria II; BD Biosciences) of individual amoebae into wells of a microtiter plate, followed by immediate addition of 175 μl of 0.22-μm-filtered (Millipore) drinking water (ftH_2O) in order to dilute the Triton-X from 0.02% to <0.01%.

To achieve a 10:1 infection ratio of bacteria to amoeba cells, 6×10^5 amoebae were incubated with 6×10^6 legionellae or else incubated individually in T25 25-cm^2 culture flasks (Corning), using ftH_2O as the assay medium. At 0 and 48 h, culture flasks containing only bacteria, only amoebae, or bacteria and amoebae were harvested using a cell scraper. As controls for each experiment, 150 bacterial cells were sorted into 24 wells of a 96-well plate containing either 100 μl of ftH_2O or 0.02% Triton-X, and individually infected amoebae were sorted into 24 wells containing 100 μl of ftH_2O.

TABLE 1 Effect of Triton-X on *L. pneumophila* cultivability and amoeba lysis[a]

Time (min)	Triton-X concentration (%)	% of cells lysed[b]				% of cultivable *L. pneumophila* cells[c]	
		A. polyphaga		*N. fowleri*			
		Mean	SD	Mean	SD	Mean	SD
0	0.02	—	—	—	—	100.0	±0.00
	0.01	—	—	—	—	100.0	±0.00
4	0.02	—	—	—	—	100.5	±1.56
	0.01	—	—	—	—	97.4	±3.72
6	0.02	—	—	—	—	11.6	±9.36
	0.01	—	—	—	—	90.5	±1.13
8	0.02	—	—	—	—	0.35	±0.20
	0.01	—	—	—	—	92.6	±1.87
10	No Triton-X	0.0	±2.20	0.0	±0.63	100.0	±0.00
	0.02	91.2	±0.93	100	±0.00	0.06	±0.04
	0.01	0.1	±1.36	4.1	±1.63	95.4	±1.47

[a] Data are from 3 independent experiments. Cells were incubated in either 0.22-μm-filtered drinking water (ftH_2O) or Triton-X for 0 to 10 min at room temperature.
[b] Amoebae were counted using a hemacytometer. —, no data.
[c] Numbers of CFU were enumerated as described previously.

For wells with only *L. pneumophila* B2 cells, averages of 79 and 84 CFU well^{-1} were observed for bacteria sorted into ftH_2O and 0.02% Triton-X, respectively, indicating that the detergent did not significantly ($P > 0.05$) affect bacterial cultivability. No legionella CFU were detected in the control wells with unlysed amoeba cells at 0 and 48 h postinfection. For each experiment, infected amoeba cells were sorted into 312 wells containing 100 μl of 0.02% Triton-X and, as stated previously, 175 μl of ftH_2O was immediately added to all wells after sorting in order to dilute the Triton-X from 0.02% to <0.01%. Table 1 shows that *L. pneumophila* B2 incubation in 0.02% Triton-X for up to 4 min results in no loss of

Received 31 October 2011 **Accepted** 29 December 2011
Published ahead of print 6 January 2012
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doi:10.1128/AEM.07392-11

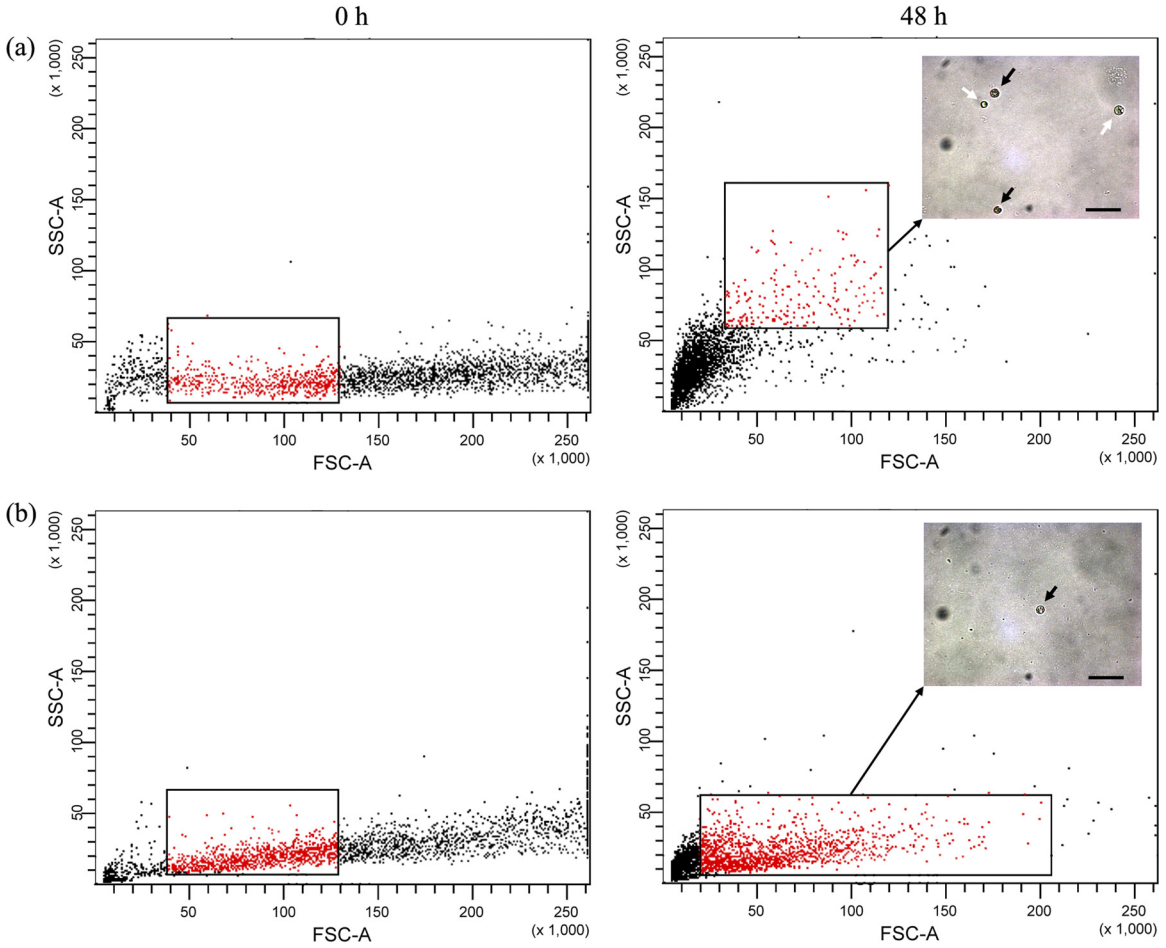

FIG 1 FSC and SSC profiles of *L. pneumophila*-infected amoeba trophozoites. Plots are representative of three independent experiments. *A. polyphaga*-infected cells (a) and *N. fowleri*-infected cells (b) at 0 and 48 h after legionella infection. Individual amoeba cells were sorted from a single region (indicated in red) for *L. pneumophila* CFU enumeration based on forward scatter (FSC-A) and side scatter (SSC-A). Images in insets are representative of sorted cells within the gated region and microscopically verified (magnification, ×400) for intracellular infection at 48 h. Infected cells and uninfected cysts/trophozoites are indicated by black and white arrows, respectively. Black bars, 50 μm.

legionella cultivability. The process of cell sorting into one 96-well plate required up to 3 min, including the time needed to dilute the Triton-X from 0.02% to <0.01%, thus having no effect on the cultivability of the released *L. pneumophila* B2 strain. A 10-μl aliquot from each well was plated onto buffered charcoal yeast extract (BCYE) agar plates (BD Diagnostics). The theoretical limit of detection (LOD) was 28 legionella CFU well^{-1} or per single infected amoeba. Negative controls consisted of wells containing noninfected amoeba cells sorted into wells containing ftH_2O.

Cell sorting was performed to capture *L. pneumophila*-infected cells from forward scatter (FSC) and side scatter (SSC) gated amoebae (Fig. 1). Initially, amoeba cells were infected for 0, 24, 48, and 72 h and collected at each time point for cell sorting. Several gates were then drawn based on their FSC and SSC profiles, and amoeba cells were collected from each gate. The sort regions (Fig. 1, red-gated cells) were chosen based on the criteria of containing both the vast majority of infected amoeba trophozoites and the highest percentage of infected amoebae 48 to 72 h postinfection. The inset in Fig. 1 shows a representative image of cells contained within the gate that were sorted into tubes and verified for intracellular infection with the aid of an inverted Nikon Eclipse T100F microscope 48 h postinfection (no infected cells were observed at 0 h postinfection). For each infected amoeba, maximums of 1,348 and 385 legionella CFU/host cell were determined using *A. polyphaga* and *N. fowleri* hosts, respectively (936 total cells sorted per amoeba host). Thus, it appears that *A. polyphaga* is more susceptible to strain B2 infection and is a better vehicle for legionella amplification than *N. fowleri* ($P < 0.001$) (Table 2). The average numbers of culturable *L. pneumophila* cells estimated within a host were 329 for *A. polyphaga* and 44 for *N. fowleri*.

Legionella cells are reported to be 1 to 3 μm in length and 0.5 to 1 μm in width (1). Assuming that the volume of a *Legionella* cell can be approximated by a sphere of equal volume, the maximum number of bacterial cells that can be contained within a single amoeba was calculated by utilizing the concept of packing density (η), which is defined as the fraction of a volume filled by a given collection of solids, which is, in this case, spheres. Random close packing of spheres in three dimensions gives a packing density of only 0.64 (8). For example, the average widths of *A. polyphaga* and *N. fowleri* trophozoites were measured to be approximately 20 μm (19.49 and 19.30 μm, respectively; data not shown), thus giving an average sphere volume of 4,190 μm^3. Dividing that by

TABLE 2 *L. pneumophila* growth ranges within single amoeba trophozoites[a]

Amoeba host	Infection temp (°C)	No. of *Legionella* CFU/host cell				% of hosts[b]:	
		Mean	Median	Maximum	SD	Infected	< LOD[c]
A. polyphaga	32	329[d]	248	1,348	±285.0	52	48
N. fowleri	30	44	28	385	±46.1	9	91

[a] Data are from 3 independent experiments. Statistical significance was determined using the unpaired, two-tailed Student *t* test, analysis of variance for multiple group comparisons using the Student-Newman-Keuls posttest, and Fisher's exact test.
[b] A total of 936 individual amoeba trophozoites were sorted.
[c] < LOD, below the limit of detection of 28 legionella CFU per host cell.
[d] $P < 0.001$.

the volume of an average-sized *Legionella* cell (2 μm in length, 0.75 μm in width) and multiplying by the random close packing density gives an estimate of 1,140 average-sized *Legionella* cells that may be packed into an average-sized *A. polyphaga* or *N. fowleri* trophozoite. In this study, the recorded maximum numbers of legionellae that were observed for infected *A. polyphaga* trophozoites (1,348 CFU amoeba^{-1}) and *N. fowleri* trophozoites (385 CFU amoeba^{-1}) are therefore plausible.

The data from this study detail the maximal potential release of *L. pneumophila* from a single infected trophozoite under conditions that may be representative of drinking water systems. A previous guinea pig aerosol infection model revealed that the median infectious dose of *L. pneumophila* was <129 bacteria while the 50% lethal dose (LD_{50}) was approximately 10^5 bacteria (3), indicating that, according to this study, as few as 1 to 75 infected amoebae within aerosols may contain enough pathogenic legionellae to cause health effects. However, significantly more are likely to be necessary, given the low expected efficacy in producing respirable aerosols containing legionellae from a shower or similar drinking water source (10). Future studies will aim to quantify both intracellular growth for a range of *L. pneumophila* strains within more ecologically relevant free-living amoebae isolated from drinking water biofilms and the density of free-living amoebae within those systems.

ACKNOWLEDGMENTS

We thank Jay Garland and Michael Ware for their critical review of the manuscript and Michael Ware for his expertise and assistance with the BD FACSAria II flow cytometer.

The United States Environmental Protection Agency's Office of Research and Development reviewed and approved this work for publication.

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