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Legionella-containing structures produced by *Acanthamoeba castellanii* - the influence of environmental factors

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Background

Intracellular replication of legionellae has been observed in various amoebae and there are indications of that in ciliates. During interaction between the protozoa and legionellae, structures such as food pellets and digestive vesicles containing legionellae can be formed. These "legionella-containing structures" (LCS) are generally around 5 micrometer in diameter or less, i.e. respirable size. As LCS can contain hundreds of viable legionellae, they could infect humans more efficiently than a single bacterium would; thus being of clinical importance. However, the conditions leading to the formation LCS are poorly understood. This study is aimed to elucidate this problem.

Methods

We performed a series of co-culture experiments testing the effects of different incubation temperatures and multiplicity of infection (MOI) on the LCS production for two strains of *Legionella pneumophila*, "Paris" and "Corby", and *Acanthameoba castellanii*. The infection efficiency of the strains was also followed and compared between the strains that had been pre-passed through *A. castellanii* 2 times (="A.c.-passed"), and "fresh" strains without former interaction with the amoeba.

- Organisms: green fluorescent protein-expressing *Legionella pneumophila* serogroup 1 strains "Corby" or *L. p.* "Paris" + *Acanthamoeba castellanii* (ATCC 30234)
- **Experiment 1:** T = 23 C (RT), 30 C, 37 C, MOI 10:1, and 100:1 (bacteria to cell ratio); 4 days follow-up: inverse microscopy of microplates
- **Experiment 2:** T = 30 C, growth medium 1:1, 1:3, and 1:10 PYG:PBS, 4 days follow-up with microplate microscopy
- **Experiment 3:** T = RT, MOI 10:1, also *L. p.* strains passed through *A. c.*, 11 days follow-up: microplates microscopy, amoebas counted with cell counting chamber, viable bacteria plated

Results and conclusions

Experiment 1: In general, the higher the incubation temperature was, the quicker was the initial infection of *A. castellanii* cells and consequent LCS formation. The increase in MOI heightened this effect. However, at the combined higher temperatures and high MOI amoebae were quickly lysed in case of the *L. p.* strain "Corby", resulting in limited numbers of LCS. The largest amounts of LCS were produced at RT for "Corby" and at 30 C for "Paris". However, these results were obtained by general viewing of the whole sample without attempting to quantify the actual LCS concentrations. To better evaluate the risk, more accurate method needs to be used.

Table 1. Observances of vesicles and other LCS from Experiment 1 in co-cultures of *Acanthamoeba castellanii* (Ac) *and Legionella pneumophila* (Lp) at different incubation temperatures and MOIs. Darker colour denotes more LCS.

Sample	Day 1	Day 2	Day 3	Day 4
Corby RT MOI 10:1	1-2 Lp /amoeba	50+ Lp in vacuoles, some cells full, Ac rounded	Some largish vesicles	Plenty of largish vesicles Ac mostly cysts
Corby 30 °C MOI 10:1	5-10 Lp /amoeba	A few vesicles, amoebal debris	Am debris, no fluorescence	Am debris, no fluorescence
Corby 37 °C MOI 10:1	Ac cells full with Lp	A few vesicles very few Ac	Some vesicles No Ac	Some vesicles No Ac
Corby RT MOI 100:1	50+ Lp in vacuoles, Ac mostly cysts	A few vesicles Ac cells full with Lp	Plenty of largish vesicles No Ac	Plenty of largish vesicles No Ac
Corby 30 °C MOI 100:1	A few vesicles Ac cells full with Lp	Some big vesicles No Ac	A few small vesicles No Ac	No vesicles No Ac
Paris RT MOI 10:1	Only some Ac infected 5-10 Lp /amoeba	Not all Ac infected 5-10 Lp /amoeba	Most Ac infected 10+ Lp /amoeba	Most Ac infected 10+ Lp /amoeba
Paris 30 °C MOI 10:1	Only some Ac infected 5-10 Lp /amoeba	A few big vesicles Only some Ac infected 5-10 Lp /amoeba some full	Some big vesicles Only some Ac infected 5-10 Lp /amoeba some full	Some small vesicles Most Ac infected 10+ Lp /amoeba some full
Paris 37 °C MOI 10:1	½ Ac infected, some with 1-2 Lp, some full	Most Ac infected ½ full, ½ with a few Lp	A few vesicles Some Ac	No vesicles No Ac
Paris RT MOI 100:1	Only some Ac infected 1-2 Lp /amoeba	Most Ac infected 50+ Lp in vacuoles, some full	A few vesicles Plenty of small clusters of Lp	Some vesicles
Paris 30 °C MOI 100:1	Only some Ac infected 50+ Lp in vacuoles	A few small vesicles Most Ac infected	Plenty of vesicles Plenty of Ac rounded but with many vacuoles	Plenty of vesicles Plenty of small clusters of Lp Some Ac

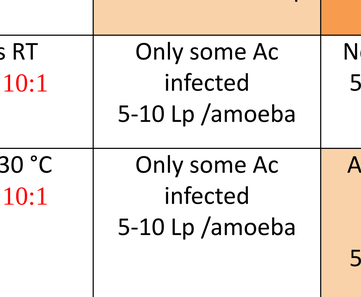

Figure 1. An example of LCS produced (arrow). *Legionella pneumophila* "Paris" (fluorescent green) and *Acanthamoeba castellanii*, day 3, 30 C, MOI 10

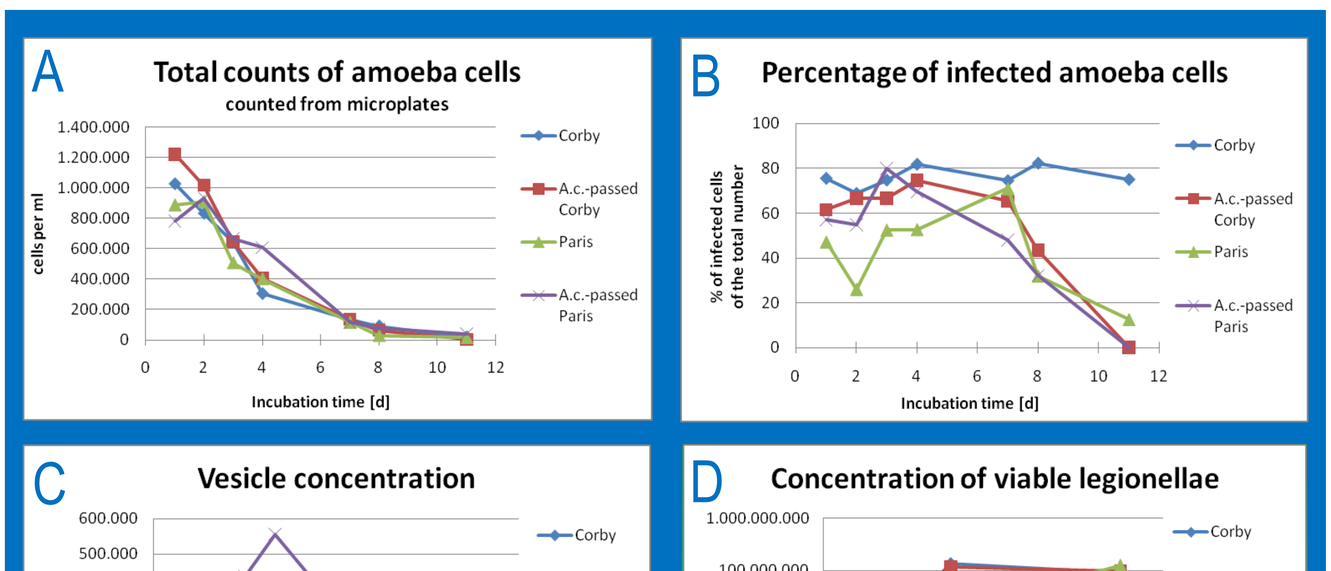

Figure 2. Results from Experiment 3. A, B and C *A. castellanii* cell and vesicle counts and percentage of *L. pneumophila* "Corby" or "Paris"-infected cells determined with direct counting from microplates under microscope with ocular counting grid; D concentrations of viable legionellae at the co-culture solution determined by plating; E and F *A. castellanii* cell counts and percentage of dead cells determined with a Fuchs-Rosenthal cell counting chamber and Trypan-blue exclusion.

Experiment 2: The nutrient content of the growth medium did not visibly affect the LCS production in *L. p.* -infected amoebae. A quick overgrowth of legionellae was observed in the less diluted PYG medium, whereas amoebae withdrew to cysts sooner in the most diluted medium. Dilution of 1:3 PYG:PBS was deemed most suitable compromise for co-culture studies between the selected strains.

Experiment 3: In this experiment we aimed for better quantitation of the vesicles, unfortunately with no great success. No significant differences were observed in the LCS production between the different strains with the method used. However, counting vesicles or unattached cells directly from the microplate turned out to be virtually impossible with any accuracy as they constantly moved in the solution. With a cell counting chamber, the determination was more repeatable. However, mechanically removing the attached cells from the microplate may disrupt the fragile infected cells leading in underestimation in numbers. Furthermore, it is in practice difficult to draw a distinguishing line between a LCS and an infected cell, as the structure sizes are in a continuum and the structural differences are not well visible under light microscope. Currently, we are testing different staining and fixing methods to overcome these problems.

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