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Permissivity of fish cell lines to three *Chlamydia*-related bacteria : *Waddlia chondrophila, Estrella lausannensis* and *Parachlamydia acanthamoebae*.

Carole Kebbi-Beghdadi[1], Claudia Batista[1] and Gilbert Greub[1*].

[1] Center for Research on Intracellular Bacteria (CRIB), Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland, [2]

Corresponding author:

Dr Gilbert Greub

Institute of Microbiology

Rue du Bugnon 48

1011 Lausanne

Switzerland

Tel: 0041 21 314 4979

Fax 0041 21 314 4060

e-mail: gilbert.greub@chuv.ch

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Abstract

Epitheliocystis is an infectious disease affecting gills and skin of various freshwater and marine fishes, associated with high mortality and reduced growth of survivors. Candidatus *Piscichlamydia salmonis* and *Clavochlamydia salmonicola* have recently been identified as etiologic agents of epitheliocystis in Atlantic Salmon. In addition, several other members of the *Chlamydiales* order have been identified in other fish species.

To clarify the pathogenicity of *Chlamydia*-like organisms towards fishes, we investigated the permissivity of two fish cell lines, EPC175 (Fathead Minnow) and RTG-2 (Rainbow Trout) to three strict *Chlamydia*-related bacteria: *Waddlia chondrophila*, *Parachlamydia acanthamoebae* and *Estrella lausannensis*. Quantitative PCR and immunofluorescence demonstrated that *Waddlia chondrophila* and, to a lesser extent, *Estrella lausannensis* were able to replicate in the two cell lines tested. *W. chondrophila* multiplied rapidly in its host cell and a strong cytopathic effect was observed. During *E. lausannensis* infection, we observed a limited replication of the bacteria not followed by host cell lysis. Very limited replication of *Parachlamydia acanthamoebae* was observed in both cell lines tested.

Given its high infectivity and cytopathic effect towards fish cell lines, *W. chondrophila* represents the most interesting *Chlamydia*-related bacteria to be used to develop an *in vivo* model of epitheliocystis disease in fishes.

Introduction

Epitheliocystis is a common infection in many fish species affecting both gill and skin epithelium and characterized by the presence of hypertrophied cells containing granular inclusions. It has been described in over 50 species of fishes from marine as well as freshwater environment and both in cultured and wild fishes, its prevalence being however greater in cultured fishes (Nowak & LaPatra, 2006). The mortality associated with this disease ranges from 4% to 100% and occurs mainly in the early life stages of cultured fishes (Nowak & LaPatra, 2006). Based on ultrastructural observations and antigenic evidence, the causative agent of epitheliocystis was thought to be a member of the *Chlamydiales* order, which comprises gram-negative bacteria exhibiting a strict intracellular life cycle and sharing a common biphasic developmental cycle including an infectious, metabolically inactive elementary body (EB) that differentiates into a metabolically active reticulate body (RB), dividing by binary fission (Moulder, 1991, Groff*, et al.*, 1996).

Draghi *et al.* reported in 2004 the first identification by molecular techniques (amplification of 16S ribosomal RNA) of the agent of epitheliocystis in Atlantic salmon (*Salmo salar*) (Draghi*, et al.*, 2004). Their phylogenetic analysis showed that this bacteria, named "*Candidatus* Piscichlamydia salmonis", branched as a new family-level lineage in the *Chlamydiales* order, an order that includes 6 other family-level lineages: the *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae*, *Criblamydiaceae* and *Rhabdochlamydiaceae* (Greub, 2009, Greub, 2010). More recently, the same authors also identified Candidatus *Piscichlamydia salmonis* in Arctic charr (*Salvelinus alpinus*) (Draghi*, et al.*, 2010). In the last 5 years, based on partial 16S rRNA gene sequences, the epitheliocystis agents of several fish species have been identified as belonging to the *Chlamydiales* order but being

phylogenetically distinct (Meijer, *et al.*, 2006, Draghi, *et al.*, 2007, Karlsen, *et al.*, 2008, Mitchell, *et al.*, 2010, Polkinghorne, *et al.*, 2010). These results suggest that the causative agent of epitheliocystis is a group of intracellular bacteria that are genetically diverse from each other and spread throughout the *Chlamydiales* order. Bacteria belonging to this order have been found to infect a wide range of terrestrial animals including mammals, marsupials, reptiles and insects (reviewed in (Horn, 2008) and several of them, particularly those belonging to the *Chlamydiaceae* family, are well-known human and animal pathogens (Everett, 2000, Hahn, *et al.*, 2002, Baud, *et al.*, 2008). In addition, some members of the *Parachlamydiaceae, Simkaniaceae* and *Waddliaceae* families are currently also considered as possible pathogenic bacteria for humans and animals (Greub & Raoult, 2002a, Dilbeck-Robertson, *et al.*, 2003, Friedman, *et al.*, 2006, Baud, *et al.*, 2007, Borel, *et al.*, 2007, Baud, *et al.*, 2009, Goy, *et al.*, 2009, Deuchande, *et al.*, 2010, Baud, *et al.*, 2011). In order to further study the pathogenesis of the *Chlamydia*-related bacteria involved in epitheliocystis, it would be valuable to establish experimental *in vitro* and *in vivo* fish models of infection. For this purpose and since none of the epitheliocystis agents identified so far could be isolated from its fish host and cultivated *in vitro* (Meijer, et al., 2006), we chose three *Chlamydia*-related bacteria, representatives of the *Waddliaceae, Criblamydiaceae* and *Parachlamydiaceae* families, to define their ability to enter and multiply in two permanent fish cell lines. More precisely, we studied the permissivity of EPC175 (epithelial cells derived from skin of Fathead Minnow) and RTG-2 (fibroblast cells derived from gonad of Rainbow Trout) towards *W. chondrophila* (*Waddliaceae* family), *E. lausannensis* (*Criblamydiaceae* family) and *P. acanthamoebae* (*Parachlamydiaceae* family) using specific quantitative PCRs

developed in our group (Casson*, et al.*, 2008, Goy*, et al.*, 2009, Lienard*, et al.*, 2011) and in-house polyclonal antibodies.

Material and methods

Cell culture and bacterial strains

Epithelioma papulosum cyprini cells (EPC-175) originally form the common carp (Fijan*, et al.*, 1983) but recently shown to be contaminated with fathead minnow (*Pimephales promelas*) skin cells (Winton*, et al.*, 2010) and rainbow trout (*Oncorhynchus mykiss*) gonad cells (RTG-2) (Wolf & Quimby, 1962) were obtained from Prof. H. Segner (Bern, Switzerland) and routinely maintained at 25°C in minimal essential medium (MEM; Gibco Invitrogen, Basel Switzerland) supplemented with 10% foetal calf serum (Biochrom, Berlin, Germany), 1% non essential amino acids (Biochrom) and 1% Hepes (BioConcept, Allschwil, Switzerland).

W. chondrophila strain WSU 86-1044 (ATCC VR-1470), *E. lausannensis* strain CRIB 30 and *P. acanthamoebae* strain Hall's coccus were grown at 32°C within *Acanthamoeba castellanii* strain ATCC 30010 in 25 cm^2 cell culture flasks (Corning, New York, USA) with 10 ml of peptone-yeast extract-glucose broth as described elsewhere (Greub & Raoult, 2002b). After 5 days, cultures were harvested and filtered through a 5 µm filter (Millipore, Carrigtwohill, Ireland) to eliminate trophozoites and cysts. Bacteria recovered from the flow-through were diluted in MEM (*W. chondrophila* 1/1000, *E. lausannensis* 1/300, *P. acanthamoebae* 1/150) and used to infect cells.

Infection procedure

The day before infection, fish cells were harvested from Corning culture flasks with 0.25% trypsin (Sigma), washed with fresh medium and seeded at $0.5x10^6$ cells per well in 24-wells microplates (Corning). Cells were infected with living or heat-inactivated (1 hour at 95°C) bacteria diluted in MEM. The dilutions used (see above) represent an MOI of 0.1-1 (MOI was estimated by counting under an epifluorescent microscope (see below) the number of bacteria and the number of cells and by dividing the first number by the second). Plates were then centrifuged at 1790 x g for 10 min at room temperature. After 15 min of incubation at 25°C, cells were washed with fresh medium to remove non-internalized bacteria and were then incubated for different periods of time at 25°C.

Confocal microscopy

At different time points, infected cells cultivated on glass coverslips were fixed with ice-cold methanol for 5 min, washed 3 times with PBS and then blocked and permeabilized in block solution (PBS, 0.1% saponin, 0.04% NaN3, 10% FCS) at 4°C. Coverslips were incubated 1 hour at room temperature with in-house polyclonal rabbit anti-*W. chondrophila* (dilution 1/1000), rabbit anti-*P. acanthamoebae* (dilution 1/200) or mouse anti-*E. lausannensis* (dilution 1/1000) antibodies diluted in PBS, 0.1% saponin, 1% BSA. After 3 washings in PBS 0.1% saponin, coverslips were again incubated 1 hour at room temperature with a 1/1000 dilution of AlexaFluor 488-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Invitrogen, Basel, Switzerland), a 1/10 dilution of Concanavalin A-Texas Red conjugate (Invitrogen, Basel, Switzerland) and 150 ng/ml DAPI (dilactate, Molecular Probes, Invitrogen, Basel, Switzerland) in PBS 0.1% saponin, 1% BSA. After washing twice with PBS

0.1% saponin, once with PBS and once with deionised water, the coverslips were mounted onto glass slides using Mowiol (Sigma-Aldrich, Buchs, Switzerland). Cells were observed under an epifluorescent microscope (Axioplan 2, Zeiss, Feldbach, Switzerland) and a confocal microscope (AxioPlan 2 LSM 510, Zeiss).

Electron microscopy

RTG-2 cells were infected with *W. chondrophila* diluted 1/500, as described above in 25 cm^2 flasks and harvested 48 hours post-infection. Cells were trypsinized, centrifuged 10 min at 1'500 rpm, washed once in PBS and fixed overnight in 4% glutaraldehyde (Fluka Biochemika, Buchs, Switzerland) at 4°C. After one more washing step with PBS, cells were prepared as described previously (Casson*, et al.*, 2006). Thin sections on grids were examined with a transmission electron microscope Philips CM 100 (Philips, Eindhoven, The Netherlands).

Quantitative PCR

At different time points after infection, cells were harvested in 1 ml medium. Genomic DNA was extracted from 100 µl of the above harvested cells using the Wizard SV Genomic DNA Purification System (Promega, Madison, USA) and eluted from the column in 250 µl volumes, according to the manufacturer's instructions. Quantitative PCR was performed using iTaq supermix with ROX (BioRad) and 200 nM of forward primer (WadF4 5'-GGCCCTTGGGTCGTAAAGTTCT-3'; EstF 5'-ACACGTGCTACAATGGCCGGT-3'; PacF2 5'-GGATGAGGCATGCAAGTCGAACGAA-3'), 200 nM of reverse primer (WadR4 5'-CGGAGTTAGCCGGTGCTTCT-3'; EstR 5'-CCGGGAACGTATTCACGGCGTT-3'; PacR2 5'-AGGTCTTGCGATCCCCCACTTTGA-3'), 100 nM of probe (WadS2 5'-FAM-

C**A**TGGGAA**C**AAGAGAAG**GAT**g-BHQ1-3';

EstS 5'-FAM-CAGC**C**A**AC**GTGAGG**G**-BHQ1-3' ; PacS2 5'-FAM-CGGGCAACCGTTTAGTGGCGGAAGGG-BHQ1-3') and 5 µl of DNA sample (Casson*, et al.*, 2008, Goy*, et al.*, 2009, Lienard*, et al.*, 2011). Cycling conditions were 95°C, 3 min followed by 40 cycles of 95°C, 15 sec; 60°C,1 min. Amplification and detection of PCR products were performed with StepOne Plus Real-time PCR System (Applied Biosystems).

Cell viability

Cell viability was determined by a Trypan Blue exclusion assay at 0h, 48h, 72h and 144 h post infection. Ten µl of cell suspension were mixed with an equal volume of Trypan blue (Gibco, Grand Island, NY) and stained 5 min at room temperature. Cells were counted in KOVA cell chamber system (Hycor, California, USA) and the number of living (unstained) and dead (stained) cells was determined in duplicate.

Results

EPC-175 and RTG-2 cells were infected with *W. chondrophila*, *E. lausannensis* or *P. acanthamoebae* and bacterial growth was monitored at different time points after infection. The number of bacterial genomic DNA copies was measured with specific quantitative PCRs (qPCRs) that had been developed in our laboratory (Casson*, et al.*, 2008, Goy*, et al.*, 2009, Lienard*, et al.*, 2011). Bacterial replication was simultaneously assessed by immunofluorescence and confocal microscopy using in-house polyclonal antibodies. For the three *Chlamydia*-related bacteria tested, the growth kinetics were very similar in EPC-175 and in RTG-2 cells. The level of bacterial replication, however, varied considerably depending on the organism

analysed. As expected, no multiplication of heat-inactivated bacteria was observed for either the organism or the cell line tested.

Growth kinetic of *W. chondrophila*

Results obtained by qPCR revealed a rapid multiplication phase, lasting about 48 hours, during which the number of bacteria increased by more than 3 logs (Fig. 1, panel a). Furthermore, confocal microscopy observations indicated that after the first round of replication, host cells were lysed and elementary bodies (EBs) were released in the medium triggering a new infection cycle (Fig. 2A). Six days post infection, most of the host cells were lysed and no more bacterial replication could be detected by qPCR.

Electron micrographs presented in Fig. 2B confirmed that 48 hours post infection, infected cells are filled with very large vacuoles containing dividing reticulate bodies (RBs) as well as re-differentiated elementary bodies (EBs) ready to be released in the extracellular medium.

Growth kinetic of *E. lausannensis*

The growth kinetic of *E. lausannensis* revealed a limited multiplication phase lasting about 48 hours during which the number of genomic DNA copies increased by approximately 1 log (Fig. 1, panel b). After 48 hours, no more bacterial replication could be detected by qPCR and the number of genomic DNA copies remained constant. Furthermore, as revealed by direct observation of the infected cells under confocal microscopy (Fig. 3) there was no lytic effect of the bacteria on the host cells and only little re-infection of new host cells. This absence of cytopathic effect was

confirmed by measuring the cell viability in a Trypan blue assay and demonstrating that percentage of viable cells only slightly decreased from 100% to 90% during the course of infection, a decrease that was also observed with heat-inactivated bacteria and which could be due to a lack of nutrients, since the culture medium was not replaced in these experiments.

Growth kinetic of *P. acanthamoebae*

The *P. acanthamoebae* growth curve assessed by quantitative PCR (Fig. 1, panel c) indicated a slight increase in the number of bacterial DNA copies (<1 log) in both cell lines. Moreover, during the first 48 hours after infection, we could observe by confocal microscopy inclusions containing one reticulate body or rarely a cluster of these metabolically active forms, suggesting that although replication is very limited, *P. acanthamoebae* may differentiate from elementary bodies to reticulate bodies after entry in fish cells.

Discussion

In this work, we demonstrated that two different members of the *Chlamydiales* order, *W. chondrophila* and *E. lausannensis* are able to enter and multiply in EPC-175 and RTG-2, two fish cell lines of different origins. Previous results obtained by our group and others have established that *W. chondrophila* and *E. lausannensis* are able to rapidly grow in various mammalian cell lines and strains of amoebae suggesting a broad host range for these two organisms which, in addition, are able to multiply at different temperatures ranging from 20°C to 37°C (Kocan*, et al.*, 1990, Henning*, et al.*, 2002, Goy*, et al.*, 2008, Kebbi-Beghdadi*, et al.*, 2011, Lienard*, et al.*, 2011). The

multiplication of *E. lausannensis* in the two cell lines tested was limited when compared to the exponential growth of *W. chondrophila*, a restricted replication that could be due to nutrient deficiency or to decreased fitness of the host cells in a system in which the culture medium was not replaced during the course of the experiment. Indeed, as revealed by genome analysis, *W. chondrophila* possess larger capabilities than *E. lausannensis* to synthesize *de novo* nucleotides, amino acids, lipids and co-factors and may therefore be less dependent on its host cell (Bertelli*, et al.*, 2010), Bertelli, *et al.,* unpublished).

P. acanthamoebae was shown to enter and multiply within human lung fibroblasts, pneumocytes and macrophages, but the level of replication was much lower in these cell lines than in amoebae which are their natural hosts (Greub*, et al.*, 2003, Greub*, et al.*, 2003, Greub*, et al.*, 2005, Casson*, et al.*, 2006, Hayashi*, et al.*, 2010, Roger*, et al.*, 2010). In the two fish cell lines used in the present study, *P. acanthamoebae* was able to enter the host cell and, in some cases, to differentiate from EBs to RBs as indicated by DAPI staining, which revealed the presence of bacteria containing decondensed nucleus, a feature characteristic of RBs (Croxatto & Greub, 2010). In some rare cases, RBs started to replicate and inclusions containing clusters of reticulate bodies could be observed during the first 48 hours post infection. However, at later time points, these replicating bacteria could not be seen, suggesting that the replication cycle had aborted. This very limited replication could explain the slight increase in the number of bacteria detected by qPCR during the first 48 hours following infection. However, minimal differences in the number of genomic DNA copies detected by qPCR could also be due to DNA extraction artifact. Indeed, we observed that DNA yield upon extraction may be lower with EBs than with RBs because of differences in their membrane composition (unpublished results). The

membrane of EBs presents highly disulfide-linked proteins that confer rigidity and stability to the cell wall and that are reduced during differentiation to RBs (Hatch, 1996, McCoy & Maurelli, 2006).

Various members of the *Chlamydiales* order are causative agents of epitheliocystis, but since the biology of these micro-organisms remains largely unknown, strategies to deal with epitheliocystis are limited to quarantining diseased fishes. In these conditions, it would be of valuable interest to have an *in vivo* fish model of infection by *Chlamydia*-related epitheliocystis agents. The development of a zebrafish model would be of peculiar interest since several genetic approaches, such as random mutagenesis or gene knockout, are available for this model organism and may be used to investigate the host determinants of the disease. In addition, the susceptibility of zebrafish embryos to chlamydial infection could also be explored during larval stages, taking advantage of the sequential staged development of the innate and adaptive immune system in this organism. Finally, such an *in vivo* model could be used to test the efficiency of preventive or therapeutic measures to control the disease. In the present study, we demonstrated that *W. chondrophila* is able to enter and very efficiently replicate in two different fish cell lines. This bacteria would thus be a good candidate to infect fishes *in vivo* and develop an animal model of epitheliocystis. Alternatively, *W. chondrophila* and *E. lausannensis,* that both replicate in EPC-175 and RTG-2, could be used *in vitro* to further study host-pathogen interactions.

Several members of the *Chlamydiaceae* family such as *C. psittaci* or *C. abortus* are known to cause zoonotic diseases (Longbottom & Coulter, 2003). In addition, seropositivity for *W. chondrophila*, *P. acanthamoebae* or *C. sequanensis* is associated with animal contacts which suggests a possible zoonotic potential for

these *Chlamydia*-related bacteria as well (Baud*, et al.*, 2007, Baud*, et al.*, 2009). Furthermore, the organisms discovered in epitheliocystis of Arctic charr from Virginia (USA) (Draghi*, et al.*, 2007) were partly identical to *Chlamydiales* previously identified in cat conjunctivitis (von Bomhard*, et al.*, 2003), which seriously raises the question of zoonotic transmission of these epitheliocystis agents. Further investigations are now required to define the possible role of fish species as reservoirs for emerging human or animal pathogens as well as the zoonotic potential of these newly described bacteria.

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Figure legends

Fig. 1 Bacterial growth within fish cells

Replication of *Waddlia chondrophila* (panel a), *Estrella lausannensis* (panel b) and *Parachlamydia acanthamoebae* (panel c) in fish cell lines. Bacterial replication is measured by quantitative PCR over 144 hours following infection of EPC-175 (black triangles) and RTG-2 cells (grey circles) either with living bacteria (plain lines) or with heat-inactivated bacteria (dashed lines)**.** The results are the mean +/- SEM of duplicates of one representative experiment.

Fig. 2 *W. chondrophila* replication within RTG-2 cells.

A Growth of *W. chondrophila* in RTG-2 cells assessed by immunofluorescence and confocal microscopy at different time points following infection. Bacteria were stained green with a polyclonal rabbit anti-*Waddlia* antibody and fish cells were stained red with Texas Red-conjugated Concanavalin A.

B Electron micrographs of RTG-2 cells infected with *W. chondrophila* 48 hours post-infection showing a very large inclusion containing RBs, some of them dividing by binary fission (white arrows) and EBs.

Fig. 3 *E. lausannensis* replication within RTG-2 cells.

Growth of *E. lausannensis* in RTG-2 cells revealed by confocal microscopy observation of cells at different time points after infection. Bacteria (green) were stained with a polyclonal mouse anti-*Estrella* antibody and fish cells (red) were stained with Texas Red-conjugated Concanavalin A.

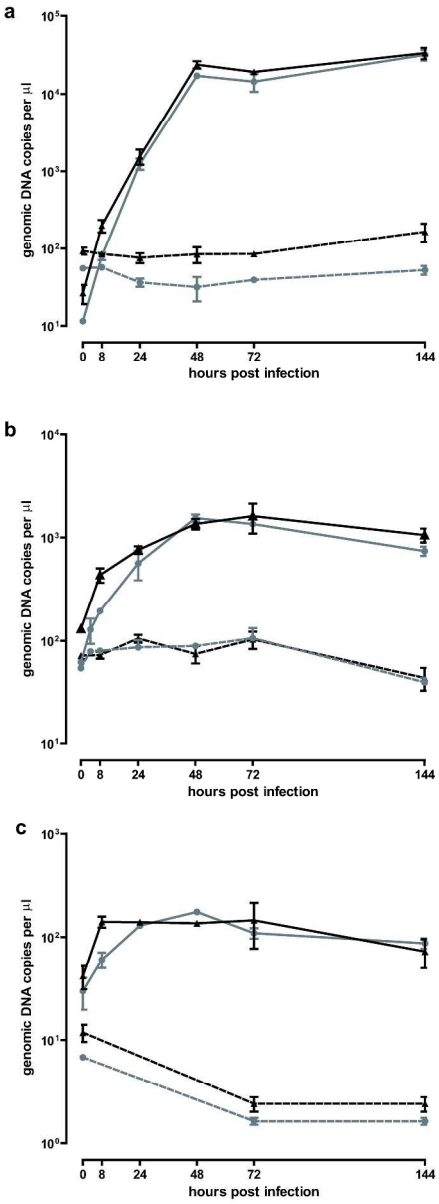

Figure 1

Bacterial growth within fish cells
Replication of Waddlia chondrophila (panel a), Estrella lausannensis (panel b) and Parachlamydia acanthamoebae (panel c) in fish cell lines. Bacterial replication is measured by quantitative PCR over 144 hours following infection of EPC-175 (black triangles) and RTG-2 cells (grey circles) either with living bacteria (plain lines) or with heat-inactivated bacteria (dashed lines). The results are the mean +/- SEM of duplicates of one representative experiment.

287x419mm (300 x 300 DPI)

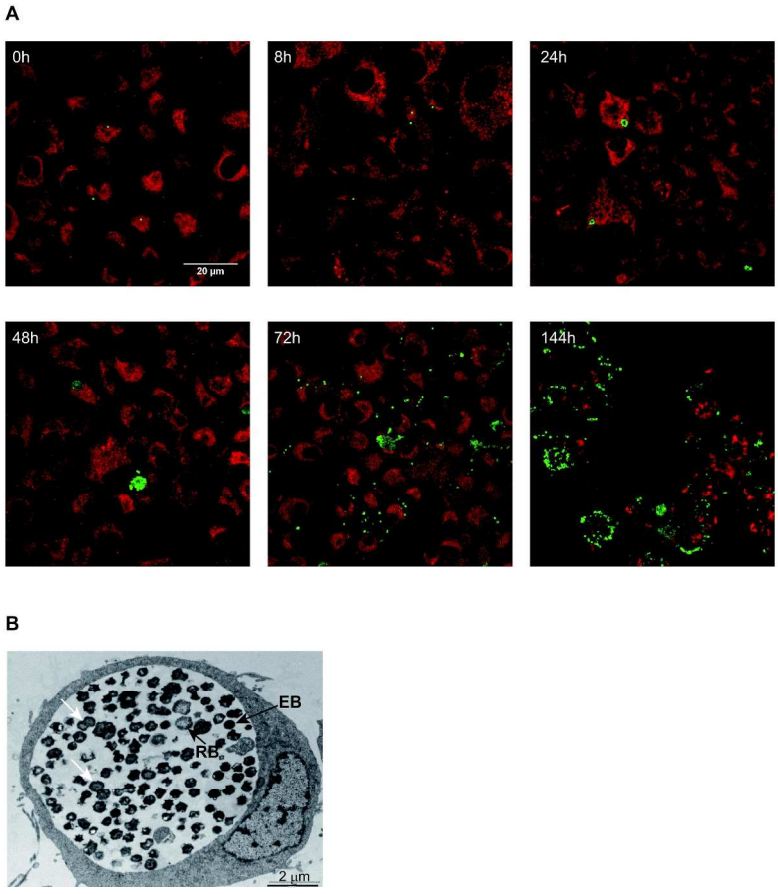

Figure 2

W. chondrophila replication within RTG-2 cells.
A Growth of W. chondrophila in RTG-2 cells assessed by immunofluorescence and confocal microscopy at different time points following infection. Bacteria were stained green with a polyclonal rabbit anti-Waddlia antibody and fish cells were stained red with Texas Red-conjugated Concanavalin A.
B Electron micrographs of RTG-2 cells infected with W. chondrophila 48 hours post-infection showing a very large inclusion containing RBs, some of them dividing by binary fission (white arrows) and EBs.

274x296mm (300 x 300 DPI)

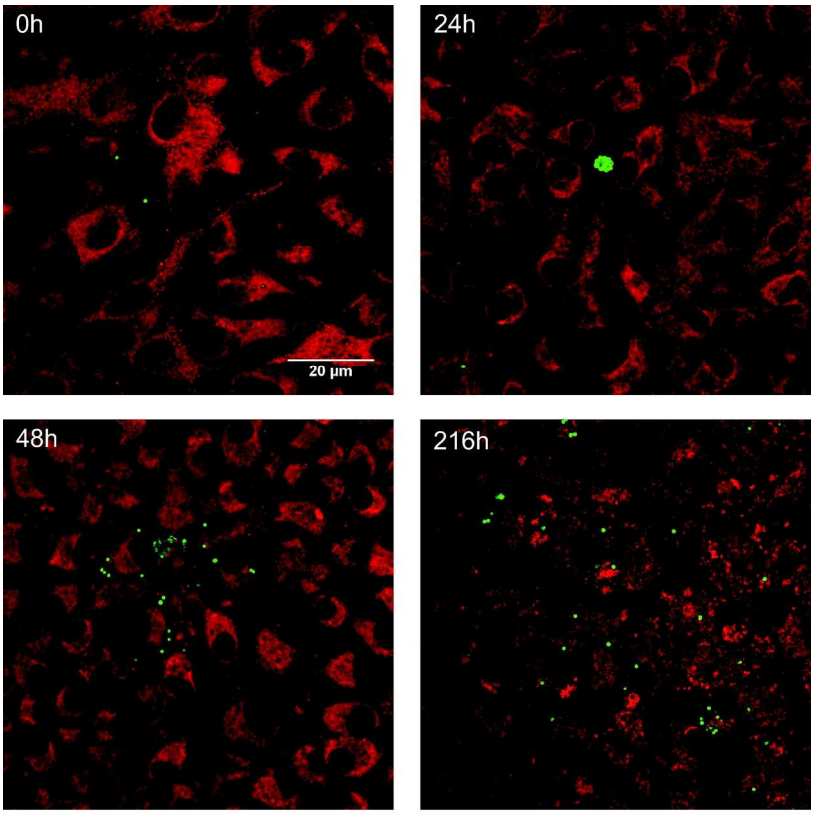

Figure 3

E. lausannensis replication within RTG-2 cells.
Growth of E. lausannensis in RTG-2 cells revealed by confocal microscopy observation of cells at different time points after infection. Bacteria (green) were stained with a polyclonal mouse anti-Estrella antibody and fish cells (red) were stained with Texas Red-conjugated Concanavalin A.

190x284mm (300 x 300 DPI)