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Short Communication

Effect of *Piscirickettsia salmonis* inoculation on the ASK continuous cell line

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Piscirickettsia salmonis (*P. salmonis*) is a Gram-negative aquatic pathogen that causes piscirickettsiosis, a contagious systemic disease which was first described in coho salmon *Oncorhynchus kisutch* (Walbaum) cultured in sea net pens (Fryer *et al.* 1990). Since then, this condition has been detected in a variety of teleost fish from distant geographical regions in the world, but so far it has been consistently more severe in salmonid species reared in the South Pacific Ocean in Chile (Arkush & Bartholomew 2011; Rojas *et al.* 2013).

First isolation of *P. salmonis* was in the chinook salmon, *O. tshawytscha* (Walbaum), embryo (CHSE-214) cell line (Fryer *et al.* 1990), and these cells were since then the most extensively used substrate for this bacterium propagation (Arkush & Bartholomew 2011; Rojas *et al.* 2013). For some years, it was thought that *P. salmonis* could replicate *in vitro* only in cell cultures, but rather recently, it was found that it can also be grown in some enriched artificial media (Mauel, Ware & Smith 2008; Mikalsen *et al.* 2008), and therefore at present, it is characterized as a facultative, instead of an obligated, intracellular organism. Although several fish cell lines in which *P. salmonis* replicates have been described, no attempts have been made to know whether the Atlantic salmon, *Salmo salar* L., kidney (ASK) cell line developed by Devold *et al.* (2000) is permissive to this bacterium. The objective of this work was to determine the effect of *P. salmonis* inoculation on ASK cells through the cytopathic effect (CPE) description and bacterial titration after cell exposure to this fish pathogen.

Monolayers of the ASK (ATCC CRL 2797) and the CHSE-214 (ATCC CRL 1681) cell lines, both cultured at 18 °C in absence of antimicrobials, were used. The ASK cells were cultured with L-15 Leivobitz medium with L-glutamine (2.05 mM) and supplemented with β mercaptoethanol (38.5 μM) and foetal calf serum (FCS) at 10% (all from Gibco, Life Technologies, Carlsbad, CA). The CHSE-214 cells were grown in Eagle′s minimal essential medium with Earle′s salts (Automod Sigma-Aldrich, Lenexa, KS), L-glutamine (2 mM) and FCS (10%).

An isolate of *P. salmonis* obtained from an Atlantic salmon suffering clinical piscirickettsiosis, sampled at a sea-site of the south of Chile, was used. It was cultured in CHSE-214 cells. Infectious supernatant was harvested when CPE reached 100%, and it was titred by end-point dilution assay using 96-well microplates containing monolayers of CHSE-214 cells, with 6 wells per dilution, employing the method of Reed and Müench (1938) to estimate the tissue culture infectious dose 50% per mL ($TCID_{50}$ mL^{-1}). Finally, the inoculum used to expose the cells in the infectivity assays explained below was a bacterial suspension having $10^{5.7}$ $TCID_{50}$ contained in 0.2 mL.

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In the infectivity assays, which were conducted in duplicate, fresh monolayers of ASK cells cultured in 25-cm^2 flasks were exposed to the *P. salmonis* inoculum and observed daily under inverted microscope for 20 post-inoculation (*p.i.*) days. Concurrently, for comparison, CHSE-214 cells were infected and treated in the same way as the ASK cells. Non-infected ASK and CHSE-214 cells were also included as experimental controls. At the end of the assays, methanol-fixed smears from supernatants of the infected and non-infected cell cultures were obtained. These smears were stained with Gram and Giemsa and also labelled to detect *P. salmonis* by indirect immunofluorescence test (IFAT) according to Lannan, Ewing & Fryer (1991).

Titration of ASK cell supernatants post-*P. salmonis* infection was carried out once CPE approached 100% in these cells, which occurred at 20 *p.i.* days. Supernatants were titrated in microplates containing CHSE-214 cells as previously explained. Titration was also conducted after a second passage of *P. salmonis* in ASK cells which, in turn, were infected with 0.2 mL of supernatant of infected ASK cells with *c.* 100% of CPE.

Monolayers of ASK cells exhibited a conspicuous and distinctive CPE after *P. salmonis* infection. First morphological changes were evident quite early, starting at day 3 *p.i.*, and were characterized by the presence of rounded intracytoplasmatic vacuoles in few cells. As time elapsed, the number of affected cells and the vacuoles in them progressively increased in such a way that in some cells, more than 50 vacuoles of different size and morphology could be counted. The smaller vacuoles were almost circular with a diameter starting from *c.* 1 μm. Some of the larger vacuoles showed the same shape of the smaller ones, but others were more asymmetrical, some of them reaching *c.* 5 μm in their longer axis, as shown in Fig. 1 (b and c). Some vacuoles contained structures consistent with *P. salmonis* morphology, while others looked empty. In time, a progressive ASK cell detachment occurred homogeneously without leaving the discrete large spaces observed in the CPE when *P. salmonis* infects CHSE-214 cells (Fryer *et al.* 1990). The CPE difference between ASK and CHSE-214 cells infected with *P. salmonis* can be seen in illustrations *c* and *d* (Fig. 1) at 5 *p.i.* days and in *e* and *f* (Fig. 1) at 11 *p.i.* days. At day 20 *p.i.*, detachment of the infected ASK cells approached 100%. Detachment was clearly higher in ASK than in CHSE-214 cells at a given time, as shown in pictures *g* and *h* (Fig. 1).

In advanced infection stages, *P. salmonis* compatible particles, extracellularly suspended, were seen with inverted microscope in a higher number and larger size in ASK compared with CHSE-214 cultures. In turn, observation under standard optical microscopy of smears of ASK cells infected with *P. salmonis*, stained with either Gram or Giemsa or IFAT labelled, showed the typical morphological features widely described for this bacterium (Fryer *et al.* 1990; Lannan *et al.* 1991; Fryer & Hedrick 2003; Arkush & Bartholomew 2011).

The replication ability of *P. salmonis* in ASK cell monolayers shown here was expected for at least two reasons. The first one is that these cells originate from Atlantic salmon and it is widely known that salmonid fish are highly susceptible to *P. salmonis* infection in natural and under experimental conditions (Fryer *et al.* 1990; Garcés *et al.* 1991; Fryer & Hedrick 2003; Arkush & Bartholomew 2011). The second reason is that the ASK line is derived from kidney cells (Devold *et al.* 2000) which are one of the main target tissues of *P. salmonis* (Branson & Nieto Díaz-Muñoz 1991; Venegas *et al.* 2004; McCarthy *et al.* 2008). It is worth mentioning that there are 11 cell lines so far reported as permissive to *P. salmonis*, nine of them from teleosts (salmonid and non-salmonid fish), and the remaining two, from an amphibian and an invertebrate species. Salmonid cell lines, besides CHSE-214, are CSE-119 (coho salmon embryo), RTG-2 (rainbow trout gonad), CHH-1 (chum salmon heart) (Fryer *et al.* 1990), RTS11 (rainbow trout spleen) (Rojas *et al.* 2009) and SHK-1 (salmon head kidney) (Vera *et al.* 2012). From non-salmonid fish are EPC (*epithelioma papulosum cyprini*), FHM (fathead minnow) (Fryer *et al.* 1990) and BB (brown bullhead) cells (Almendras *et al.* 1997). The cell lines of amphibian and insect origin are XTC-2 and Sf21 from *Xenopus laevis* (Daudin) and *Spodoptera frugiperda* (J.E. Smith), respectively (Birkbeck *et al.* 2004).

The titre obtained from supernatants of infected ASK cells was $10^{5.5}$ $TCID_{50}$ mL^{-1}. Same results were achieved when *P. salmonis* inocula for infecting the ASK monolayers came from infected CHSE-214 or ASK cell supernatants. These titres are approximately ten times lower than the values reached in CHSE-214 cells in which normal titres range from 10^6 to 10^7 $TCID_{50}$ mL^{-1} (Fryer *et al.* 1990; Cvitanich, Garate & Smith 1991). The

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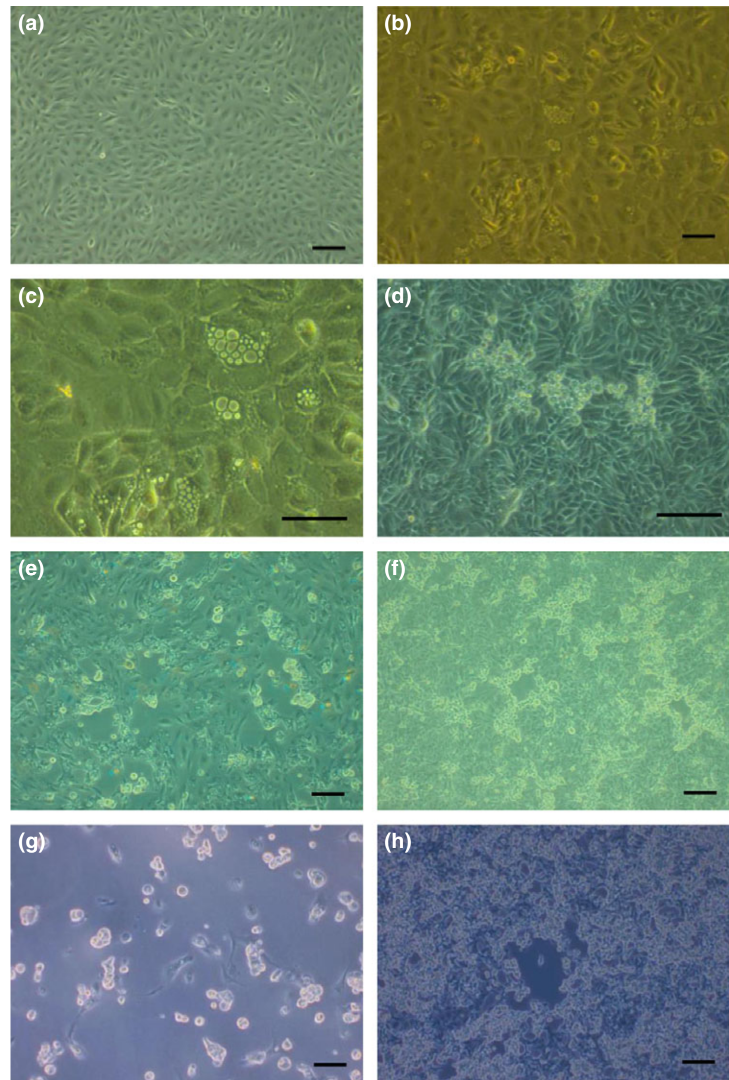

Figure 1 Monolayers of ASK [(a), (b), (c), (e) and (g)] and CHSE-214 cells [(d), (f) and (h)]. Inverted microscope with phase contrast. (a) Uninfected ASK cells. 100x. Illustrations (b) to (h) show cells after different post-inoculation (*p.i.*) days with *Piscirickettsia salmonis*. (b) ASK cells after 5 *p.i.* days. 100x. Some cells show intracytoplasmatic vacuoles. (c) and (d), ASK and CHSE-214 cells, respectively, 5 *p.i.* days. 200x. (c) Intracytoplasmatic vacuoles are observed with higher magnification than in (b). (d) Typical clusters of rounded refringent cells observed at the beginning of the cytopathic effect (CPE) caused by *P. salmonis* in CHSE-214 cells. (e) and (f), ASK and CHSE-214 cells, respectively, 11 *p.i.* days. 100x. (e) Extensive cell vacuolization and detachment of some of them. (f) Typical CPE in CHSE-214 cells with discrete foci of cell detachment surrounded by rounded refringent cells. (g) and (h), ASK and CHSE-214 cells, respectively, 14 *p.i.* days. 100x. (g) Detachment of more than 60% of the ASK cell monolayer. (h) Massive presence of rounded refringent CHSE-214 cells, but with less than the 15% of detachment of the cell monolayer. Bars ≈ 20 μm.

relatively low titres obtained in ASK cells indicate that these cells would not be a good option for high production of viable *P. salmonis*.

In conclusion, ASK cells are permissive to the *P. salmonis* infection, and therefore, they expand the cell line repertoire that can be used for primary isolation as a diagnostic tool and, most importantly, for a wide scope of research studies, such as those focussed to understand the host–parasite interactions.

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