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Additional evidence of the facultative intracellular nature of the fish bacterial pathogen *Piscirickettsia salmonis*[#]

Evidencia adicional de la naturaleza intracelular facultativa del patógeno bacteriano de peces *Piscirickettsia salmonis*

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RESUMEN

Piscirickettsia salmonis es un microorganismo altamente contagioso y virulento que afecta a la salmonicultura mundial desde el último tercio del siglo pasado y del cual sus mecanismos de sobrevivencia intracelular son completamente desconocidos. Después de algunos reportes recientes en donde se cuestiona su condición de intracelular obligado, hemos decidido mostrar evidencia adicional para cambiar este paradigma, llevando a cabo experimentos tanto clásicos como moleculares que confirman su naturaleza de intracelular facultativo. En este reporte se demuestra inequívocamente que la bacteria recuperada desde cultivos celulares infectados, de placas de agar o de medio líquido, es el mismo organismo, el cual cumple con los postulados de Koch. Además, análisis genéticos y proteómicos confirman que la bacteria obtenida de diferentes fuentes de crecimiento corresponde a la misma cepa tipo LF-89, la que fue originalmente descrita por Fryer en 1992. Sin embargo, el crecimiento de la bacteria, tanto en medios libres de células como en cultivo celular, es subóptimo, por lo cual se requieren más análisis para entender completamente la biología del patógeno. Interesantemente, en este trabajo se logró la mantención de la bacteria en medio líquido, pero a una tasa muy baja de crecimiento. En conclusión, y sumado a reportes anteriores, hemos confirmado la naturaleza intracelular facultativa de este patógeno de peces de cultivo.

Palabras clave: *Piscirickettsia salmonis*, intracelular facultativo, genómica.

Key words: *Piscirickettsia salmonis*, facultative intracellular, genomic analysis.

INTRODUCTION

Piscirickettsia salmonis is the etiological agent of the Salmonid Rickettsial Septicaemia (SRS) or Piscirickettsiosis, which is an aggressive infectious disease affecting salmonid fish since the late '80s (Bravo and Campos 1989; Graggero *et al* 1995, Marshall *et al* 2007). Additionally, non-salmonids have been affected by *P. salmonis* or *P. salmonis*-like organisms. Recently, the presence of this bacteria has been detected in specimens of white Seabass (*Atractoscion nobilis*) on the Southern California coast (Arkush KD *et al* 2005). European Seabass (*Dicentrarchus labrax*) in Greece have been affected by a pathogen similar to *P. salmonis* (Athanasopoulou *et al* 2004); also in Hawaii, Tilapia populations (*Oreochromis mossambicus* and *Sarotherodon melanotheron*), both free-living as well as reared fish have suffered a Piscirickettsiosis-type disease (Mauel *et al* 2003), which suggests that the expansion of this agent to other fish species of commercial importance has already begun (Marshall *et al* 2007).

P. salmonis was described as the first Gram-negative intracellular bacterial pathogen isolated from fish and it is a significant cause of mortality in the Chilean salmon industry (Fryer and Hedrick 2003). The bacteria is characterized as: non-motile, non encapsulated, pleomorphic but generally coccoid with a variable size between 0.5 and 1.5 µm in diameter (Bravo *et al* 1989, Kuzyk *et al* 1996). Taxonomically, *P. salmonis* has been placed within the group Gamma-proteobacteria and is one of the few species belonging to the family Piscirickettsiaceae. In fact, it is not related to the family Rickettsiaceae as previously described (Mauel *et al* 1999).

Due to its intracellular nature, the *in vitro* maintenance of the bacteria was routinely carried out in fish cell lines. However, two recent reports have suggested that the bacteria is able to grow on artificial cell-free media (Mikalsen *et al* 2008, Mauel *et al* 2008). The former has described an artificial media based on heart-brain infusion, hemoglobin and sheep blood. The identity of the LF-89 prototype strain was validated by using PCR against the ribosomal DNA 16S and Internal Transcribed Spacer region (ITS). The latter has described a rich agar blood media in which three isolates of *P. salmonis* were used for testing, where the identity was also confirmed by ribosomal DNA PCR procedures. Both groups did genetic and phenotypic characterization

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of the colonies, concluding that they corresponded to *P. salmonis*. Preliminary studies suggested that the virulence of the bacteria had been retained as shown by newly Atlantic salmon fish infection in a controlled laboratory trial (Mikalsen *et al* 2008). None of these reports have been firmly conclusive and a number of questions regarding the obligate intracellular nature condition of the pathogen still remain without answer.

In this study, we address these remaining questions which over a decade have held back the research into the biology of the agent, in order to promote the design of strategies for its control. The prototype strain LF-89 has been successfully grown and cloned as single colonies on Sheep Blood Agar plates (BFCG) as well as on Fish Blood Broth media (BB). The identity of the colonies was determined by PCR against the ITS region of the ribosomal DNA and also against the coding sequence of the ChaP.s functional protein (Marshall *et al* 2007). These results were further confirmed by western blot analysis protein profiles using a wide array of anti-*P. salmonis* antibodies and also by direct and indirect immunofluorescence analysis. The infectivity of the bacterial clone was established by productive infection over CHSE-214 and RTS 11 cell lines.

MATERIALS AND METHODS

GROWTH OF *P. SALMONIS* ON FISH CELL LINES

P. salmonis type strain LF-89 (ATCC VR 1361) was used to infect both the CHSE 214 salmon embryo cell line (ATCC CRL-1681) and the trout macrophage-monocyte RTS11 cell line (kindly donated by Dr. Niels Bols; University of Waterloo, Canada). The cell lines were monitored for Cytopathic Effect (CPE) every 24 h for 7 days. Monolayers of CHSE-214 cells were routinely propagated at 17 °C in 25 cm² culture flasks containing minimal essential medium (MEM, Gibco), supplemented with 7.5% heat-inactivated fetal bovine serum (FBS) and adjusted to pH 7.2 with 10 mM sodium bicarbonate and 15 mM HEPES. Monolayers/suspensions of RTS11 cells were grown at 20 °C in 25 cm² culture flasks containing Leibovitz L-15 media (Gibco) for its propagation, supplemented with 15% FBS as it had been described earlier (Ganassin R C and Bols N 1998). For infection experiments both cells lines were grown in 24 well plates.

P. SALMONIS CULTURE ON ENRICHED BLOOD AGAR PLATES

P. salmonis from different origins (DMSO frozen vials at -80 °C and/or directly from infected cell cultures with 80 to 90% visible CPE) were streaked out onto Blood Fetal Cysteine Glucose media agar plates (BFCG), containing 5 % sheep blood supplemented with 3% FBS, 0.1% L-cysteine and 1% glucose (Mauel *et al* 2008) and incubated for 10 days at 17 °C.

P. SALMONIS CULTURE ON LIQUID MEDIA

To test if *P. salmonis* was capable of growth in liquid media, a single colony was picked and suspended in 500 µl sterile phosphate buffer (1x PBS). 50 µl were used to inoculate 5 ml of Blood Broth (BB) (Tryptone 10 g/l, Peptone 2 g/l, Yeast Extract 2 g/l, NaCl 5 g/l, 5% of Fish Blood lysate v/v and 0.1% of L-cysteine). Cultures were incubated with gentle shaking for 14 days at 17 °C, and the growth evaluated every 24 hours per 13 days at OD₆₂₀, in order to create a *P. salmonis* growth curve.

The blood used for the BB medium preparation was collected from *P. salmonis* free populations of rainbow trout (*Oncorhynchus mykiss*) (Salmonicultura Río Blanco, Región de Valparaíso, Chile). The whole blood was sonicated at 11 root mean square (RMS), centrifuged at 11,000 rpm for 30 min to produce the lysate, the supernatant recovered and filtered through 0.22 µm filters and the lysate stored at 4 °C.

GENETIC CHARACTERIZATION

In order to ensure that the bacteria culture was *P. salmonis*, 10 colonies were analyzed by PCR using the ITS specific primers RTS1 and RTS4 (Marshall *et al* 1998). Additionally, we used the functional gene from the ChaP.s protein as molecular marker to confirm the colony identity (Marshall *et al* 2007, Rojas *et al* 2008). The amplification of ChaP.s COOH-extreme (carboxyl end) was carried out with the specific primers F13 (5'-GATGAAAGAGAAGAAAGACCGC-3') and R8 (5'-ATGGGCGGCATGGGCGGCATGATG-3'), generating a fragment of 475 bp. PCR products were cloned into pCR2.1 TOPO TA cloning vector (Invitrogen) and submitted to sequence to Macrogen Inc. (Korea).

To validate the *P. salmonis* growth in liquid media, 150 µl from the BB culture were used for DNA extraction by the Chelex method (Walsh *et al* 1991) and analyzed by PCR with the same primers described above. PCR products were visualized on 2% agarose gel electrophoresis stained with Ethidium Bromide.

IMMUNODETECTION

Serological characterizations of bacterial agar and broth cultures were achieved by direct and indirect immunofluorescence microscopy. For indirect fluorescence, glass coverslips containing a 1:1000 dilution of *P. salmonis* from blood agar plates were incubated for 1 h at 37 °C with 1:100 dilution of anti-ChaP.s chicken IgY (Marshall SH, personal communication). Then, the samples were washed three times with 1x PBS and incubated for 45 min with 1:100 dilution of rabbit Anti-Chicken IgY Alexa Fluor 488 conjugate (Invitrogen). For direct fluorescence microscopy we have used commercial *P. salmonis* Fluoro test (CFT) according to manufacturer instructions (BiosChile

I.G.S.A.). Coverslips were mounted onto glass slides using fluorescent mounting medium (DAKO Corporation). All procedures were done in the dark.

PROTEIN PROFILE ANALYSIS

Protein profile of *P. salmonis* from BFCG media was analyzed and compared with *P. salmonis* infected CHSE-214 cells using SDS-PAGE procedures (Sambrook J 2001). Western blot analysis was carried out with anti-*P. salmonis* purified-lysed rabbit polyclonal antibodies (anti-*Ps*-L) (Marshall *et al* 2007) and rabbit anti-ChaP.s epitope P-57 (Marshall, personal communication) as first antibodies. Anti-rabbit IgG HRP conjugated (Pierce-Thermo Corporation) was used as second antibody. Reactivity was determined by 3,3'-diaminobenzidine (DAB) (Pierce, Thermo Corporation) colorimetric reaction.

KOCH'S POSTULATES

We tested and validated the virulence of the bacteria grown on BFCG media by inoculating the microbe on the fish cell lines CHSE-214 and RTS11. A single colony was suspended in 500 µl sterile 1x PBS, serially diluted from 10^{-1} to 10^{-4} and used as inoculums to infect both cell lines in 24 well plates. Cells were analyzed 5 days post-infection in order to observe the typical CPE produced by *P. salmonis*. Supernatant of both cell lines were used to initiate cultures on BFCG media plates and incubated at 17 °C by 10 days.

RESULTS AND DISCUSSION

We choose to cultivate *P. salmonis* on the rich agar blood media described by Mauel *et al* 2008 instead of using the one described by others researchers (Mikalsen *et al* 2008). Initially, we started cultures with two different bacterial inoculums, one from a *P. salmonis* frozen vial and the other from supernatant of *P. salmonis* infected CHSE-214 cell culture. 10 days post-incubation at 17 °C, the agar plates showed the appearance of distinctive grey-white color, opaque center and translucent slightly undulating margin colonies (figure 1a), same as previously described by Mikalsen in 2008. Ten of these colonies were analyzed by PCR with specific primers directed against the ITS region and also with specific primers targeted to the immunogenic protein ChaP.s COOH-extreme. Both amplifications were positives, showing the expected amplicon sizes of 284 bp for the ITS region and 475 bp for ChaP.s (figure 1b). Upon sequence analysis of PCR products, we confirmed that the colonies grown on agar plates were *P. salmonis* strain LF-89.

Both media described require intact blood cells for efficient *P. salmonis* development. To date, there are no reports which describe a cell-free liquid medium for *P. salmonis*, suggesting that there is some dependency of the bacterium with respect to the eukaryotic blood cells to allow its multiplication. Interestingly, in this work we designed a liquid medium named BB, which in theory is very similar to the blood agar described by Mauel *et al* 2008 but instead of sheep blood we used lysed rainbow

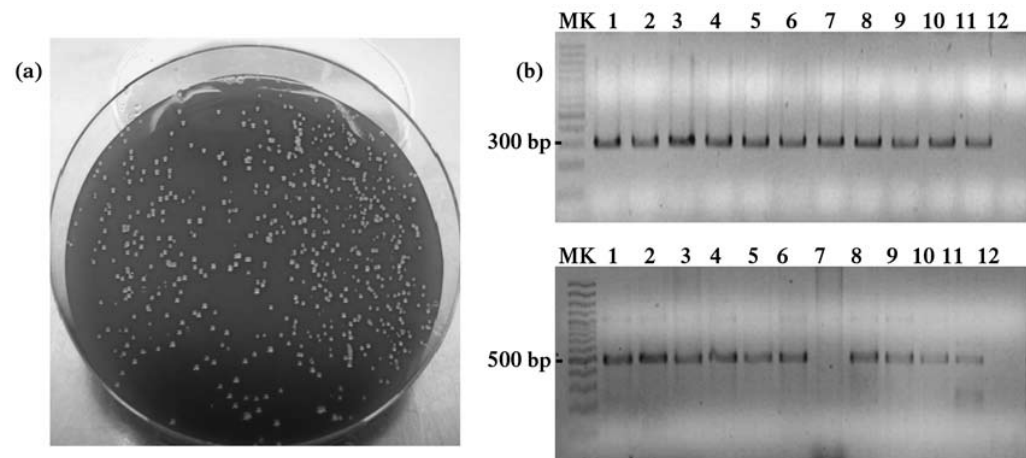


Figure 1. *P. salmonis* derived from supernatant of infected CHSE-214 cells. (a) *P. salmonis* grown on BFCG agar plates. (b) Colony PCR analysis, upper panel shows amplification with primers against ITS region and lower panel shows PCR amplification against ChaP.s COOH-extreme. MK: 100 bp ladder (Winkler Ltda.); lanes 1-10 corresponds to different *P. salmonis* colonies; lane 11: *P. salmonis* 214 infected CHSE-214 cells; lane 12: PCR negative control.

P. salmonis derivada desde sobrenadantes de células CHSE-214 infectadas. (a) *P. salmonis* crecida en medio BFCG. (b) Análisis de colonias por PCR; el panel superior muestra la amplificación con cebadores dirigidos a la región ITS y el panel inferior muestra la amplificación de ChaP.s extremo-COOH. MK: marcador de DNA 100 pb (Winkler Ltda.); Carriles 1-10: diferentes colonias de *P. salmonis*; carril 11: Células CHSE-214 infectadas con *P. salmonis*; carril 12: Control negativo de PCR.

trout blood with membrane and organelles components removed, but cytoplasmic cell contents still remain. BB media batch cultures were initiated with 1/25 vol. of *P. salmonis*, and incubated at 17 °C with gentle agitation for 13 days. *P. salmonis* identity was confirmed by PCR analysis (figure 2a). A bacterial growth curve was achieved with the BB media (figure 2b). The obtained results were unexpected since the bacterial density was not too high, reaching its maximum after 13 days of incubation, with a value of 0.25 at OD₆₂₀. This value is very low compared with other bacterial species such as *Escherichia coli*. For this reason, it is imperative to optimize the growth conditions. The low growth rate obtained with BB medium could be mainly related to the depletion of some limiting nutrients. In order to optimize the conditions, we might

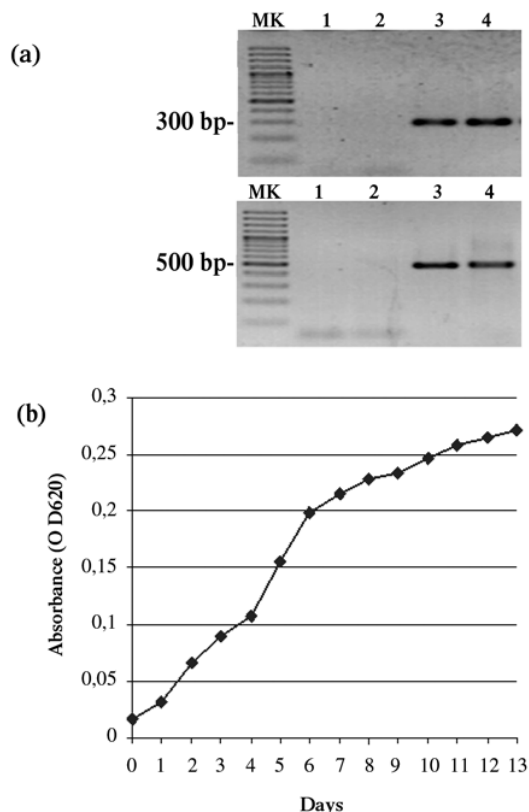


Figure 2. *P. salmonis* grown in liquid cultures (BB medium). (a) PCR analysis 13 days of growth, the upper panel shows ITS amplification and the lower panel shows ChaP.s protein COOH-extreme amplification. (b) Growth curve of *P. salmonis* in BB medium. MK: 100 bp ladder (Winkler Ltda.); lanes 1-2: PCR negative controls; lane 3: *P. salmonis* infected CHSE-214 cells; lane 4: *P. salmonis* grown in BB media.

Crecimiento de *P. salmonis* en cultivos líquidos (Medio BB). (a) Análisis por PCR del cultivo a 13 días de crecimiento; el panel superior muestra la amplificación de la región ITS y el panel inferior muestra la amplificación de ChaP.s extremo-COOH. (b) Curva de crecimiento de *P. salmonis* en medio BB. MK: marcador de 100 pb (Winkler Ltda.); carriles 1-2: controles negativos de PCR; carril 3: células CHSE-214 infectadas con *P. salmonis*; carril 4: *P. salmonis* crecida en medio BB.

try other means of cultivation such as the fed-batch, as an alternative to the batch culture system developed in this study. Nevertheless, in this report we have been focused on highlighting that *P. salmonis* is capable of growth in bacteriological solid and liquid media. This assumption was corroborated with the significant growth obtained in both media tested.

The identity of the *P. salmonis* colonies was also confirmed by serological characterization using *P. salmonis* specific antibodies. One colony was analyzed by indirect immunofluorescence microscopy using chicken anti-ChaP.s IgY as first antibody (figure 3a), these results were compared with *P. salmonis* infected CHSE-214 cell line (figure 3b). High fluorescence signal was observed in both preparations meaning that the bacterial growth from agar plates was *P. salmonis*. The same samples were validated by direct immunofluorescence microscopy using a commercial *P. salmonis* detection kit (Fluoro Test, BiosChile I.G.S.A), which is specific to *P. salmonis* detection. These results confirmed the previous assay that the colonies grown on BFCG agar plates were *P. salmonis* strain LF-89 (figure 3c and figure 3d).

Protein profile analysis was evaluated using conventional 12% SDS-PAGE and western blot. Protein profiles for *P. salmonis* grown on BFCG agar plates and for *P. salmonis* infected CHSE-214 were equivalent (figure 4a, lanes 1, 2 and 3) and the comparison with non-infected CHSE-214 cell (figure 4a, lane 4) confirmed that there are a number of distinctive proteins that belong to *P. salmonis*. Western blot analysis with the anti-*P.s*-L as first antibody showed a distinctive 45 kDa protein in both *P. salmonis* samples but completely absent in non-infected CHSE-214 cells (figure 4b). The western blot also showed reactivity against a protein of 17 kDa which might be the OspA antigen described by Kuzyk *et al* in 2001, but not seen on non-infected cells (figure 4b, lane 4). Western blot analysis with specific rabbit IgG anti-ChaP.s epitope P57 as first antibody showed specific reactivity with the immunogenic protein ChaP.s with the *P. salmonis* samples, but no reaction with non-infected CHSE-214 (figure 4c). In figure 4c the ChaP.s protein (57 kDa) appears under the 66 kDa because the molecular weight range in SDS-PAGE is reduced. These results definitely confirmed that the organism growing on BFCG agar was *P. salmonis*, and reaffirm the hypothesis concerning the intracellular facultative nature of this bacterium.

To determine the infectivity of *P. salmonis* obtained from BFCG agar we infected both CHSE-214 and RTS11 cell lines with different bacterial serial dilutions. CHSE-214 cells demonstrated CPE at 5 days post infection for all serial dilutions (figure 5), typical to the normal process observed on *P. salmonis* infected CHSE-214, consisting on the formation of clusters of rounded and vacuolized cells that eventually cause cell lysis which detach the monolayer (Fryer *et al* 1990). Figure 5a shows infected CHSE-214 with bacterial dilution 10⁻¹, cell lysis has occurred as well as the loss of the monolayer. Bacterial infection dilutions

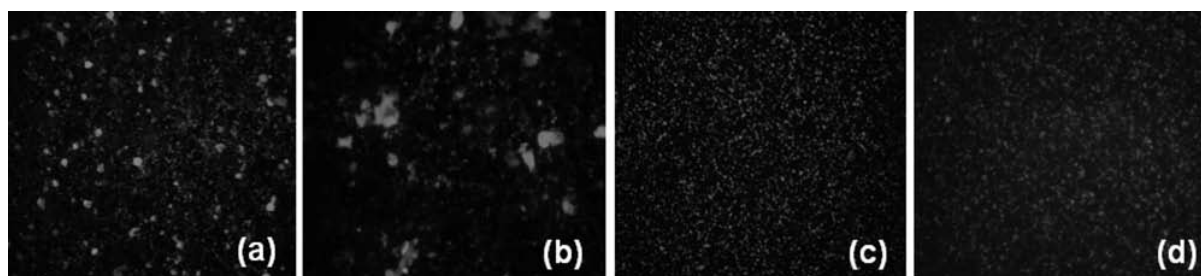


Figure 3. Immunofluorescence analysis of *P. salmonis*. (a) Indirect immunofluorescence of *P. salmonis* grown on BFCG plates using Chicken IgY anti-ChaP.s antibody. (b) Indirect immunofluorescence of *P. salmonis* from CHSE-214 infected cells, using Chicken IgY anti-ChaP.s. (c) Direct immunofluorescence of *P. salmonis* grown on BFCG plates, using a commercial *P. salmonis* detection kit. (d) Direct immunofluorescence of *P. salmonis* from CHSE-214 infected cells, using a commercial *P. salmonis* detection kit.

Análisis por inmunofluorescencia de *P. salmonis*. (a) Inmunofluorescencia indirecta de *P. salmonis* crecida en agar BFCG, usando un anticuerpo IgY anti-ChaP.s. (b) Inmunofluorescencia indirecta de *P. salmonis* desde células CHSE-214, usando un anticuerpo IgY anti-ChaP.s. (c) Inmunofluorescencia directa de *P. salmonis* crecida en agar BFCG, usando un kit comercial de detección. (d) Inmunofluorescencia de *P. salmonis* desde células CHSE-214 infectadas, usando un kit comercial de detección.

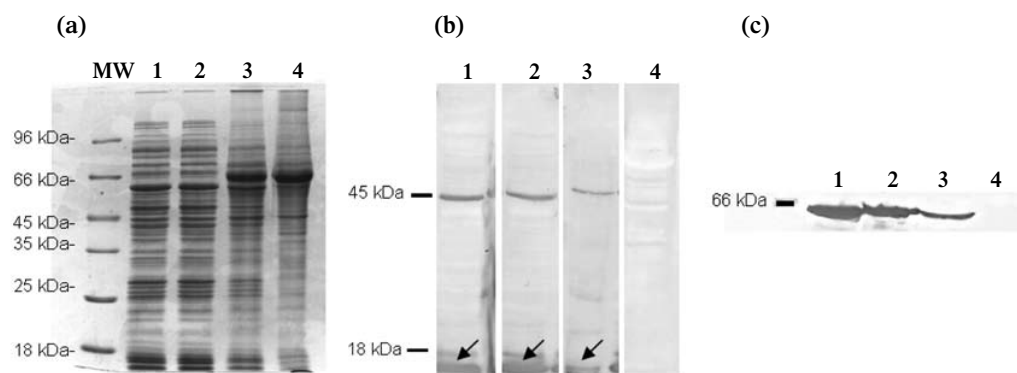


Figure 4. Protein profile comparison. (a) 12% SDS-PAGE. (b) Western Blot using rabbit IgG anti-*P.s-L*. (c) Western Blot using rabbit IgG anti-ChaP.s epitope P57. MW: Molecular weight markers; lane 1: *P. salmonis* colony 1 from BFCG medium; lane 2: *P. salmonis* colony 2 from BFCG medium; lane 3: *P. salmonis* infected CHSE-214 cells; lane 4: non-infected CHSE-214 cells. Arrows indicate the OspA protein.

Comparación de perfiles proteicos. (a) SDS-PAGE al 12%. (b) Análisis por Western Blot utilizando un anticuerpo IgG anti-*P.s-L*. (c) Análisis por Western Blot utilizando un anticuerpo IgG anti-ChaP.s epítipo P57. MW: Marcador de pesos moleculares; carril 1: colonia 1 de *P. salmonis* desde medio BFCG; carril 2: colonia 2 de *P. salmonis* desde medio BFCG; *P. salmonis* desde células CHSE-214 infectadas; carril 4: células CHSE-214 no infectadas. Las flechas indican la proteína OspA.

10^{-2} to 10^{-4} showed rounded and vacuolized cells (Figures 5b, 5c and 5d) while non-infected cells did not present these features (figure 5e). *P. salmonis* infected RTS11 cells did not show evident CPE, however the suspended monocytes cells tended to form clusters, and finally at day 15 post-infection suffered lysis. Figure 6a, left panel, shows infected RTS11 cells at day 5 post-infection with a typical cell aggregation caused by the presence of bacterial lipopolysaccharide, not seen in non-infected cells (Figure 6a, right panel). Due to the lack of CPE, a PCR evaluation was necessary using the specific primers RTS1 and RTS4 that targeted to the ITS region (figure 6b). In the PCR results we observed a gradient of product quantity representing the infected RTS11 cells with different bacterial dilutions. These results demonstrate that *P. salmonis* maintained its infection capacity for salmonid cell cultures after growth on artificial media.

Supernatants from both *P. salmonis* infected CHSE-214 and RTS11 cells were used to start new cultures on BFCG agar. *P. salmonis* characteristic colonies were observed after 10 days of incubation at 17 °C. Analysis by PCR verified the presence of *P. salmonis* within the colonies (data not shown). Based upon the results presented in here, Koch's postulates have been fully accomplished since: (i) the *P. salmonis* microbe has been found abundantly in both infected cell lines, but not in non-infected cells, ii) the pathogen has been isolated from infected cells and growth in pure culture (figure 1), iii) the cultured microorganism has caused infection when introduced in new non-infected cell cultures (figure 5 and figure 6), and (iv) the *P. salmonis* has been re-isolated from the inoculated experimental host (CHSE-214 and RTS11) and identified as identical to the original specific causative agent.

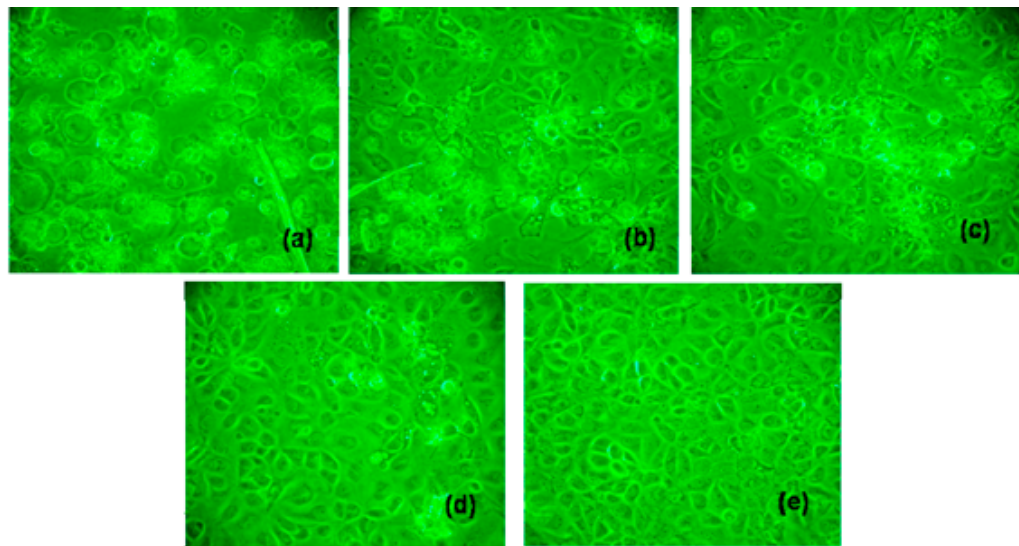


Figure 5. *P. salmonis* infected CHSE-214 cell line at 5 days post-bacterial inoculum (a) *P. salmonis* 10^{-1} dilution. (b) *P. salmonis* 10^{-2} dilution. (c) *P. salmonis* 10^{-3} dilution. (d) *P. salmonis* 10^{-4} dilution, (e) Non infected CHSE-214 cell line (control).

Células CHSE-214 infectadas con *P. salmonis* a 5 días del inóculo bacteriano. (a) Dilución *P. salmonis* 10^{-1} . (b) dilución *P. salmonis* 10^{-2} . (c) Dilución *P. salmonis* 10^{-3} . (d) Dilución *P. salmonis* 10^{-4} . (e) Células CHSE-214 no infectadas (control).

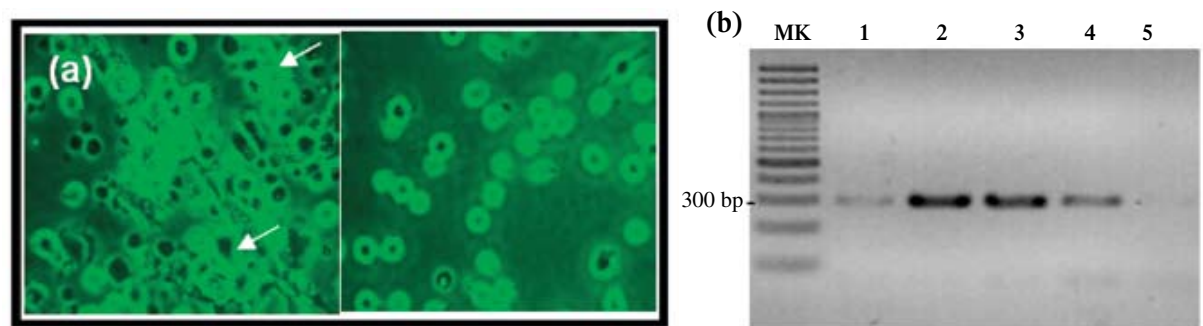


Figure 6. *P. salmonis* infected RTS11 cell line. (a) RTS11 cell line infected with *P. salmonis* at 5 days post-infection the left panel corresponds to infected RTS11 cells and the right panel corresponds to non-infected RTS11 cells. (b) PCR analysis with specific primers against ITS region. MK: 100 bp ladder (Winkler); lane 1: *P. salmonis* infected CHSE-214 cells (PCR positive control); lane 2: *P. salmonis* 10^{-1} dilution; lane 3: *P. salmonis* 10^{-2} dilution; lane 4: *P. salmonis* 10^{-3} dilution; lane 5: *P. salmonis* 10^{-4} dilution. The white arrows indicated cells clusters produced by LPS.

Línea celular RTS 11 infectada con *P. salmonis*. (a) Fotografía de células RTS11 infectadas con *P. salmonis* a 5 días post-infección; el panel derecho corresponde a células sin infectar y el panel izquierdo corresponde a células infectadas. (b) Análisis por PCR con cebadores específicos sobre la región ITS. MK: marcador de 100 pb; carril 1: Células CHSE-215 infectadas (control positivo); carril 2: infección con dilución 10^{-1} de *P. salmonis*; carril 3: infección con dilución 10^{-2} de *P. salmonis*; carril 4: infección con dilución 10^{-3} de *P. salmonis*; carril 5: infección con dilución 10^{-4} de *P. salmonis*.

The liquid cell-free media (BB) along with the BFCG agar media allowed the replication and maintenance of the bacteria similar to both CHSE-214 and RTS11 cell line, validating the facultative intracellular nature of this pathogen. In addition, the growth in artificial media, improved the recovery of cells per ml, free of eukaryotic contaminants and at low cost, allowing the genetic manipulations of the bacteria for further biotechnological applications.

SUMMARY

Piscirickettsia salmonis is a highly virulent and contagious microorganism that affects net pen-reared salmonid fish worldwide

since the last third of the past century, with little knowledge about its intracellular survival mechanisms. Following a number of recent and non-conclusive reports which questioned its obligate intracellular condition, we decided to show additional evidence to challenge this well-established paradigm carrying on both basic biology as well as classical molecular experiments that confirm its facultative intracellular nature. In this report, we unequivocally demonstrate that the bacteria recovered from tissue culture amplification, *in vitro* grown agar plates or grown in liquid cultures, were the same organism and all of these isolates equally fulfils Koch's postulates. In addition, genomic and proteomic analyses confirmed that bacteria from different growth source conditions belonged to the same LF89 prototype strain as originally described by Fryer in 1992. Notwithstanding, growth of the bacteria both in cell-free media as well as in tissue culture cell lines were definitively suboptimal, and much more analysis are required to fully understand the biology of

the pathogen. Interestingly, we were able to grow the bacteria in liquid media but at very slow rate. We conclude that, in addition to previous reports, our results confirmed the facultative intracellular nature of this fish pathogen.

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