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Prevalence, geographic distribution and phenotypic differences of *Piscirickettsia salmonis* EM-90-like isolates

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Abstract

Early reports accounted for two main genotypes of *Piscirickettsia salmonis*, a fish pathogen and causative agent of piscirickettsiosis, placing the single isolate EM-90 apart from the prototypic LF-89 and related isolates. In this study, we provide evidence that, contrary to what has been supposed, the EM-90-like isolates are highly prevalent and disseminated across Chilean marine farms. Molecular analysis of 507 *P. salmonis* field isolates derived from main rearing areas, diverse hosts and collected over 6 years, revealed that nearly 50% of the entire collection were indeed typed as EM-90-like. Interestingly, these isolates showed a marked host preference, being recovered exclusively from Atlantic salmon (*Salmo salar*) samples. Although both strains produce undistinguishable pathological outcomes, differences regarding growth kinetics and susceptibility to the antibiotics and bactericidal action of serum could be identified. In sum, our results allow to conclude that the EM-90-like isolates represent an epidemiologically relevant group in the current situation of piscirickettsiosis. Based on the consistency between genotype and phenotype exhibited by this strain, we point out the need for genotypic studies that may be as important for the Chilean salmon industry as the continuous surveillance of antimicrobial susceptibility patterns.

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Introduction

With around 3.6 billion US dollars of incomes from export in 2015, salmon farming represents an important economic activity for southern Chile (Subsecretaria de Pesca, Subpesca 2016). However, infectious diseases, especially Salmonid rickettsial septicemia (SRS) or piscirickettsiosis, are a major concern for the development and sustainability of this industry. SRS is caused by the Gram-negative bacterium *Piscirickettsia salmonis* (Cvitanich, Garate & Smith 1991; Fryer & Hedrick 2003), an intracellular facultative pathogen responsible for a septicemic disease with high mortality in seawater-reared salmonids (Mauel, Ware & Smith 2008; Mikalsen *et al.* 2008). Although *P. salmonis* was first isolated from coho salmon (*Oncorhynchus kisutch*) (Bravo & Campos 1989; Fryer *et al.* 1992), today, the disease is broadly disseminated in the three salmonid species reared in Chile. The Chilean National Fisheries and Aquaculture Service (Servicio Nacional de Pesca y Acuicultura, Sernapesca) recognized that about 79% and 83% of the mortality ascribed to infectious diseases in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), respectively, were indeed caused by piscirickettsiosis in 2015 (Sernapesca 2016a). Available vaccines have resulted ineffective to prevent the disease and merely delay the first outbreak after seawater transfer (Jakob *et al.* 2014). As a consequence of

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persistent SRS outbreaks, the use of antibiotics has increased (Sernapesca 2015a). This situation has most likely affected the population of *P. salmonis* and has triggered the selection of resistant types. In this regard, our laboratory has recently reported the emergence and spread of isolates resistant to quinolones, florfenicol (FFC) and oxytetracycline (OTC) (Henriquez *et al.* 2016). The heavy economic losses incurred in controlling this resilient pathogen are persistently jeopardizing the sustainability of the Chilean salmon industry.

The classification of *P. salmonis* resulted conflicting from the very beginning, as the bacterium was related erroneously to the order of Rickettsiales (Cvitanich *et al.* 1991). However, standard phylogeny based on the sequencing of the 16S rRNA gene demonstrated that *P. salmonis* belongs to the *Gammaproteobacteria* class of the phylum *Proteobacteria* (Fryer *et al.* 1992). Later, genetic reports also accounted for differences at the level of classic typing markers, namely 16S-23S rRNA and internal transcribed sequence (ITS), among *P. salmonis* isolates (Mauel, Giovannoni & Fryer 1996, 1999; Casanova *et al.* 2001). These analyses allowed for grouping the isolates into two categories: LF-89-like, that is related to the type strain, and the outlier EM-90 strain, which has been recovered from a diseased *S. salar*. In this context, we have recently sequenced the genome of representative members of both strains, the A1-15972 (EM-90-like) and B1-32597 (LF-89-like) isolates (Bohle *et al.* 2014). The comparison of their respective genomes disclosed differences in the presence of virulence factors like type IV secretion systems, among others, thus showing for the first time the extensive genetic heterogeneity of both genogroups. A study performed on 19 isolates from Chile and Canada added information on the previous phylogenetic studies, suggesting that outbreaks of piscirickettsiosis in Chile are caused by isolates derived from at least two genogroups (Otterlei *et al.* 2016). More recently, the amplification of 16S rDNA combined with the use of restriction enzymes has been used for *P. salmonis* typing, leading to the same conclusion (Mandakovic *et al.* 2016). Nevertheless, neither experimental evidence supporting the strain dichotomy hypothesis nor epidemiological data that shed more light on the relevance of EM-90-like isolates has been given.

This study provides an update on the characterization of our *P. salmonis* strain collection. Profiles of susceptibility to different antimicrobials were extended from 292 to 507 specimens, and isolates were genotypically assessed. In contrast to what has long since been assumed, our results demonstrate that both LF-89-like and EM-90-like isolates are highly and ubiquitously prevalent. Additionally, the latter group of isolates was shown to have a narrow host range, and, to date, presumably affects exclusively *S. salar*. Remarkably, and despite the fact that the majority of LF-89-like isolates present some degree of antibiotic resistance, specimens classified as EM-90-like were all susceptible to tested antimicrobials. Subsequent phenotyping of a panel of isolates depicted differences in traits possibly related to antibiotic susceptibility and the high number of recently recorded SRS outbreaks caused by EM-90-like pathogens.

Materials and methods

Bacterial isolation, growth kinetics and minimal inhibitory concentration assessment

Pools of tissues (kidney, liver and brain) were aseptically collected for colony isolation on ADL-PSA agar plates, a solid derivative of ADL-PSB (Henriquez *et al.* 2016). Bacteria were recovered from three different salmonid species during routine analyses of SRS outbreaks between 2010 and 2015, but also in a field campaign that took place in 2015. To allow bacterial growth, plates were maintained at 18 ± 1 °C for 7–8 days. The respective isolates were kept at −80 °C in appropriate cryovials containing a mixture of 80% ADL-PSB and 20% DMSO (Merck) until further use.

Evaluation of growth kinetics was carried out in a microplate reader EPOCH2 (BioTek). Frozen bacteria were inoculated into 3 mL of ADL-PSB and maintained statically for 4 days at 18 °C. Bacterial cells were washed with saline-buffered solution (SBS; 0.15 M NaCl, 7.3 mM KH_2PO_4, 11.5 mM K_2HPO_4, pH 6.0), supplemented with 4% lactose and adjusted to an optical density (OD) = 1.0 at a wavelength of 600 nm in the same solution. About 30 µL of a 1:10 dilution of this cell suspension was used to inoculate wells of flat-bottom 96-well microtiter plates prefilled with 120 µL of ADL-PSB or serum-free basal medium BM4 (Henriquez *et al.* 2013), reaching a final concentration of ~3×10^5 colony-forming units (CFU) per mL. The microplates were incubated

at 18 or 22 ± 0.1 °C for 3 days, with 1 min of shaking before reading the absorbance at 600 nm each hour.

The isolates were phenotypically characterized for susceptibility to quinolones (oxolinic acid, OA; flumequine, FLU), florfenicol (FFC) and oxytetracycline (OTC) (all purchased from Sigma-Aldrich), according to the protocol previously described (Henriquez *et al.* 2016). *Escherichia coli* ATCC 25922 was used as positive control. Reported epidemiological breakpoints for antimicrobials were taken into account for the classification of each isolate in wild-type (WT) or non-wild-type (NWT) categories (Henriquez *et al.* 2016). Detailed information regarding *P. salmonis* isolates is shown in Table S1.

Molecular testing

Genomic DNA samples were obtained from bacteria cultured in agar plates using the E.Z.N.A™ Bacterial DNA Kit following the instructions given by the manufacturer (Omega-Biotek). *P. salmonis* identification was conducted by means of a PCR method previously described (Kuzyk *et al.* 2001). The sequence of the 16S rRNA gene of selected isolates was determined using Sanger chemistry with primers ADL_EubA and ADL_EubB (5′-AAGGAGGTAATCCAGCCGCA-3′, 5′-AGAGTTTGATCCTGGCTCAG-3′, respectively). For ITS, the primer pair ITS_Ext_F and R (5′-CCTGCGG CTGGATTACCT-3′, 5′-TAGATCTTTCAGTT CCCC-3′) was used. Fragments were sequenced in a 310 Genetic Analyzer (Applied Biosystems). The 16S-ITS sequence of the EM-90 isolate corresponded to a consensus using U36940, U36944, AF205384, AF212839 sequences previously reported (Mauel *et al.* 1996; Casanova *et al.* 2001). The maximum parsimony method was used to infer the evolutionary history of the taxa analysed. The respective tree was obtained using the Close-Neighbour-Interchange algorithm, with a bootstrap consensus including 1,000 replicates. Evolutionary analyses were conducted in MEGA5 software (http://www.megasoftware.net; Tamura *et al.* 2011). For genotyping, a modified PCR approach that has previously been used to this end was applied (Mauel *et al.* 1996). Primers PS2S (5′-CT AGGAGATGAGCCCGCGTTG-3′) and ADL_ LF89 (5′-GCACTTCCGCATCTCTGCAG-3′) were employed for typing of LF-89-like isolates, while primers PS2S and ADL_EM90 (5′-GCA-CATCAATATCTCTATCA-3′) were utilized for EM-90-like specimens. PCR rounds were carried out in a final reaction volume of 25 μL. The reaction mixture contained 6 pmol of each primer, 3 nmol of each deoxynucleotide triphosphate, 1 mM $MgCl_2$, 1.0 U of *Taq* DNA polymerase (Invitrogen), and 1.0 μL of DNA template. The thermal profile for PCR was as follows: preheating at 95 °C for 3 min; followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; and a final extension step at 72 °C for 3 min, using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems).

Bactericidal action of *Salmo salar* naïve serum

Fish blood samples were kindly donated by Camanchaca S.A. salmon farm. Respective fish were raised and maintained in freshwater and had never been exposed to *P. salmonis* according to molecular testing and clinical records. Blood was collected by caudal venipuncture from Atlantic salmon with an average body weight of 6 kg, and naïve serum (NS) was obtained by allowing the blood to clot for 2.0 h at 4 °C and subsequent centrifugation at 1000 g for 5 min. NS samples were pooled and stored at −20 °C in aliquots of 1 mL. Before testing, NS aliquots were filtered through a polyethersulfone membrane with a pore size of 0.22 μm. For assessment of bacterial survival, we adapted a previously utilized method to a 96-well deep-well plates format (Boesen *et al.* 1999). Briefly, fresh bacterial culture aliquots were adjusted to $OD_{600} = 1.0$ by dilution in SBS. Then, 10 μL of bacterial inoculum was mixed with 75 μL of NS and 15 μL of SBS. Bacteria were incubated at 18 °C for 3 h. Complement activity was stopped by addition of 900 μL SBS. Subsequently, $\log_{10}$ serial dilutions in SBS of these samples were prepared for colony counts on ADL-PSA agar plates maintained at 18 °C for 7 days. Bacteria treated with heat-inactivated NS (44 °C for 20 min) were used as a negative control. Colony counts were normalized to CFU mL^{-1} in sole SBS, which was considered to be 100%. Results were tested for normal distribution applying the Shapiro–Wilk test and were subsequently analysed performing the nonparametric Mann–Whitney rank-sum test.

Results

An extensive collection of 507 *P. salmonis* isolates encompassing specimens obtained from 163 marine farms located in 45 *barrios*, that is subdivisions of geographic macrozones, was considered for this study (Table S1). Molecular typing of the entire collection revealed no significant differences between the overall proportion of either genotype ($n = 247$ for LF-89-like and $n = 260$ for EM-90-like). However, a predominance of LF-89-like types in the period 2010–2014 was noted (Fig. 1a). This situation reversed in 2015, when a peak of EM-90-like incidence was recorded (Fig. 1a). With respect to distinct geographic regions, an asymmetric distribution of genotypes was observed (Fig. 1b). In fact, EM-90-like isolates were more prevalent in Region de Aysen (~63%), while the LF-89-like strain was predominant in Region de Los Lagos (~55%). The distribution of genotypes by species was also investigated. Strikingly, the EM-90-like isolates were recovered exclusively from samples derived from *S. salar*, while the ones grouped as LF-89-like were isolated from all salmonid species farmed in Chile (Table 1).

With regard to the genotypes' susceptibilities to antibiotics, we found all 260 isolates classified into the EM-90-like group to be susceptible to quinolones, FFC and OTC. On the contrary, less than 11% of LF-89-like isolates presented this phenotype. The majority of LF-89-like specimens showed resistances to at least one of the antibiotics tested (Table 1). In terms of distribution of antibiotic resistances by host, 77% of isolates derived from *S. salar* corresponded to WT, while a minor proportion resulted to be NWT (Fig. 2a). The opposite situation was found for *O. mykiss,* because only 5% of isolates were susceptible to tested drugs (Fig. 2b). All isolates recovered from *O. kisutch* depicted a reduced susceptibility to quinolones ($n = 26$), including some types showing additional intermediate resistance to FFC.

To gain further insights into phenotypic traits linked to varying incidence and antibiotic susceptibility patterns observed in the EM-90-like group, we evaluated growth kinetics under two different temperatures and in distinct culture media and also assessed the pathogen's resistance to NS using a panel of isolates (Table 2). The results unveiled striking differences regarding these traits (Figs 3 and 4). EM-90-like isolates were able to grow in ADL-PSB, regardless of the temperature and medium tested (Fig. 3a,b), whereas the LF-89-like proved to have higher nutritional demands and to be more dependent on temperature, growing only at a permissive temperature of 18 °C in a rich medium (Fig. 3c). On the other hand, the EM-90-like types were more susceptible to the

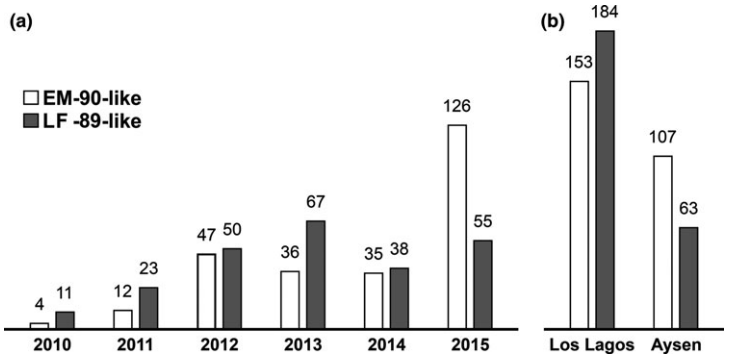

Figure 1 Temporospatial distribution of *P. salmonis* strains between 2010 and 2015 (a), by geographic region (b).

Table 1 Relationship between *P. salmonis* strains, antibiotic susceptibility patterns and host

	Phenotype				Host		
Strain	SUS	QUI^R	QUI^R/FFC^I	OTC^R	*S. salar*	*O. mykiss*	*O. kisutch*
LF-89-like[a]	27 (10.9)	176 (71.3)	24 (9.7)	20 (8.1)	110	111	26
EM-90-like	260	–	–	–	260	–	–

[a]Numbers in parentheses represent percentages. Phenotype nomenclature: SUS, susceptible to quinolones, FFC and OTC; QUI^R, resistant to quinolones; QUI^R/FFC^I, resistant to quinolones/intermediate susceptibility to FFC; OTC^R, oxytetracycline resistant.

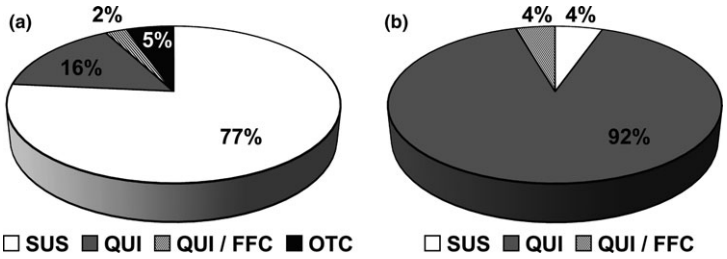

Figure 2 Distribution of susceptibilities of *P. salmonis* to determined antibiotics, for isolates recovered from *S. salar* (a) and *O. mykiss* (b).

Table 2 Selected isolates for phenotypic testing

ID	ID (Table S1)	Region	Origin (Barrio)	Strain	Host	Year of isolation	GenBank accession number[a]
PM-15972	3	Aysen	28A	EM-90-like	*Salmo salar*	2010	KX524938
PM-21657	25	Aysen	18C	EM-90-like	*Salmo salar*	2011	KX524939
PM-23019	29	Los Lagos	17B	EM-90-like	*Salmo salar*	2011	KX524940
PM-24231	36	Aysen	18C	EM-90-like	*Salmo salar*	2011	KX524942
PM-26862	46	Los Lagos	2	EM-90-like	*Salmo salar*	2011	KX524941
PM-22180	27	Aysen	32	LF-89-like	*Oncorhynchus mykiss*	2011	KX524945
PM-31429	118	Los Lagos	17B	LF-89-like	*Oncorhynchus mykiss*	2012	KX524943
PM-32597	125	Los Lagos	17B	LF-89-like	*Oncorhynchus kisutch*	2012	KX524946
PM-58386	356	Los Lagos	16	LF-89-like	*Salmo salar*	2015	KX524944

[a]16S-ITS sequence length of about 2200 nt.

bactericidal action of complement components present in NS, thereby contrasting the refractory behaviour of the LF-89-like genogroup (Fig. 4).

The phylogenetic relationship among isolates was determined by sequencing of the 16S rRNA gene (Fig. 5a). Clustering analysis splitted the isolates into two groups. The same result was obtained using the ITS sequences (Fig. 5b). Additionally, we explored the relationships among specimens of our panel of isolates by contrasting their 16S-ITS sequences with those of the G2 clade isolates reported by Otterlei *et al.* (2016). An interesting finding was that the EM-90-like isolates were clustered in the same group with those belonging to the G2 clade (Fig. S1).

Discussion

By means of this field study, we provide evidence on the relevance of a neglected *P. salmonis* strain in the context of the epidemiology of piscirickettsiosis: the EM-90-like strain. We took advantage of reported polymorphisms in the 16S rRNA gene, which is preferentially used for a first-glance assessment of phylogenetic relationships and as a genomic signature for strain differentiation (Mauel *et al.* 1996). Totally unexpected was the fact that EM-90-like isolates account for about 50% of our collection, which contains more than 500 isolates. This clearly indicates that this genotype plays a much more important role than that of an anecdotic case reported in close temporal proximity to the isolation of the type strain LF-89. Our results support the notion that members of the EM-90-like genogroup are widely disseminated and responsible for a major proportion of piscirickettsiosis cases. Isolates pertaining to that group do not only share a common genotype, but also present similar phenotypic features that demonstrate inner group homogeneity and distinction from the LF-89-like genogroup. One such remarkable phenotypic trait was that the EM-90-like isolates were susceptible to all antibiotics tested. In contrast, few LF-89-like isolates could be shown to be susceptible; the majority of them depicted impaired susceptibility patterns. This finding resulted puzzling, considering that both strains have probably been subjected to the same selective pressure for years. It thus seems plausible to infer that the susceptible phenotype of the EM-90-like strain depends on intrinsic factors. For instance, it seems more susceptible to the bactericidal action of fish NS, which suggests that such bacteria are prone to be cleared by innate immune mechanisms, but both strains may also differ at the level of external membrane components (Boesen *et al.* 1999). Since all antibiotics tested reach the bacterial cytoplasm, one possibility is that members of the

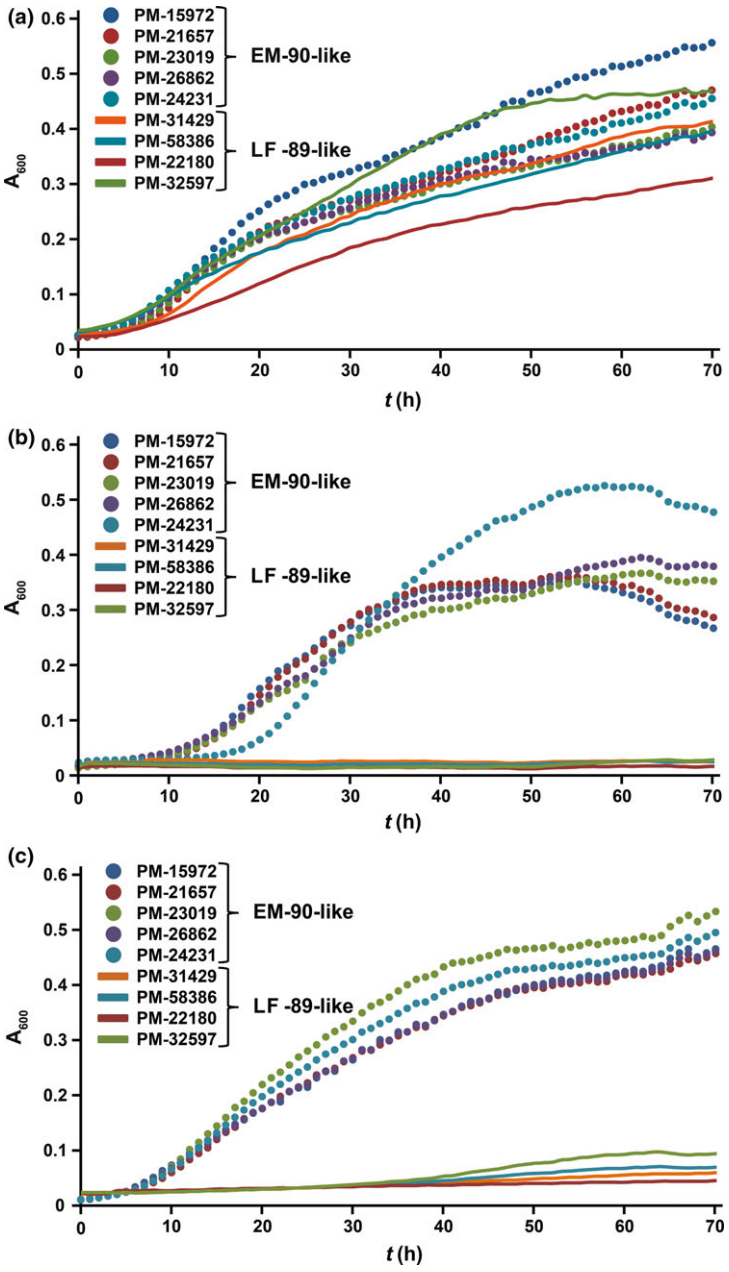

Figure 3 Growth kinetics in ADL-PSB at 18 °C (a) and 22 °C (b). Kinetics performed in BM4 broth at 18 °C (c). [Colour figure can be viewed at wileyonlinelibrary.com]

EM-90-like genogroup are lacking determined genes encoding for membrane proteins that have recently been associated with antibiotic resistance (Sandoval *et al.* 2016). More research is needed to confirm this hypothesis.

Concerning the host range, all EM-90-like isolates were recovered from *S. salar*, while LF-89-like isolates exhibited a wider host range and affected the three salmonid species reared in Chile. The host specificity depicted by the EM-90-like strain does not necessarily mean that the respective pathogens would not be able to cross the species barrier. Recent findings support this hypothesis (Otterlei *et al.* 2016). In the cited study, 10 isolates were clustered into what the authors named the G2 clade that matched the here described EM-90-like group (Fig. 5), and one of them corresponded to an isolate recovered from *O. mykiss*. Moreover, in a genetic characterization performed on wild fish other than salmonids, the EM-90 genomic signature was found in samples of pejerrey *Odontesthes regia* (Contreras-Lynch *et al.*

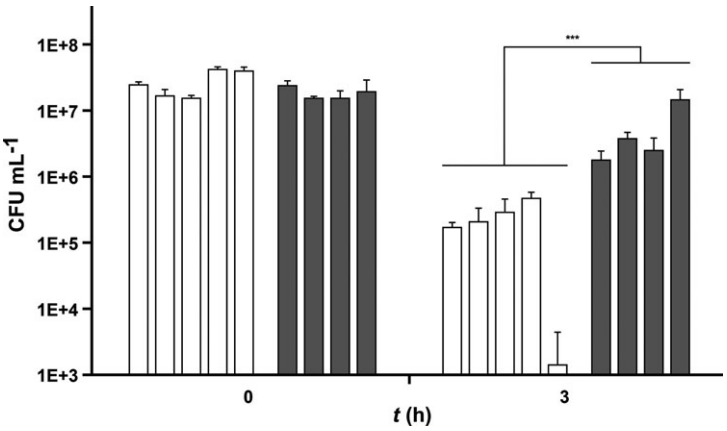

Figure 4 Survival of *P. salmonis* isolates after exposure to naïve *S. salar* serum. White bars represent colony counts of EM-90-like isolates, while black bars correspond to the LF-89-like ones. Isolate ID follows the same order as presented in Fig. 3. Data have been obtained in three independent experiments. The significant difference between groups, as assessed by means of the Mann–Whitney rank-sum test, is shown as *** ($p < 0{,}001$).

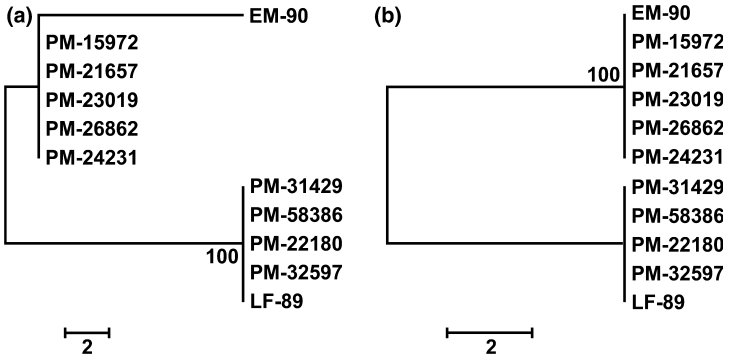

Figure 5 Phylogenetic tree based on maximum parsimony representing 1523 positions for the 16S rRNA gene (a) and 315 positions for ITS (b). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The trees are drawn to scale, with branch lengths calculated using the average pathway method and the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated.

2015). Although the biologic significance of these findings is not yet clear, both these results and the presumably high pathogen load in the environment reinforce the idea that the EM-90-like strain will not be permanently constrained to a specific salmonid host.

Similar to Otterlei *et al.* (2016), we found *P. salmonis* strains to exhibit distinct growth requirements, both with regard to the complexity of culture media and temperature. The authors reported that isolates of the G2 clade were less demanding in this context, which is in accordance with the results obtained here for the EM-90-like strain. In fact, only members of this strain were the able to growth at a high temperature and in a serum-free medium. Taking into account the results of growth kinetics analysis, the peak of incidence of EM-90-like isolates in 2015 may be ascribed to the increase in the so-called climate anomaly informed by the National Oceanic and Atmospheric Administration (NOAA), which globally impacted the sea temperature and provoked an average rise in water temperature of ~ 0.74 °C, the warmest year ever recorded (https://www.ncdc.noaa.gov/sotc/global/201513). In fact, the climate change has already been proven to introduce a factor of unpredictability in persistence and dissemination of several bacterial pathogens (Hellberg & Chu 2015). We note that the higher incidence of outbreaks caused by the EM-90-like types in 2015 was independent of the respective geographic location, thus reflecting absence of a bias linked to this factor, even though in the six-year period of collection, the EM-90-like types predominated in Region de Aysen. This last observation could be explained by the host tropism shown by this strain and the fact that salmon industry in Aysen has relied basically

on the deployment and culture of Atlantic salmon smolts in marine farms (Sernapesca 2015b). Consistent with the increasing number of EM-90-like cases observed in 2015, an elevated incidence of piscirickettsiosis outbreaks in the warmer seasons was documented in the corresponding official report (Sernapesca 2016a).

It is worth commenting the information collected on the antimicrobial susceptibility patterns. In comparison with the data previously reported by our group (Henriquez *et al.* 2016), an important reduction in the number of QUI-resistant types was recorded (decrease from 49.3 to 34.7%). However, we observed an augmented incidence for QUI-resistant/FFC intermediate and OTC-resistant types in 2015 (from 3.1 to 4.7% and from 1.7 to 3.9%, respectively). This situation is in agreement with the amounts of FFC and OTC utilized for SRS treatments in 2014 and 2015 (Sernapesca 2015a, 2016b). This trend constitutes a sanitary warning because of the reduced alternatives for therapies that veterinarians currently have to manage the disease, but an increasing incidence of piscirickettsiosis caused by EM-90-like isolates may paradoxically help to revert this trend.

In summary, we have clearly demonstrated that SRS outbreaks in Chile are caused by two strains with nearly identical prevalence and distribution. Based on the features that distinguish members of the EM-90-like strain from the LF-89-like group at genetic level, with regard to host preference, growth requirements, susceptibility to antimicrobials and culture characteristics, we propose this strain to be considered as a subspecies within the taxonomic classification of the genus *Piscirickettsia*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree based on Maximum Parsimony for 16S-ITS reflecting 2188 positions.

Table S1. Dataset.

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