Short communication

Patterns of *Piscirickettsia salmonis* load in susceptible and resistant families of *Salmo salar*

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ABSTRACT

The pathogen *Piscirickettsia salmonis* produces a systemic aggressive infection that involves several organs and tissues in salmonids. In spite of the great economic losses caused by this pathogen in the Atlantic salmon (*Salmo salar*) industry, very little is known about the resistance mechanisms of the host to this pathogen. In this paper, for the first time, we aimed to identify the bacterial load in head kidney and muscle of Atlantic salmon exhibiting differential familiar mortality. Furthermore, in order to assess the patterns of gene expression of immune related genes in susceptible and resistant families, a set of candidate genes was evaluated using deep sequencing of the transcriptome. The results showed that the bacterial load was significantly lower in resistant fish, when compared with the susceptible individuals. Based on the candidate genes analysis, we infer that the resistant hosts triggered up-regulation of specific genes (such as for example the LysC), which may explain a decrease in the bacterial load in head kidney, while the susceptible fish presented an exacerbated innate response, which is unable to exert an effective response against the bacteria. Interestingly, we found a higher bacterial load in muscle when compared with head kidney. We argue that this is possible due to the availability of an additional source of iron in muscle. Besides, the results show that the resistant fish could not be a likely reservoir of the bacteria.

1. Introduction, methods and results

The bacterium *Piscirickettsia salmonis* (*P. salmonis*) was first isolated in Chile, as the causative agent of Pisciricketsiosis [1]. *P. salmonis* produces a systemic aggressive infection that involves several organs and tissues such as kidney, spleen, liver, intestine, brain, skin, ovary and gills [2—4], producing severe mortalities in different salmonid species (with losses up to US$ 150 million per year [5]). This bacterium is a gram-negative, non-motile, aerobic and pleiomorphic (ranging in diameter 0.5—1.5 μm). The phylogenetic analysis based on sequencing of the 16S rRNA gene placed it in a new family of *Piscirickettsiaceae* within the class of γ-proteobacteria, most closely related to *Coxiella*, *Francisella* and *Legionella* [5,6].

There are few studies concerning evaluation of genetic resistance of the host to this pathogen. McCarthy et al. [7] detected the bacteria in cytoplasmic vacuole of macrophages in head kidney and spleen obtained from Salmon Rickettsial Syndrome (SRS) affected fishes. Therefore, the head kidney has been considered a key organ for studying the host response to this pathogen, and this has been validated at the transcriptomic level [8,9]. However, little is known about several aspects of host-pathogen interaction, such as for example, the bacterial load in resistant versus susceptible fish. Furthermore, this is a relatively new pathogen in Atlantic salmon (salmonids are only native to the northern hemisphere) hence the time frame for host-pathogen co-evolution is limited. Consequently, the relatively short evolutionary time is not enough for increasing the host fitness under constant exposure to the bacterium in aquaculture production. However, we have shown that significant genetic variation exists for disease resistance in different salmon populations [10], yet it is unknown how the different genetic backgrounds (associated with disease resistance) are related to bacterial load.

The aim of this study was: (a) to measure the bacterial load in head kidney and muscle of susceptible and resistant fish; (b) to estimate the transcriptional response of a set of immune related candidate genes associated with disease resistance. This will give important information about the distribution of the bacteria in different organs, which is a key issue when studying this disease from a genetic-epidemiological point of view.

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A challenge test was carried out, using a virulent isolate (LF-89) of *P. salmonis* maintained in the Unit of Aquatic Animal Pathology, University of Chile. The bacteria are grown in the cell line CHSE-214 to maintain its virulence. The inoculum of the bacteria was $0.2 \times 10^{4,8}$ TCID 50%/mL, and the individuals were inoculated with intra-peritoneal injection (IP) dose of 0.1 ml/fish. The challenge was carried out using IP in order to ensure that an equal dose of the bacteria was inoculated in each fish. Therefore, estimates of disease resistance (measured as mortality and bacterial load) are not expected to be confounded, with the initial dose of the bacteria. Due to the fact that IP is one of the routes that cause high mortalities [4], the challenge test applied in this experiment was quite stringent for finding resistant individuals within the population. Furthermore, previous studies have shown that even before 24 h after intact skin exposure, the bacteria can be detected in head kidney [4], probably due to the virulence of the bacteria, thus resistance measured using horizontal transmission (cohabitation) is expected to yield very similar results in terms of the familiar mortality (see below).

The animals used in the experimental challenge came from 29 different families obtained from a commercial Atlantic salmon population. This population is a good representative of the genetic variability observed in different Atlantic salmon stocks used for aquaculture [11]. A total of 85 individuals per family were marked individually using Pit-Tags, seven weeks before the disease challenge. They were maintained in fresh water following standard bioethical and husbandry practices. The mean body weight when the challenge started was 42 g (SD; 11 g). The individual mortality was registered until 52 days of post-inoculation, and the surviving fish were sacrificed on day 55. The heritability for disease resistance in this population was equal to 0.4, which was quite substantial, when compare to other diseases [10]. The susceptible fish was obtained as moribund fish with clinical signs of the disease, and resistant fish comprised those that survived at the end of the experiment. All individuals were subjected to euthanasia using an increase dose of the anesthetic tricaine methanesulfonate (MS-222) for relieving fish suffering. The bioethics and welfare committee of the Faculty of Veterinary Science, University of Chile, approved all procedures.

From the complete experiment we use two datasets; the data set A was generated from families representing two extreme groups in terms of disease resistance (genetically resistant versus susceptible families). These groups are composed of: 10 susceptible fish (moribund animals during 22–24 days after the incubation period) and 10 resistant fish (animals that survived at 52 days of incubation period). This dataset was used to identify differences in bacterial load between susceptible and resistant fish, in head kidney and muscle, obtained from the same individuals. The data set B consisted of additional samples (muscle) taking from dying individuals (n = 29) (susceptible fish) at different time point across the challenge test (21–45 days) or individuals survived at the end of the experiment (n = 15). This data set was used to assess differences between the individuals that die across all the experiment and the survivors of the distribution. The samples from head kidney and muscle were stored in RNA*later*® (Invitrogen) or ethanol for RNA and DNA extraction respectively until processing.

The DNA was extracted from samples of both tissues using a DNeasy Blood & Tissue Kit (Qiagen), and the RNA was extracted from head kidney with the GeneJET RNA Purification Kit (Thermos Fisher Scientific). A qPCR was performed using primers and hydrolysis probe of the *P. salmonis* 23 s [12], which has been modified according to the consensus sequence of a Chilean strain (LF-89) and our own *P. salmonis* genomic sequence (data not presented). The forward (TCTGGGAAGTGCGGCGATAGA), the reverse (TCCCGACCTACTCTTGTTTCATC) primers and the hydrolysis probe (56-FAM/TG

ATA GCC CCG TAC ACG AAA TGG CAT A/36-TAMSp) was used for amplification of 23 s of *P. salmonis*.

The reverse transcription of RNA was performed using the SuperScript II First-Strand Synthesis System (Life Technologies) with random primers. The qPCR reaction was made with the SensiMix™ II Probe Kit (Bioline). Each 10 μL of qPCR reaction included 2× SensiMix (including deoxynucleotide triphosphate, $MgCl_2$, and Taq polymerase), 0.5 μM of forward primer, 0.75 μM of reverse primer, 0.15 μM of hydrolysis probe and 50 ng of gDNA or 15 ng of cDNA. qPCR cycling conditions included a 10 min denaturation step (95 °C), followed by 50 amplification cycles (95 °C 10 s, 59 °C 60 s). In a separate reaction we amplified 23S fragment, using DNA isolated from *in vitro* cultured *P. salmonis*. Subsequently, this amplified fragment was purified from the agarose gel (GeneJet Gel Extraction kit; Thermo Scientific), quantified using fluorometry (with QUBIT protocol; life Technologies) and used for serial dilution to obtain a standard curve. Based on amplicon size (77 pb) and sequence, we calculated the copy number of the standard curve using the molecular weight of the amplicon (47620.82 Da), that is equivalent to 47620.82 g, as follows: 1 ng of amplicon contain $1 * 10^{-9} * 6.02 * 10^{23}/47620.82$ g $= 12641529482$ (copies), where $6.02 * 10^{23}$ is the Avogadro's number. The copy numbers in the samples were estimated based on the standard curve using a linear regression equation that relates Ct value and the amount of total DNA (ng) in the standard curve.

Raw Ct data and copy number was log transformed. The bacterial load was modeled based on the fact that significant genetic variation for SRS resistance, which is estimated based on differences between mean mortality between families. Alternatively, it could be assumed that the susceptible and resistant fish were two independent factors explaining the bacterial load. In all samples (kidney and muscle) the 23S genomic region was amplified and the expression of 23S gene was studied only in head kidney samples [11]. All the analyses were carried out using INFOSTAT [13].

To evaluate the transcriptomic response of susceptible and resistant individuals in response to the bacteria, we used a set of candidate genes (Table 1). These genes were differentially expressed in head kidney from an independent cohabitation challenge test with *P. salmonis* in seawater (Dettleff et al., In preparation). To measure gene expression we used the data generated from an RNA-seq experiment using head kidney (Martinez et al., in preparation). In this paper we used a sub-sample that is composed of 8 individuals (4 susceptible and 4 resistant), which were also used in the data set A. RNA sequencing was carried out in the Roslin institute, following the standard Illumina library preparation protocol (101bp paired-end). Quality reads obtain from the individuals were mapped (CLC genomics workbench, version 7.5) on the sequence of the candidate genes obtained from NCBI (Table 1). The differential expression of these genes was modeled using EdgeR [14] with a false discovery rate < 0.05 and absolute fold change >2.

The corresponding analysis of the 23S hydrolysis probe was used to evaluate the differential bacterial load given by the resistance of the host. The susceptible individuals showed a significantly higher bacterial load (p < 0.05) as compared to the resistant fish in all samples analyzed including muscle and head kidney. The mean differences of Ct between groups were 3.23 and 3.06, for muscle and head kidney respectively (Table 2). In contrast, the muscle showed a significantly higher bacterial load as compared to head kidney in all groups (irrespectively whether the fish is resistant or susceptible). The correlation between muscle and head kidney Ct was equal to 0.84 (Fig. 1). This is the first report demonstrating that the muscle is a critical tissue for bacterial aggregation. Considering that the *Piscirickettsiosis* syndrome showed severe muscle lesions (including cavernous lesions) it is somewhat expected that the muscle tissue could have been presenting a higher bacterial load.

Table 1
EdgeR analysis of mRNA abundance in head kidney for candidate genes in resistant and susceptible families after challenge with *Piscirickettsia salmonis*. Positive values for the fold change indicate increased expression in the susceptible group.

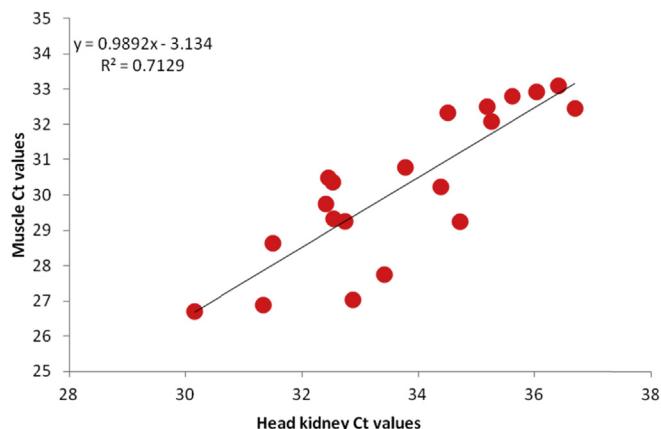| NCBI accession number	Candidate gene	P-value	Fold change	FDR p-value correction
LOC105030768	PREDICTED: *Esox lucius* haptoglobin-like, mRNA	2.67E-06	18.87	4.13E-05
BT057477.1	*Salmo salar* Serum amyloid A-5 protein putative mRNA, complete cds	2.19E-06	15.94	4.13E-05
LOC105027099	PREDICTED: *Esox lucius* putative ferric-chelate reductase 1, mRNA	9.58E-05	11.76	9.90E-04
AJ223954.1	*Oncorhynchus mykiss* mRNA for interleukin-1-beta	1.14E-03	7.42	7.07E-03
BT047979.1.	*Salmo salar* Tumor necrosis factor-inducible protein TSG-6 precursor putative mRNA, complete cds	4.83E-03	4.77	0.02
KC508833.1	*Hucho taimen* c-type lysozyme (LysC) mRNA, complete cds	7.49E-03	−2.62	0.03
FR734084.1	*Oncorhynchus mykiss* partial mRNA for complement factor I (cfi gene)	9.00E-03	7.44	0.03
BT059981.1	Hemoglobin subunit alpha-4 putative mRNA, complete cds	0.11	−2.16	0.23

Table 2
Average of Ct and $\log_{10}$ of copy number, and p-value of the t-test, for the dataset A (susceptible and resistant groups) in muscle and head kidney of fish challenged with *P. salmonis*.

Tissue	Group	Number of samples	Ct average	$\log_{10}$ of copy number average	p-value (t-test)
Muscle (DNA)	Resistant	10	31.84	1.93 (0.43)	<0.0001
	Susceptible	10	28.61	3.14 (0.44)	
Head kidney (DNA)	Resistant	10	35.26	0.64 (0.34)	<0.0001
	Susceptible	10	32.20	1.72 (0.35)	
Head kidney [11]	Resistant	10	32.73	—	0.0011
	Susceptible	10	22.78	—	

The expression level of the 23S gene in head kidney of resistant fish was significantly higher ($p < 0.05$) than the susceptible group (the Ct difference being equal to 9.95) (Table 2), indicating a higher bacterial replication, in the latter.

The mean family mortality and the copy number were linearly related, the correlation was 0.71 and 0.54 in muscle and head kidney, respectively (Fig. 2). The difference in the bacterial load of muscle in the data set B (individuals that die across the experiments and the survivors) was significant (Table 3). However, there was a greater heterogeneity in the susceptible group compared with the resistant group as seen in the coefficient of variation (Table 3). This is probably due to the individual susceptibility to the bacteria.

All the candidate genes except hemoglobin subunit alpha-4 appeared to be differentially expressed (DE) in the head kidney between the resistant and susceptible group. Out of DE genes, all showed increased gene expression levels in the susceptible group, except for the c-type lysozyme (LysC). Recent studies proved that this gene has a protective function towards bacterial infections, and it is expressed particularly in macrophages [15]. The gene with the highest fold-change was the Haptoglobin (Fold change: 18.87), whose expression is expected to be more related to acute stress and general inflammatory response [15].

2. Discussion

Until now, there is little information regarding the mechanism by which resistant Atlantic salmon respond to infection of *P. salmonis*. In our experiment, the family mortality was an important factor explaining the bacterial load of susceptible fish. Families showing a more susceptible genetic background (with a higher mean familiar mortality) exhibited a significantly larger bacterial load. Correlation analysis between mean familial mortality and bacterial load indicate that the genetic background for disease resistance of the host is a good predictor of bacterial load and that the resistant fish clear the bacteria from the different tissues examined.

Although the resistance mechanisms triggered by the fish are unknown, we showed that resistant fish were capable of mounting an effective mechanism of bacterial clearance, so that the bacteria remained almost undetectable in target tissues. On the other hand, the susceptible fish was not able to mount an efficient immune response, due to an exacerbated, yet inefficient, immunological response. Similar findings have been reported previously [8,9]. This could be one of the possible factors involved in the relative low rate of success when using antimicrobial drug treatments for controlling *P. salmonis* [16].

The clearance of pathogens in resistant Atlantic salmon has been observed on challenge tests with Infectious salmon anemia virus (ISAv) due to a specific T-cell response [17]. The susceptible fish generated an increased innate immune response, but failed to
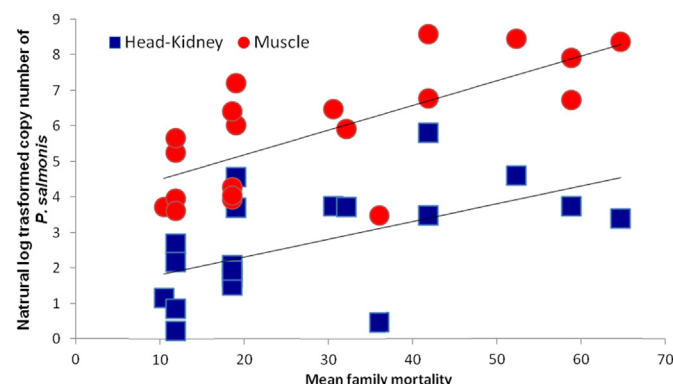

Fig. 1. Relationship between bacterial load (Ct) in kidney and muscle.

Fig. 2. Relationship between family mortality and copy number of *P. salmonis*.

Table 3
Average of Ct and $\log_{10}$ of copy number, coefficient of variation and p-value of the t-test, for set B (dead and survivor) in muscle of fish across the challenge with *P. salmonis*.

Group	Number of samples	Ct average	$\log_{10}$ of copy number average	Coefficient of variation	p-value
Dead (case)	29	28.56	3.74 (0.76)	20.36	<0.0001
Survivor (control)	15	34.18	2.11 (0.28)	13.17	

provide protection. Wang et al. [18] showed that catfish susceptible to *Edwardsiella ictaluri* presented increased acute phase responses, but the disease itself, could not be controlled.

Macrophages, monocyte-like cells and the endothelium are important for explaining the body dynamics and containment of *Piscirickettsia salmonis* [19,20]. Our results show that the monocyte-macrophage system associated gene LysC was up regulated in resistant fish, which is consistent with previous findings. This gene has been demonstrated to be highly inducible to lipopolysaccharide (LPS) of *Escherichia coli*, when compared to g-type lysozyme gene [21]. Meanwhile, the susceptible fish demonstrated an up-regulation of pro-inflammatory genes such as the cytokine IL-1b and acute phase response related genes such as haptoglobin and serum amyloid A, which could not seem to be an effective response against the bacteria. Similar observations have been reported by Refs. [18] and [22], where the exacerbated response of susceptible fish might be responsible for triggering more tissue damage mediated through cell apoptosis and oxidative damage.

Muscle is one of the tissues where the bacteria had been found and in some instances is severely affected by this pathogen [23]. It has been shown that skin is the main route of entry for the bacteria, even when the skin is without lesions [4]. Besides, severe compromise of muscle has been observed in several SRS outbreaks (Marcos Godoy, personal communication). However, in this study the skin was not considered as a direct route of infection, because the fish were intra-peritoneally inoculated with the bacterium. This was done in order to control the confounding effects of bacterial dose and mortality in cohabitation challenge tests. Cohabitation and IP challenge have been tested in other studies and it has been found that the genetic correlation between the mortality of the families between tests, was not significantly different from one [24]. Therefore, we assumed that the IP challenge test is a good predictor of mortality occurring under salmon production environment. Hence, we expect that the transcriptional response of genes implicated in disease resistance mechanism may be similarly affected under both challenge tests, which is indeed the case for the candidate genes tested.

On the other hand, it is important to note that iron is a key nutrient for the survival of the bacteria [25], and the bacteria could have developed different mechanisms for obtaining iron from the host. Still, this is an area of active research in bacterial transcriptomics (Alvaro Machuca, personal communication). The *heme* group constitutes the major iron source in animals and it is found in hemoproteins (hemoglobin and myoglobin). Studies in *Bordetella bronchiseptica* and *Lactobacillus sakei* showed that myoglobin can be an important source of iron because the heme-group is used as a source in a specific metabolic pathway of iron of these bacteria [26,27]. *P. salmonis* could grow in a cell free agar supplemented with hemoglobin [28]. Considering the above evidence where the heme-group play a key role as a source of iron for the pathogen survival, we infer that the significantly higher bacterial load in muscle could be explained by the potential use of the heme-group from myoglobin as a source of iron for *P. salmonis*.

Mortality is a well-recognized approach to measure disease resistance, such as for example, in several studies to evaluate the genetics behind of disease resistance or when measuring vaccination effectiveness. This paper shows the importance of measuring indicator traits such as the bacterial load of individuals for predicting disease resistance. We further observed that resistant fish could not be a likely reservoir of the bacteria after exposure to the pathogen that may be of paramount importance from an epidemiological point of view.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VM conceived and designed the study and contributed to the analysis of the results of RNA sequencing. PD and CB process the samples, perform the qPCR and carry out the molecular analysis. PD, CB, AP and VM contributed to the discussion of the results and preparation of the manuscript. VM provide the funding through the research project FONDECYT 1120608. All authors read and approved the final manuscript.

Acknowledgments

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