From the viral perspective: Infectious salmon anemia virus (ISAV) transcriptome during the infective process in Atlantic salmon (Salmo salar)

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ABSTRACT

The infectious salmon anemia virus (ISAV) is a severe disease that mainly affects the Atlantic salmon (Salmo salar) aquaculture industry. Although several transcriptional studies have aimed to understand Salmon-ISAV interaction through the evaluation of host-gene transcription, none of them has focused their attention upon the viral transcriptional dynamics. For this purpose, RNA-Seq and RT-qPCR analyses were conducted in gills, liver and head-kidney of S. salar challenged by cohabitation with ISAV. Results evidence the time and tissue transcript patterns involved in the viral expression and how the transcription levels of ISAV segments are directly linked with the protein abundance found in other virus of the Orthomyxoviridae family. In addition, RT-qPCR result evidenced that quantification of ISAV through amplification of segment 3 would result in a more sensitive approach for detection and quantification of ISAV. This study offers a more comprehensive approach regarding the ISAV infective process and gives novel knowledge for its molecular detection.

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1. Introduction

Infectious salmon anemia (ISA) is a viral disease that has been reported in different countries (Thorud and Djupvik, 1988; Rowley et al., 1999; Lovely et al., 1999; Bouchard et al., 2001; Godoy et al., 2008), and within the Atlantic salmon (Salmo salar) aquaculture industry, it has brought severe economic impacts, thus becoming a continuous concern for the industry. The etiological factor of ISA is the infectious salmon anemia virus (ISAV), an Orthomyxoviridae family member with a segmented genome composed of eight different single-strand RNA fragments of negative sense encoding for 10 different proteins (Clouthier et al., 2002; Merour et al., 2011; Mjaaland et al., 1997). Of these, segments 1, 2, and 4 have been reported coding for proteins of the polymerase complex PB2, PB1, and PA respectively (Clouthier et al., 2002; Snow et al., 2003; Ritchie et al., 2001). Moreover, it has been suggested that segment 3 encodes for the nucleoprotein (NP), while segment 5 and 6 for the viral surface proteins, fusion protein (F) and hemagglutinin esterase (HE) respectively (Aspehaug et al., 2005). Based on the cloning of the open reading frames (ORF) from segment 7, it has been proposed that this segments is involved in the expression of three different proteins, NSP1, NSP3, and NEP (Kibenge et al., 2007; Ritchie et al., 2002), while segment 8 is believed to encode for two matrix proteins, M1 and M2 (Clouthier et al., 2002; Cottet et al., 2011). Furthermore, based on the nucleotide variation of segments 2, 6, and 8, it has been proposed that different ISAV isolates can be classified into European (Norway, Scotland, and Chile) and North American (Canada and United States) genotypes (Cottet et al., 2011; Blake et al., 1999; Devold et al., 2001; Kibenge et al., 2001).

Due to the high impact that ISA outbreaks have on the S. salar aquaculture industry, many studies have been aimed at understanding the infective process of ISAV, as well as the host immune response, through the discovery of genes regulated during the infection. Thus, microarray-based high-throughput transcriptional studies have been focused on the transcription of host genes and the relevance of this regulation during the infective process (Jorgensen et al., 2008; LeBlanc et al., 2010, 2014). However, given that all of these studies place emphasis on the transcription of the host’s endogenous genes, the transcriptional dynamics of viral genes during the infection has not been addressed yet. In addition to the conformation of novel proteins that will be implicated in the replication and structure of the novel assembled virus, it has also been suggested that ISAV segments could act as antagonist for the interferon mediated immune response (Garcia-Rosado et al., 2008; McBeath et al., 2006). Thus, understanding the transcriptional dynamics of ISAV genes through host-infected tissues could provide novel insights into the modulation of the S. salar immune response during infection. Taking advantage a high-throughput transcriptome sequencing data yielded previously for S. salar exposed to ISAV, the present study
assessed the fraction of the *S. salar* transcriptome that have so far been overlooked in the use of microarrays-based approaches and that are related with the transcriptome of ISAV segments into host tissues. For this, RNA-seq analysis was conducted over different tissues of *S. salar* challenged by cohabitation with ISAV. The results revealed temporal patterns of viral accumulation in the gills, liver, and head-kidney tissues, in addition to a variation in transcription dynamics for different ISAV segments. These results offer more comprehensive approach regarding the ISAV infective process, new perspectives in the molecular detection of ISAV and its relevance for further immune studies in *S. salar*.

### 2. Materials and methods

#### 2.1. Experimental individuals

One thousand four hundred Atlantic salmon individuals (108 ± 7.74 g) from a commercial farm (Hornopirén, Puerto Montt, Chile) were transferred to the “Centro de Investigación y Transferencia Acuícola” Aquainnovo S.A (Lenca, Puerto Montt, Chile). Individuals were acclimated for 26 days and sanitarily checked to discard the presence of *Piscirickettsia salmonis* bacteria (SR5), the infectious pancreatic necrosis virus (IPNV), and the infectious salmon anemia virus (ISAV). After quarantine, *S. salar* individuals were randomly divided into four tanks (0.5 m$^3$) containing 160 individuals each.

#### 2.2. Experimental design

Just before the beginning of the challenge, head-kidney, liver and gills were sampled from eight randomly selected individuals, used as control group (T0). Then, for each tank, 12 fish (30%), henceforth called trojans, were Pit-tagged and received an intraperitoneal (IP) injection of 10$^7$ TCID$_{50}$ of the virulent HPR7b ISAV strain isolated by Novartis (NPV-090). The remaining 28 individuals (70%), were considered cohabitants and were used for tissue sampling. The mortality produced by the ISAV infection was molecular and clinically confirmed, with an ISA outbreak declared after three consecutive days of dead trojans. Then, head-kidney, gills and liver were sampled from healthy cohabitants 3 (T1), 7 (T2), and 14 (T3) days after the ISAV outbreak was declared, for a total of 4 sampled points. Eight individuals were randomly sampled per point, and tissues were stored in RNAlater® solution (Ambion, USA) at −80 °C until total RNA extraction.

#### 2.3. RNA isolation and illumina sequencing

From 0.3 mg of each tissue, total RNA was isolated using the RiboPure™ Kit (Ambion, USA) according to the manufacturer’s instructions. RNA concentration and purity were estimated using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). Furthermore, the RNA integrity number (RIN) was evaluated using the 2200 TapeStation (Agilent technologies, USA) with the R6K screen tape. Samples with RIN values above eight and with a 260/280 ratio equal to 1.8 were used for library construction.

Twelve different libraries were constructed with total RNA pooled from equal amounts of five randomly selected individuals per time and tissue sampled. Library construction was carried out using the Truseq™ RNA Sample Preparation Kit v2 (Illumina, USA) according to the manufacturer’s instruction. Two libraries were pooled in equal amounts and sequenced on six independent runs with the Miseq® Illumina Sequencer (Illumina, USA) at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), University of Concepción, Chile.

#### 2.4. Annotation of ISAV segments

Sequencing data analysis was performed using the CLC Genomics Workbench software (CLC bio, Denmark). Raw data obtained from six sequencing runs were filtered by quality and adapter/index trimmed. CLC bio’s *de novo* assembly algorithm was used to create a contig list from previously filtered reads using a mismatch cost = 2, insertion cost = 3, deletion cost = 3, length fraction = 0.8, similarity fraction = 0.8, and a minimum contig length = 250. Finally, contigs were adjusted by mapped reads, and end gaps where treated as mismatches. ISAV segments annotation was performed based on nucleotide similarity, thus, contig sequences were blasted against non-redundant (nr) nucleotide database (BLASTn) using a word size = 3, gap cost existence = 11, extension = 1, and a BLOSUM62 matrix. Contigs corresponding to the ISAV transcriptome were identified and used for further analysis.

#### 2.5. RNA-seq and RT-qPCR analysis

Using previously annotated ISAV segments as a reference, RNA-Seq analysis were performed including a minimum read length fraction = 0.8, minimum read similarity fraction = 0.8, and unsppecific read match limit = 10, in relation to the reference. Expression values were estimated as Read Per Kilobase of exon model per Million mapped reads (RPKM) and then normalized by totals using state numbers in reads per 1,000,000. ISAV segments were clustered according to their expression patterns and grouped in heat maps. Hierarchical clustering was applied with RIN values above eight and with a 260/280 ratio equal to 1.8 were used for library construction.
was performed estimating the Manhattan distance and by selecting an average linkage as a clustering strategy.

RT-qPCR was conducted over the 8 different segments of ISAV genome. Thus, specific primers for each segment are presented in Table 1. The qPCR runs were performed with StepOnePlus™ (Applied Biosystems, Life Technologies, USA) using the comparative ΔΔCt method. Each reaction was conducted with a volume of 10 μL using the Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA). The amplification conditions were as follows: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s and each sample was measured considering biological and technical triplicates for each sample. Five putative housekeeping genes (HKG), Elongation factor 1-alpha, β-actin, β-2-microglobulin, GAPDH, 18 s rRNA and S20 were statistically analyzed by NormFinder algorithm to assess their transcriptional expression stability. Here, ELF was selected as HKG for gene normalization. Expression values were estimate with ΔΔCt comparative method and statistical differences were assessed through Student’s t test. P-values < 0.05 were considered statistically different. RT-qPCR Primers used for the amplification of for each segment are presented in Table 1.

3. Results and discussion

Although several high-throughput transcriptional studies have been performed in S. salar exposed to ISAV, none have placed focus on the scope that the transcriptional regulation of viral genes has during the infective process (Jorgensen et al., 2008; LeBlanc et al., 2010, 2014). For this purpose, from the 196,846 contigs generated through de novo assembly (data not shown), it was possible to identify the eight different segments that constitute the ISAV genome based on nucleotide similarity (BLASTn). The results are summarized in Table 2, showing E-values equal to zero for eight different contigs generated by de novo assembly, therefore supporting an accurate identification of the ISAV genome (Clouthier et al., 2002). Considering that the lowest E-values were obtained with different segments from Norwegian and Chilean strains (data now shown), results corroborate that the ISAV strain used in this study corresponded to the European genotype. According to our results, just two different ORFs were identified in the Contig annotated to segment 7; meanwhile segment 8 evidenced the presence of the two ORFs. To our knowledge, this is the first report on all of the different segments of the ISAV genome for a South American strain.

Furthermore, RNA-Seq analysis was conducted using as reference the different ISAV characterized segments for the strain used in this study. Taking into account the number of reads matched against each segment, the percentage of viral reads among the total reads for each tissue and time was estimated. Regardless of the time and tissue analyzed, results evidenced that a low percentage of the complete transcriptome obtained by sequencing corresponded to the ISAV transcriptome (>0.1%). Similar results have been observed when comparing the percentage of viral Epstein-barr virus reads within total reads obtained from different RNA-Seq experiments (Arvey et al., 2012). In relation to the variation in the percentage of viral reads within total viral reads for each tissue and time analyzed (Fig. 1B), results showed that at T1, the gills had the largest percent of viral reads (7%), compared with the 4% and 1% reached at the same point in head-kidney and liver respectively. These results support that ISAV was transmitted from IP injected to healthy fish through cohabitation and confirms previous reports indicating that the gills are one of the first tissues affected during ISAV infection (Weli et al., 2013). On the other hand, regardless the tissue analyzed, the larger percentage of viral reads was reached at T2 (Fig. 1B). These results were ratified by RT-qPCR analyses, in which the larger levels of relative quantity (Rq) for each segment were shown at T2 (Fig. 3). In addition, differences in transcription values were evidenced in segment 8 evaluated through RT-qPCR and RNA-Seq, being higher in RNA-Seq data (Fig. 3). These results could be explained by the fact that libraries...
were constructed from purified mRNA from total RNA, meanwhile RT-qPCR was developed from total RNA. As a result, RT-qPCR expression values include both viral genome segments and mRNA.

Taking together the stabilized mortality curve in T3 (Fig. 1A) and the decrease in virus abundance at T3, results evidenced that resistant mechanism in host were triggered in response to viral infection, thus explaining the stabilized mortality and the decrease of viral loads assessed both by RNA-Seq and RT-qPCR.

In regards to individual viral gene transcription, different patterns were associated with each ISAV segments between all tissues and times analyzed (Fig. 2). According to the RPKM values, two main groups were identified through hierarchical clustering (Fig. 2). Segments coding for the polymerase complex (1, 2, and 4) evidenced lower transcription values when compared with the NP, HE, NSP, F and M proteins (3, 6, 7, 5 and 8 respectively) (Fig. 2). These results can be correlated to a previously reported analysis for the protein composition of the virion from other Orthomyxoviridae family members (Table 3), showing that PB2, PB1, and PA are the less abundant proteins while the nucleoprotein and the matrix proteins are more abundant. In relation to surface virion proteins, it has also been suggested that the HE protein is at least four times more abundant than the F protein (Lamb and Choppin, 1976; Lamb and Krug, 2001). The present results showed that transcription values of ISAV segments were in direct relation to the protein abundance previously shown for Orthomyxoviridae virions (Table 3), suggesting the possible existence of regulatory mechanisms for the ISAV transcriptome.

By analyzing the individual transcription values for each segment assessed through RT-qPCR, significant differences \((p < 0.05)\) were found in the transcription level of segment 3 and segment 8 (Fig. 4). Commonly, the molecular detection of ISAV is based on the

<table>
<thead>
<tr>
<th>Transcription values</th>
<th>RPKM</th>
</tr>
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<tbody>
<tr>
<td>PB2</td>
<td>28.543</td>
</tr>
<tr>
<td>PB1</td>
<td>36.462</td>
</tr>
<tr>
<td>PA</td>
<td>40.089</td>
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<tr>
<td>F</td>
<td>77.216</td>
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<td>NSP</td>
<td>136.787</td>
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<td>HE</td>
<td>200.313</td>
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<td>Mx</td>
<td>234.726</td>
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<tr>
<td>NP</td>
<td>245.865</td>
</tr>
</tbody>
</table>

Table 3

| Transcription values | Rq ± SE values obtained by sequencing Gills (G), Liver (L) and Head-kidney (K) in 3 (T1), 7 (T2), and 14 (T3) days after the ISAV outbreak. | S3 NP | S8 M |
|----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| Protein              | Virion structure                                                                                                                                         | Protein | Abundance |
| PB2                  | 51 PB2                                                                                                                                                | PB2    | 30–60     |
| PB1                  | 52 PB1                                                                                                                                                | PB1    | 30–60     |
| PA                   | 53 PA                                                                                                                                                | PA     | 30–60     |
| F                    | 54 Na                                                                                                                                                | Na     | 100       |
| NSP                  | 55 NSP                                                                                                                                                | NSP    | 130–200   |
| HE                   | 56 HE                                                                                                                                                | HE     | 500       |
| Mx                   | 57 Mx                                                                                                                                                | Mx     | 1,000     |
| NP                   | 58 NP                                                                                                                                                | NP     | 3,000     |

Fig. 3. Comparative of the expression values for the different ISAV segments obtained by qPCR and RNA-Seq, \(R_q \pm SE\) values obtained for the different segments of ISAV virus and RPKM values obtained by sequencing Gills (G), Liver (L) and Head-kidney (K) in 3 (T1), 7 (T2), and 14 (T3) days after the ISAV outbreak.

Fig. 4. Comparison of transcription values obtained from Segment 3 and segment 8 obtained by RT-qPCR, \(R_q \pm SE\) values obtained for segment 3 (blue) and segment 8 (red) assessed by RT-qPCR. Samples not connected with the same letter indicates significant differences \((p < 0.05)\) evaluated by Student’s \(t\) test.
amplification of the viral segment 8 through RT-qPCR [Snow et al., 2006]. However, based on the higher transcription values obtained for segment 3 in all tissues at early time points, the authors of the present study suggest that this segment should be evaluated as a more sensitive detection method that can be coupled into the standard techniques for the molecular detection of ISAV.

Conflict of interest statement
The authors declare that they have no competing financial interests.

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