# RNA-seq Analysis of Transcriptome Response to VHS-V Infection in Two Target Tissues of Resistant vs Susceptible Trout Clonal Lines.

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**ABSTRACT:** Viral diseases constitute a major threat for aquaculture industry. The Viral Hemorrhagic Septicemia Virus (VHSV) is a *rhabdovirus* reported to cause economic losses in salmonid farms. To investigate the molecular mechanisms of viral resistance/susceptibility, we undertook a whole-genome transcriptome analysis of susceptible versus resistant trout clonal lines by RNA seq analysis. We investigated the gene expression in spleen and rayed fin, two critical tissues in the disease progress. In spleen, for susceptible fish, we identified 19,324 differentially expressed (DE) genes whereas for resistant fish, 593 genes were found differentially expressed. For fin, the number of DE genes was dramatically lower, with 47 DE genes in susceptible fish and 28 in resistant fish. By using RNA-seq technology, we will characterize differences in the gene expression profiles between the two lines in a control and infected context. The final aim of this study is to decipher the genetic mechanisms underlying the resistance/susceptibility to VHS virus in trout.

**Keywords:** RNA sequencing; virus resistance; fish genetics

## Introduction

Viral diseases constitute a major threat for aquaculture industry. In particular, Viral Hemorrhagic Septicemia Virus (VHS-V) although historically being identified in farmed rainbow trout (*Oncorhynchus mykiss*), in Europe, is widely spread in wild and domestic marine or freshwater fish species. VHS-V is a negative-sense, single-stranded RNA virus belonging to the *Novirhabdovirus* genus of the family *Rhabdoviridae*. VHS is among the most studied fish viral diseases, especially in rainbow trout, where high rates of mortality have been observed during natural outbreaks.

One of the most thoroughly studied fish species is the teleost rainbow trout a major model organism for the study of carcinogenesis, toxicology, immunology, physiology, nutrition and evolutionary process (Review in Thoorgard et al. 2002). The amenability of fish to chromosomal manipulations makes the rainbow trout, among all agricultural species, ideal for genetic studies. Several clonal lines of trout were obtained by gynogenesis (induced all-maternal inheritance) or androgenesis (induced all-paternal inheritance). These lines offer nearly the same benefits than inbred and congenic lines in mice which are a powerful genetic model for the genetic dissection of traits.

Clonal lines of doubled haploid individuals exhibiting a wide range of susceptibility to VHS-V were produced (Quillet et al., 2007a). One major QTL for survival to VHS-V after waterborne infection was identified using doubled haploid individuals (Verrier et al. (2012a)). Two clonal lines having opposite response to VHS-V infection were selected to study antiviral mechanisms. By using RNA-seq technology, we will characterize differences in the gene expression profiles between the two lines in a naive context but also in response to waterborne infection. The final aim of this study is to decipher the genetic mechanism underlying the resistance/susceptibility to VHS virus in trout.

## Materials and Methods

**Doubled haploid fishes and virus challenge.** Homozygous clones of rainbow trout were produced as described in Quillet et al. (2007a). We selected two clonal lines with opposite survival rate after waterborne infection: one Susceptible S (0 % survival) and one Resistant R (~ 91 % survival).

Waterborne challenges were carried out with 12 to 15-months old juveniles (weight range from 105 to 115 g) using the VHS-V strain 07–71 (serotype 1) as described in Verrier et al. (2012a). Waterborne infection was performed as follow: Ten Fish were distributed into 10 liter aquaria and were infected by incubation for 2 h in a $4.5x10^4$ $pfu.ml^{-1}$ virus suspension in static water with vigorous aeration. Virus infected fishes and untreated fishes (control) were lethally anesthetized with 2-phenoxyethanol. Rayed fins (pelvic or anal) and spleens were taken after respectively 1 day and 6 days post infection, immediately, frozen in liquid nitrogen and stored at -80°C for RNA extraction.

**RNA extraction.** Three biological replicates (i.e. fish) for each line were used for extraction, with the exception of R rate at day 6 where 2 fish only were available. Total RNA was isolated from tissues by mechanical lysis in TriZol reagent (GibcoBRL, Invitrogen) followed by a cleanup procedure (RNeasy mini kit, Qiagen) including a treatment with RNAse-free DNase I (Qiagen) for 20 min at room temperature. The total RNA concentration and the quality of extracted RNA was determined with an Agilent 2100 Bioanalyzer and the RNA 6000 LabChip® kit (Agilent Technologies) according to the manufacturers' instructions.

**Illumina library preparation and sequencing.** The mRNA-Seq libraries were prepared using the TruSeq™

RNA Sample Preparation Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Briefly, Poly-A RNA were purified from 4 µg of total RNA using oligo(dT) magnetic beads, fragmented and retro-transcribed using random primers. cDNA were end repaired and 3′-adenylated, indexed adapters were then ligated. Ten rounds of PCR amplification were performed and the PCR products were size select on a 2% agarose E-Gel (Thermo Scientific). Libraries were checked on Agilent High Sensitivity DNA Kit and quantified with the QPCR NGS Library Quantification kit (Agilent Technologies). Tagged cDNA libraries were pooled in equal ratios and sequenced in pair end 2x100bp on Illumina HiSeq2000 with TruSeq™ v3 Kit.

**Mapping reads and gene expression counts.** The read quality has been checked with FastQC in the ng6 environment (Mariette et al. (2012)). The reads were then spliced-aligned to the trout reference genome using TopHat v2.0.5 software (Trapnell et al. (2009)). All the resulting bam files were merged to produce a unique reference alignment on which the discovery of new genes and transcripts was performed using Cufflinks 2.0.0 (Trapnell et al. (2012)). The resulting GTF file was used as reference for the quantification step. All samples were quantified on the same reference. The GTF file was also used to extract the fasta sequence of the newly discovered transcripts in order to annotate them.

The quantification was performed using a locally modified version of cufflinks (sigcufflinks, available upon request on sigenae.org) which produces, per sample, an additional file containing the counts of aligned reads and read pairs. All the count files were merged to produce the expression table. The INDELs and SNPs are called on all the files using GATK software (McKenna et al. (2010)).

**Statistical analyses.** Concordance among biological replicates was done by principal component analysis with R software (version 3.0.2). A filtering procedure between replicates was applied by using HTSfilter R Package (Rau et al. (2013)). Differentially expressed genes (DEG) were identified using DESeq2 R package (Anders et al. (2010)) with an adjusted p-value less than 0.01 and an absolute log2 Fold Change =1.

## Results and Discussion

A total of 3.119 billions reads were produced. The numbers of reads which passed the quality filtering step was 1.483 and 1.635 billions for fin and spleen respectively (Table 1). Principal Component analysis using significantly differentially expressed genes (DEG) showed high concordance among triplicates for each line and clear separation between infected and control samples (Data not shown).

The number of DEG was higher in the susceptible line compared to resistant one whatever the tissue (Table 2). In susceptible line, an extensive modification of the transcriptome was observed in the spleen (Figure 1), while it was modest in fins (Data not shown). The gene expression levels for DEG obtained in the resistant line was lower than in the susceptible one which is consistent with the resistance status. Gene pathway analysis is underway, preliminary results show that a significant set of interferon stimulated genes has been found induced in infected susceptible spleen, thus validating our experimental strategy.

**Table 1. Number of quality trimmed reads for each treatment.**

| Line[§] | Tissue | Treatment[¥] | N° of reads |
|---|---|---|---|
| S | Fin | CTRL | 189E+06 |
| | | WI | 542E+06 |
| | Spleen | CTRL | 283E+06 |
| | | WI | 573E+06 |
| R | Fin | CTRL | 273E+06 |
| | | WI | 477E+06 |
| | Spleen | CTRL | 278E+06 |
| | | WI | 499E+06 |

[§]S= susceptible line, R = resistant line.
[¥]CTRL= control, WI = waterborne infection by VHSV.

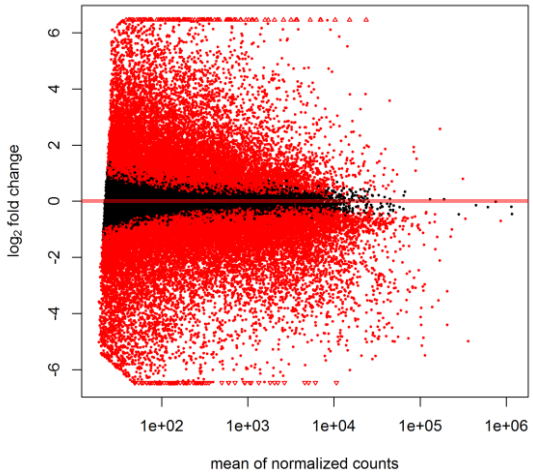


**Figure 1. MA plots displaying differentially expressed genes in spleen in control versus infected fish in the susceptible line.**

**Table 2. Numbers of differentially expressed genes in the two lines after VHS-V waterborne infection.**

| Tissue | Line S | Line R |
|---|---|---|
| Spleen | 19,324 | 593 |
| Fin | 47 | 28 |

Comparison of genes and signalling pathways according to the resistant or susceptible status will provide a whole description of the host transcriptional response to VHS-V in two relevant tissues: the spleen, major lymphoid organ involved in cellular immune responses and the fin showed to be the main portal of entry for *Novirhabdovirus* (Harmache et al (2006)). It has also been demonstrated that early viral growth in excised fin tissue is phenotypically and genetically correlated to survival (Quillet et al, 2001, 2007a; Verrier et al 2012a). We previously showed that the predominant mechanism involved in resistance should be an intrinsic or innate one, rather than a systemic immune response (Verrier et al. (2012b)). The whole comprehensive and integrated view of transcriptomic response provided here will help to describe without a priori the genes and associated pathways involved in virus response in a highly

susceptible context. We will also provide a snapshot image of transcriptome changes after viral infection in a resistant line. One of the expected deliverable is to provide new functional candidate genes for QTL mapping strategies.

We implemented here a whole-genome gene expression study by using two different clonal lines with disparate resistance to the virus. We will capitalise on using clonal lines by accumulating different and complementary datasets. As genomic resources (i.e. SNPs) are being developed in trout, data from structural polymorphism will be combined with functional data obtained here which will provide a better understanding of genetic mechanisms to VHS-V resistance.

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**Literature Cited**

Anders, S., Huber, W. (2010). Genome Biology. 11:R106.

Harmache, A., Leberre, M., Droineau, S., et al. (2006). J.Virol. 80 : 3655-59.

Mariette, J., Escudie, F., Allias, N., et al. (2012). BMC Genomics. 13, 462.

McKenna, A., Hann, M., Banks, E. (2010). Genome Res. 20: 1297-303.

Rau, A., Gallopin, M., Celeux, G. et al. (2013). Bioinformatics. 29 :2146-52.

Quillet, E., Dorson, M., Aubard, G., et al. (2001). Dis Aquat Organ. 45 : 171-82.

Quillet, E., Dorson, M., Aubard, G., et al. (2007a). Dis Aquat Organ. 76:7-16.

Quillet, E., Dorson, M, Le Guillou, S. et al. (2007b) Fish and shellfish Immunol : 22,510-19.

Thorgaard, G.H., Bailey, G.S., Williams, D., et al. (2002). Comp Biochem.Physiol B Biochem.Mol.Biol. 133 : 609-646.

Trapnell, C., Pachter, L., Salzberg, S.L. (2009). Bioinformatics. 25: 1105-11

Trapnell, C., Williams, B.A., Pertea, G., et al. (2010). Nature Biotechnology. 28:511-5

Verrier, E.R., Dorson, M., Mauger, S. et al. (2012a). Plos One. 8:e55302.

Verrier, E.R., Langevin, C., Tohry, C., et al. (2012b). Plos One, 7 :e33935.