Innate response of rainbow trout gill epithelial (RTgill-W1) cell line to ultraviolet-inactivated VHSV and FliC and rhabdovirus infection

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ARTICLE INFO

Keywords:
Microarray
VHSV
SVCV
FliC
Edwardsiella tarda
Innate immunity
Gill epithelium

ABSTRACT

Gills reportedly play a crucial role in induction of an antiviral immune response in fish. We investigated the expression of innate response genes in the rainbow trout gill epithelial cell line RTgill-W1 36 h after pretreatment with ultraviolet-inactivated viral hemorrhagic septicemia virus (UV-VHSV), flagellin C protein from Edwardsiella tarda (FliC), VHSV and SVCV using an Agilent 4 × 44k cGRASP salmonid microarray. RTgill-W1 cells pretreated with UV-VHSV, triggered an independent gene expression profile from those treated with a recombinant flagellin C protein from Edwardsiella tarda. In addition, exposure of RTgill-W1 cells to live viruses spring viremia of carp virus and viral hemorrhagic septicemia virus induced a less robust transcriptional change of 24 and 22 gene probes, respectively, when compared to 123 genes for UV-VHSV. Further the pretreatment of RTgill-W1 cells with (UV-VHSV) significantly reduced VHSV genome copy number at 6 d post infection (dpi) relative to the FliC-treated and untreated control. A quantitative PCR was used to study the transcriptional modulation of a set of 25 innate immune-related genes highlighted by the microarray data and a panel of 7 established antiviral genes in the protected cells. Notably, the expression of ifn1, ifn2, mx1 and mx3 were expressed more in untreated cells than in UV-VHSV-treated cells where virus replication was inhibited. The results from this study shed light on the mechanisms and pathways used by teleost gill epithelium innate immunity in combating viral and bacterial infection.

Introduction

Viruses in the family Rhabdoviridae such as viral hemorrhagic septicemia virus (VHSV) from the genus Novirhabdovirus and spring viremia of carp virus (SVCV) in the genus Sprivivirus (International Committee on Taxonomy of Viruses 2014) can infect a range of fish species and result in serious disease [1].

The role of the innate immune system in combating rhabdoviridae infections in fish has been fairly well studied and shown to play a critical role in controlling disease progression [2,3]. The stimulation of innate immunity in the absence of virus-specific antigen by plasmid DNA or CpG both leads to decreased VHSV III load in infected turbot Scophthalmus maximus [4] and to induction of antiviral gene markers such as type I interferon (ifn-I) and major histocompatibility complex class I (MHC-I) expression in vaccinated rainbow trout Oncorhynchus mykiss [5].

Despite the fact that the gills are the first tissue to encounter most infectious agents of fish, the contribution of the gill epithelial cells in immunity against rhabdoviruses has been the focus of only a few studies. In disk abalone Haliotis discus infected with VHSV IVa by waterbath there were 88 immune-related genes upregulated in gill tissue including transcription factors, tumor necrosis factor superfamily members, Fas ligands, interferon regulatory proteins and detoxification proteins (glutathione peroxidase) [6]. Also, in rainbow trout after water borne infection with VHSV I the expression of several chemokine receptor genes such as CCRI7, CCR9, CXCR3B and CXCR4 were upregulated at 1 day post infection and attracted leukocyte populations to the gill tissue soon after exposure [7]. Gills are a blood-rich tissue and recent studies have also demonstrated that nucleated red blood cells activate the complement cascade in blood and type I interferon signaling in head

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https://doi.org/10.1016/j.fsiarep.2021.100043
Received 1 September 2021; Received in revised form 8 December 2021; Accepted 8 December 2021
Available online 10 December 2021
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kidney after VHSV la infection in rainbow trout [8]. In rainbow trout infected with VHSV Ivb by intraperitoneal injection the virus is immuno-localized in necrotic gill epithelium for up to 38 d, indicating the involvement of this tissue in viral shedding [9]. The rainbow trout gill cell line (RTgill-W1) [10] has long been used in investigation of the role of the gill epithelium during viral infection. The innate immune response of this cell line differs depending on the subgenotype of VHSV; VHSV IVa infection results in greater expression of ifn-1 and ifn-II than infection with Ivb [11]. Pretreatment of RTgill-W1 cells with heat-inactivated VHSV (HA-VHSV) Ivb induced temporary protection from very low multiplicity of infection with VHSV as evidenced by a significant decrease in viral loads six days after infection and a two-day delay in cytopathic effect (CPE) [12], highlighting the potential of an innate immune response of the gill epithelium to reduce the impact of viral infection. In addition, there was no significant change in expression of ifn1 and ifn2 and Mx1 in pretreated cells [12].

In the present study, gene expression of RTgill-W1 cells exposed to ultraviolet irradiation killed VHSV (UV-VHSV), VHSV and SVCV, and Edwardiella tarda flagellin protein C (Flc) was investigated by two-way Agilent 4 x 44k cGRASP salmonid microarray at 36 h post-exposure. Flc is a TLR-5 ligand and a major virulence gene of E. tarda [13,14], which is expected to trigger different innate immune system pathways. There is also a growing interest in Flc as a vaccine adjuvant since it induced protective immunity against E. tarda [15]. The effect of pretreatment of RTgill-W1 cells with UV-VHSV and Flc 36 h before infection on VHSV-load post infection at 1, 3, and 6 d was studied to correlate the viral load after infection with the gene expression of a focused set of genes highlighted by the microarray study and the established major innate antiviral genes like IFNs and Mx.

Materials and methods

Cell lines

A low passage number (n = 25) RTgill-W1 cell line [10] was cultured in 175 cm² tissue culture flasks (Corning Incorporated, Corning, NY) and 6-well plates (Falcon®, Corning Incorporated – Life Sciences, Durham, NC). Leibovitz’s 15 Medium (L15) supplemented with L-glutamine (Gibco® Life Technologies Corporation, Grand Island, NY) was used. The pH of the culture medium was adjusted to 7.6 then filtered (0.2 μm); Thermo Scientific™ Nalgene™ MF75™; Waltham, MA) and supplemented with 2% or 10% fetal bovine serum (FBS) (Gibco®) and 1% antibiotic-antimycotic (Gibco®). In all conditions, cells were incubated at 15 °C.

Epitheliotha papulosum cyprini (EPC) cells [16] were used to propagate both VHSV and SVCV as above, except the medium was supplemented with 2% FBS.

UV-inactivated VHSV (UV-VHSV)

VHSV was purified from infected EPC cell culture supernatant on a sucrose gradient [17] and quantified by RT-qPCR [12]. Briefly, the virus supernatant was centrifuged (90,000 x g; 3 h; 4 °C; SW28 rotor; Beckman Coulter Canada, Mississauga, ON) over a cushion of 25% glycerol (FisherBiotec, Fair Lawn, New Jersey BP229.1) in TBS buffer (300 mM NaCl; 100 mM Tris–HCl; pH 7.4). Pellets were resuspended in 100 μl TBS buffer for 24 h at 4 °C. The collected suspension was centrifuged (90,000 x g; 18 h; 4 °C) on a continuous sucrose gradient (from 60% to 10%) in TBS buffer (SW41; Beckman Coulter). The virus band was collected, diluted with 10 ml TBS, and re-pelleted (90,000 x g; 4 h; 4 °C; SW41) and re-suspended in 10 ml TBS buffer and stored at –80°C. The purified virus was then inactivated before use by exposure to two rounds of UV radiation 1 Joule cm⁻² using Crosslinker (Electronics Corporation, Westbury, New York, USA) [18].

VHSV and SVCV propagation

EPC cells were infected at multiplicity of infection (MOI) = 0.01 with VHSV Ivb (U13653) [19] and SVCV (HHOcarp06) [20]. The infected cells were incubated with the virus for 1 h, washed with sterile PBS (1x; 0.0067 M PO4; Hyclone®, Hyclone Laboratory Inc., Logan, Utah), and incubated at 15 °C and 21 °C for VHSV and SVCV, respectively, until 70% cytopathic effect (CPE) was reached.

The supernatant was clarified by centrifugation (4000 x g for 30 min) and the virus titer was determined using plaque assay (Batts & Winton 1989). Briefly, EPC cells in a 6-well tissue culture plate (Falcon®) were infected with ten-fold serial dilutions of the virus for 1 h incubation then washed with PBS (Hyclone®). After infection and washing, each well was topped with L15 Medium (2% FBS, Gibco®, and 1% antibiotic-antimycotic, Gibco®; and 0.9% methylcellulose, Sigma-Ulrich Co, St. Louis MO) and incubated at a suitable temperature (above) for 1-4 d until adequate size plaques developed. Cells were fixed and stained with 0.1% crystal violet in 10% buffered formalin. The virus titer was expressed in plaque forming units (PFU)/ml.

Flc

Flc, an Escherichia coli recombinant Edwardsiella tarda flagellin protein C, was cloned in E. coli using a translation vector (PET43). Then, it was co-translated with a tag protein, NusA, in the form of Flc—NusA complex (Flc, here on) to allow purification using ion affinity chromatography (IMAC). The translated tag-protein alone, NusA, was used as a negative control for Flc treatment. The recombinant proteins were filtered through endotoxin removal resin column (Thermo Scientific™ Pierce™ High-Capacity Endotoxin Removal Column) using the manufacturer’s protocol followed by quantification using Nanodrop (ND-100; Nanodrop Technologies, DE, USA). The sizes of the purified proteins were confirmed by SDS-PAGE. Briefly, proteins were mixed with Laemmli buffer [21], and heated at 95 °C for 10 min then separated on 15% polyacrylamide gel.

Total RNA extraction from RTgill-W1 cells

Before trypsinization (Gibco® TrypLE Express, life Technologies Corporation, Grand Island, NY) cells were washed with verseine (130 μM NaCl; 1.47 μM KH₂PO₄; 2.68 μM KCl; 20.42 μM Na₂HPO₄·12H₂O). The collected cells were pelleted by centrifugation (4000 x g for 5 min). Total RNA was extracted using Trizol (Ambion® by Life Technologies; Carlsbad, CA) and a standard protocol. Briefly, cells were homogenized in 0.3 ml Trizol then topped with another 0.7 ml more. After 5 min, 0.2 ml of chloroform (ACROS Organics, New Jersey) was added and mixed gently for 10 min. The mix was centrifuged (13,000 x g, 15 min, 4 °C) and the aqueous phase was collected into a clean tube. An equal amount of ice-cold isopropanol (Fisher Scientific, Fair Lawn, New Jersey; ~ 0.6 ml) was added to the aqueous phase and centrifuged (13,000 x g, 30 min, 4 °C). The supernatant was decanted, and the RNA pellet was washed two times with ice-cold 75% ethanol (Commercial Alcohols, Brampton Ontario). Finally, the RNA pellet was suspended in 30 μl of RNase-Free water (Sigma).

RNA was DNase-treated with RNase-Free DNase Set (Qiagen®, Toronto, ON). Enzymatic reactions were performed in solution following the kit manual in 100 μl volume for 10 min followed by clean-up of the RNA by RNasey Mini Kit (Qiagen®, Toronto, ON) and according to the manufacturer’s protocol. Extracted RNA concentration and purity were measured using a Nanodrop and stored immediately at –80 °C. For all RNA used for microarray, the RNA integrity was assessed by 2100 Bioanalyzer (Agilent) and was confirmed to have an RNA integrity number (RIN) higher than 9.
Quantitative real-time PCR

cDNA was prepared from RNA samples using a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Ottawa, Ontario). RNase inhibitor (RNasin®, Promega, Wisconsin, USA) was added to the cDNA master mix during the processing. The preparation of cDNA was performed in a 20 µl volume and following the standard protocol. The cycling condition for cDNA synthesis was 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and on hold at 4 °C. Finally, the cDNA was stored at −20 °C for future use.

Primers used for gene expression analysis were designed using Primer3Plus (Version 2.4.0) [22]. qPCR was performed in a 10 µl reaction volume in 96-well plates (LightCycler® 480 Multiwell Plate 96, Ottawa, Ontario) using Evagreen® SoFast™ Supermix (BioRad, Mississauga, Ontario) and following standard protocols. The cycling conditions, using the Light Cycler (Roche LC480®, Ottawa, Ontario), were adjusted to 98 °C for 3 min followed by 40 cycles of 98 °C for 5 s, 60 °C for 5 s, and 72 °C for 1 s and finally a melting curve from 65 °C to 95 °C.

VHSV RT-qPCR

VHSV nucleoprotein gene transcripts (hereafter viral load) were quantified using a one-step absolute quantification RT-qPCR [12]. The product is a 101 bp sequence from the VHSV nucleoprotein gene. The PCR reaction was performed in a total volume of 10 µl containing 100 ng of template RNA using Roche LightCycler 480 Master Hydrolysis Probes (Roche Applied Science) and following standard protocol. The cycling conditions were reverse transcription 63 °C for 3 min; denaturation, 95 °C for 30 s; and amplification (37 cycles) 95 °C for 12 s, 60 °C for 45 s at, and 72 °C for 1 s.

Gene expression profile of RTgill-W1 cells at 36 h post-exposure

Experimental design

Microarray assay was used to investigate differential gene expression in pretreated (exposed) RTgill-W1 cells compared to a common reference (untreated RTgill-W1 cells; 2% FBS; 1% antibiotic antimycotic, Gibco). The common reference was applied to all arrays. The cells were exposed to pretreatments in 6-well cell culture plates (Falcon™) in which, each well represented a separate unit. For every one biological replicate, six wells on each plate were trypsinized and pooled. Three or four biological replicates were used for each treatment. At 36 h post-treatment of RTgill-W1 cells, with VHSV; SVCV; UV-VHSV; FliC; and NusA cells, they were collected for RNA extraction. In VHSV and SVCV infections, cells were infected at 0.1 MOI. In the UV-VHSV pretreatment, cells were exposed to 10 MOI inactivated virus. FliC was diluted in L15 (2% serum; 1% antibiotic antimycotic, Gibco) to a final concentration of 50 µg ml⁻¹ and similarly, NusA was diluted to 38.8 µg ml⁻¹. All treated groups were compared to untreated non-infected control.

Analysis of microarray data

The universal reference design was used to compare all treatments to a common reference. The data from a total of 17 arrays were analyzed. The arrays included three biological replicates of VHSV, SVCV, and NusA; and 4 biological replicates of UV-VHSV and FliC all distributed into at least 3 of 5 microarrays slides. All treatments were compared against a pooled control reference. The statistical analysis of the microarray data was performed using R (ver. 3.2.0) and linear models for microarray analysis (LIMMA) package [23]. The background was corrected by subtracting the background median signal intensities from foreground in each spot with an offset of 16, meaning that data from spots that had values less than 16 were deleted. Within-array variations were corrected using global loess normalization method. Between-array normalization was performed using the “T-quantile” function that ensured that all A-Values for each group had the same distribution across arrays without changing the M-values (log-ratios) [24]. The common reference samples were dye-swapped to enable the normalization of dye bias.

Differentially expressed probes were annotated using the cGRASP publicly available annotation file. The remaining, non-annotated, probes were manually annotated by blasting the probe or contig sequences to the database using Megablast to search for highly similar sequences in Atlantic salmon or rainbow trout.

Gene ontology (GO) analysis and pathway analysis were performed using the database for annotation visualization and integrated discovery (DAVID, version 6.7) [25]. The functional annotation analysis was performed for all Entrez-Gene ID lists using the modified Fishers exact P value (EASE) score of 0.1 to determine all significantly enriched GO terms and pathways. A significantly enriched GO term identifies that gene members in under this category represent a significant value from the total gene members in this group. The GO analysis of genes lists was performed using InnateDB for VHSV and SVCV.

Validation of microarray data analysis by PCR

Relative gene expression analysis was performed for 10 selected genes that demonstrated differential gene expression by microarray; these were APOA1BP, CLU, ENOSF1, IL-11, LITAF, MALT1, METB, MMP13, PLK2, and TPM1. Primers used and the accession codes of the target gene are listed in Table 1. Pearson correlation coefficient was estimated using STATA 14. To correlate PCR data with microarray, similar cutoffs for expression values were used i.e. only values with significant adjusted P values and differential expression higher than 0.75 log2 fold change were tested by Pearson correlation.

Viral load and gene expression of RTgill-W1 at 1, 3 and 6 d post-infection with VHSV in cells pretreated for 36 h before infection with UV-VHSV, FliC, and untreated control

Experimental design

RTgill-W1 cells were either untreated then infected or pretreated for 36 h with FliC, NusA, and UV-VHSV then infected with VHSV (MOI=0.1). An additional control group was cells that were neither treated nor infected. The NusA is the carrier protein for FliC and was included as a treatment control of FliC pretreatment. The infected cell monolayer was collected at 1, 3, and 6 dpi for evaluation for viral load and gene expression in parallel with the untreated non-infected cells. The UV-VHSV was applied in a final concentration of 10 MOI and the FliC was applied at a final concentration of 50 µg ml⁻¹. Moreover, the vector’s NusA was diluted to a final concentration of 38.8 µg ml⁻¹ for use on cells as a treatment control.

Exposures were applied in 6-well cell culture plates (FalconTM) in which, each well represented a separate unit and every biological replicate included six randomly pooled wells. Three independent biological replicates were used for each pretreatment.

Viral load

VHSV load was quantified in all pretreated groups using RT-qPCR (above) at 1, 3, and 6 dpi and compared to untreated infected control.

Differential gene expression

The differential gene expression was studied in all pretreatments and untreated groups using quantitative real-time PCR (above) and both were compared to untreated non-infected control.

The gene expression of a focused microarray-highlighted innate...
Table 1 (continued)

| Orthologue Name | Direction | Sequence | Accession |
|-----------------|-----------|----------|-----------|
| SRGN            | Forward   | GAGGGTCTGCCACAGGCA  | XM_036956252.1 |
| TPR1           | Reverse   | GGCAAGGATCTACAGGCA  | BT045316.1 |
| TRIM16          | Forward   | ATTCCAGGAGCCCTGCTG  | XM_021619011.2 |
| Type 1 interferon1 | Reverse   | AAGCAGCTGCAGGCTGCTG  | AY788890.1 |
| Type 1 interferon2 | Forward   | AAGCAGCTGCAGGCTGCTG  | AJ582754.2 |

responders panel of 25 genes was tested and also an established innate antiviral gene panel including, mx1, mx2, mx3, ifn1, ifn2, md25 and lgp2a. Primers and accession codes of targeted genes are listed in Table 1.

Statistical analysis

For absolute quantification of VHSV nucleoprotein by RT-qPCR, all CT values were compared to those of the known standards (gBlocks™, IDT, Iowa, USA). The quantification values were compared between pretreatment groups using a Student's t-test (STATA 14). Comparisons of viral load between treatments with P values less than 0.05 were considered statistically significant.

For microarray, the ratios of differential expression values were fitted in a generalized least square linear model followed by an empirical Bayes method to calculate the differential expression. Differential expression values that had less than a 0.7 log fold change were not considered biologically significant. Only differential expression values that had a false discovery rate (FDR) adjusted P values less than 0.05 were considered statistically significant.

For gene expression using qPCR, all gene expression CT values were normalized to the average CT values of the internal control β-actin, which was stable with no significant differences in CT values between treatments for each timepoint. Differential gene expression between UV-VHSV and untreated cells was calculated from expression values 2^ΔCT values compared using t-test after accounting for equal variance or Kruskal-Wallis if data did not follow normal distribution (Shapiro Wilk Test). Expression values that had P values less than 0.05 were considered statistically significant.

Results

Microarray analysis of differential gene expression in RTgill-W1 after stimulation

RNA integrity testing and quality control of the microarray hybridization indicated that all arrays met acceptable standards and data represent the differential gene expression after analysis. The normalized microarray data diagnostics is presented graphically using a box plot and MA plot. The coefficients quantiles of the fitted model were plotted against the theoretical quantile of a Student’s t-distribution to demonstrate graphically the differentially expressed probes and highlight that the majority of the data was normally distributed. Only differential expression with a log2 fold change more than 0.75, and an adjusted P value less than 0.05 were considered significant. Only biologically important functional annotation groups are presented. Clusters of groups are also classified based on similarity of biological meaning indicated by shared genes within these groups.

To validate the microarray results a separate qPCR assay was developed for 10 genes selected from the microarray results. A Pearson correlation coefficient of 0.9063 was calculated for the comparison
between the significant log2 expression values that had more than 0.75 log2 fold change by microarray and by RT-qPCR (Fig. 1). The expression profiles induced by both UV-VHSV and FliC highly matched in both PCR and microarray in nine out of ten genes. Generally, gene expression by qPCR had higher expression values and was more sensitive (Fig. 2). RTgill-W1 cells expressed distinct gene clusters when exposed to PAMPS from the viral and bacterial origins (Fig. 3).

The treatment of RTgill-W1 with the UV-VHSV, VHSV, SVCV, and FliC resulted in significant regulation of 121, 12, 35 and 190 individual probes, respectively, from the untreated control (Appendix 1.)

**RTgill-W1 gene expression 36 h post exposure to UV-SVHSV**

The treatment of RTgill-W1 with UV-VHSV resulted in the significant regulation of 121 probes (103 identified genes) of which, 48 were upregulated and 73 were downregulated. The available functional annotation information on these probes allowed the mapping of 95 genes. All significantly enriched GO terms are presented (Appendix 2), however, only a few are highlighted (Table 2.)

**RTgill-W1 gene expression 36 h post exposure to VHSV and SVCV**

After VHSV and SVCV infections, a total of 12 (11 annotated genes) and 35 (32 annotated genes) probes were regulated, respectively. The regulated genes were predominantly upregulated, 9 & 26 genes versus 3 and 9 downregulated, in VHSV and SVCV, respectively.

Due to a smaller number of regulated genes, gene ontology analysis of these groups was performed for individual molecules using InnateDB. There were some molecules of particular interest in both infections. Genes that were upregulated included TPM1, ENOA, and S100-A5-like after both VHSV and SVCV infections; PMSD3 and RPS23 was upregulated only after VHSV infection; and, COTI1, INSIG1, TXN, and FLNA were upregulated only after SVCV infection.

Genes downregulated included CUEDC1 after both VHSV and SVCV infections; ID1 and LITAF only after VHSV infection; and MMP13, IL-11, DUSP6, NT5DC1, PKM, IRES only after SVCV infection.

**RTgill-W1 gene expression 36 h post exposure to FliC**

The pretreatment of RTgill-W1 cells with FliC resulted in significant regulation of 190 individual probes (128 identified genes) of which, 125 were upregulated and 65 were downregulated. The functional annotation of regulated probes using DAVID resulted in 158 significant functional annotation categories that were clustered based on similarity among their gene members (Appendix 3). Due to the scope of the study only three GO terms are described (Table 3). Interestingly, NusA also resulted in modulation of gene expression in 51 probes (44 annotated genes) of which thirty probes (27 annotated genes) were shared with FliC.

**Viral load and gene expression of RTgill-W1 at 1, 3 and 6 d post-infection with VHSV in cells pretreated for 36 h before infection with UV-VHSV-FliC, and untreated control**

**VHSV load**

The pretreatments of RTgill-W1 cells induced a reduction in VHSV load. At 3 dpi, only FliC-pretreated cells had a significant reduction in viral load. At 6 d, however, the all pretreatments significantly induced a reduction in viral load. The UV-VHSV-pretreated cells had the highest reduction of 2.15 log2 fold less VHSV than in the untreated controls. There was no significant difference between the reduction caused by FliC and NusA alone at 6 dpi (Fig. 4).

**Differential gene expression in UV-VHSV treated cells and controls**

The differential expression in infected RTgill-W1 cells at 1, 3, and 6 d post-VHSV infection of 25 selected genes highlighted by microarray results was detected by qPCR in contrast to untreated non-infected controls. The genes tested were APOH, BLMH, CDK1, CEPEB, CFH, CIDE, DCN, EIF4E, ENOA, HSPA4, HSPA90AA1, IL-11, IL-8, KPNA2,
LMBRD1, MAP2K4, MEF2C, PEBP1, PGAM1, PREP, PSMD11, RNASEK, RTN3, SRGN, and TRIM16. All 25 selected genes were differentially expressed at one or more time points between the pretreated and untreated cells after infection (Fig. 5). In addition, the innate antiviral gene panel selected (above) was also tested and only MDA5 and LGP2a were more highly expressed at 6 d in the UV-VHSV treated cells than in untreated cells (Fig. 6.)

**Discussion**

Pretreatment of RTgill-W1 with HA-VSHV induced a significant reduction of VSHV load and CPE after infection [12]. The present study has replicated these results using UV-VHSV. Therefore at least some of the viral structures that stimulate an innate immune response resulting in a reduced viral load after infection are both UV- and heat-stable. The use of UV irradiation to inactivate VHSV [26] by altering its RNA may have maintained better integrity of viral structures, including glycoprotein G, since the MOI of VHSV for infection in the present study was higher than that used previously [12], yet the protection phenomenon was still demonstrated. Vaccination of Japanese flounder, Paralichthys olivaceus with a glycoprotein DNA vaccine (pCMV-VHSg), but not with recombinant glycoprotein, conferred significant protection after experimental infection with VHSV [27].

In the present study, validation of the microarray data by qPCR indicated that there was better agreement in gene modulation induced by UV-VHSV and FliC (nine out of ten genes) than those induced by both VHSV or SVCV (five out of the ten genes). This difference is likely due to the less sensitive microarray and the relatively inefficient detection of the diminished gene modulation produced in cells infected with a live virus.

**Pretreatment of RTgill-W1 with UV-VHSV**

Many genes were differentially expressed by each pretreatment. UV-VHSV regulated 121 probes and those of potential importance to the innate antiviral response, like heat shock protein (HSP), are discussed. Several HSP, including hsp70-like, hsp70b, hsp70a, and HSP90AA, were downregulated by UV-VHSV pretreatment. HSP70 is a major heat shock-like protein in mammals but is also expressed in rainbow trout as heat-shock-cognate (hsc71) [28]. HSP70 could influence VHSV at viral entry due to its’ predicted interaction with fibronectin (FN) 1, actins (ACTB, ACTG, F-actin) and gelsolin (GSN) and fibronectin is a known receptor for VHSV in rainbow trout [29]. Reduced HSP70 expression was also correlated with a reduction of VHSV titers in infected fathead minnow cells [30].

A group of translation initiation factors (GO term ‘Host-virus Interaction’), were upregulated including EIF4E (if4ea), EIF4G1 and EIF4H (eIF4H-like). Vesicular stomatitis virus (VSV), another rhabdovirus, alters host protein synthesis by dephosphorylating the eIF4E protein cap thereby reducing its’ association with eIF4G [31]. Also, the inhibition of eIF4H using siRNA inhibited the ability of herpes simplex virus (HSV) virion host shutoff protein to degrade host mRNA [32]. To evade the immune system, viruses such as VSV can induce phosphorylation of eIF2α to block the transcription of ISGs; during VSV infection host gene phosphorylation was induced only after viral protein translation was completed [33]. An elongation factor that was not regulated in the present study, eIF5A, was constitutively expressed in healthy flounder but it’s expression was increased in VHSV-infected cells before CPE developed [34]. These initiation factors require further investigation for their role in VHSV infection.

Reticulon 3 (RTN3) (GO term ‘Host-virus Interaction’) was downregulated in the present study with reduced expression of RTN3 in UV-VHSV treated cells at day 1 post infection. Reticulon 3 was associated with markedly decreased synthesis of enteroviruses [35] and hepatitis C.
Fig. 3. Interactions between differentially expressed genes after pretreatment of RTgill-W1 cells with UV-VHSV to VHSV, SVCV, NusA, and FliC at 36 h post-treatment.

| Term                                   | Gene Members                                                                 | %   | P-Value | Enrichment Fold | Clustered Categories*                                                                  |
|----------------------------------------|------------------------------------------------------------------------------|-----|---------|-----------------|----------------------------------------------------------------------------------------|
| hsa04612 Antigen processing and       | HSP70a; HSP70b; HSPA1A-Like; HSP90AA1                                        | 4.04| 0.0203  | 6.62            | GO:0,051,789—response to protein stimulus; GO:0,006,986—response to unfolded protein; IPR013126—Heat shock protein 70 |
| presentation                           |                                                                              |     |         |                 |                                                                                        |
| SP_PIR_Kewords Host-virus             | EIF4H; EIF4F; EIF4G1; RTN3; Itgb1; KPNA2                                      | 6.06| 0.012   | 4.35            | GO:0,016,281—eukaryotic translation initiation factor 4F complex                        |
| interaction                            |                                                                              |     |         |                 | GO:0,003,743—translation initiation factor activity;                                    |
| GO:0,009,611 Response to              | APOH; IL-8; CLU; CFH; CEBPB; IL-11; IFGBP4; TPM1; PEBP1                     | 9.09| 0.0103  | 2.94            | GO:0,006,955—immune response; GO:0,045,087—intrins immune response;                    |
| wounding                               |                                                                              |     |         |                 | GO:0,048,584—positive regulation of response to stimulus; defense response              |
| GO:0,010,627—regulation of            | PLK2; MALT1; IL11; PEBP1; LTAF                                                | 5.05| 0.053   | 3.48            | GO:0,010,740—positive regulation of protein kinase cascade;                            |
| protein kinase cascade                 |                                                                              |     |         |                 | GO:0,043,122—regulation of I-kappaB kinase/NF-kappaB cascade;                           |
| GO:0,006,916 Anti-apoptosis            | MALT1; CEBPB; MEF2c; CDC2; CLU; ATF5; HSPA1A-Like                           | 7.07| 0.0011  | 5.89            | GO:0,043,066—negative regulation of apoptosis                                            |
|                                        |                                                                              |     |         |                 | GO:0,010,941—regulation of cell death                                                   |

*Clustered Categories denote biological processes associated with the enrichment of genes in the indicated categories.
virus (HCV) [36]. Reticulin interacts with viral proteins associated with the formation of host membrane vesicles required for enterovirus replication. The use of RNA interference to reduce RTN3 markedly reduced enterovirus 71 plaque formation and apoptosis [35]. Two additional genes in the same GO category were also modulated: \textit{LMBRD1} was upregulated; and \textit{KAPNA2} was downregulated. \textit{LMBRD1} regulated endocytosis by insulin receptors [37] and \textit{KAPNA2} is an inducer of a nuclear transport protein that mediates HIV latency by facilitating the nuclear export and RNA processing of the virus [38]. The roles of the above three gene products during VHSV infection is unknown.

The lipopolysaccharide induced TNFα factor (\textit{LITAF}; GO term ‘Regulation of protein kinase cascade’), a regulator of TNFα transcription, was downregulated in the present study. A virus-encoded \textit{LITAF} homolog was associated with iridovirus evasion in Singapore grouper through the modulation of cell proliferation, apoptosis, and viral replication [39,40]. \textit{LITAF} is also involved in the pathogenesis of other viruses, including avian influenza virus [41], baculovirus [42] and frog virus 3 [43]. Several critical protein kinase genes that are involved in innate immunity including mucosa-associated lymphoid tissue lymphoma translocation gene 1 (\textit{MALTI}), polo-like kinase 2 (\textit{PLK2}) and tripartite motif 16 (\textit{TRIM16}) were also downregulated. \textit{TRIM16} was identified as a VHSV-induced transcript in rainbow trout [44]. \textit{TRIM16} is a member of the TRIM subfamily in teleosts, finTRIM, which shares a protein domain (B30.2) with NOD-like receptors suggesting a role in viral recognition in rainbow trout [44,45].

### qPCR of selected genes

Expression analysis of 25 selected genes revealed that 17 of these transcripts, representing the selected functional annotation groups identified by microarray, were significantly upregulated in the UV-VHSV pretreated cells compared to VHSV-infected untreated cells, and 16 of these were so at 6 days. These differentially expressed genes are potential contributors to VHSV reduction in pretreated cells. The

| Table 3 | Selected functional annotation categories for gene expression after treatment of RTgill-W1 cells with FliC–NusA complex. |
|---------|------------------------------------------------------------------------------------------------|
| GO Term | Gene Members                                                                                   |
| B cell activation | IL11; ITGB1; MALTI; MSH6                                                                 |
| Fold Enrichment | 3.27 0.0177 7.19                                                                            |
| Clusters Categories | GO:0,030,183–B cell differentiation; GO:0,002,520–immune system development; GO:0,012,502–induction of programmed cell death; GO:0,010,941–regulation of cell death; GO:0,009,612–response to mechanical stimulus; GO:0,009,628–response to abiotic stimulus |

#### qPCR of selected genes

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number of genes differentially upregulated at 6 days was unexpected. However, this was the same time point at which the greatest decrease in virus load occurred in the present study and in a previous one using pretreatment of RTgill-W1 with HA-VHSV [12]. All of these selected genes were regulated by the UV-VHSV pretreatment in the microarray experiment.

**Infection of RTgill-W1 with live VHSV and SVCV**

Infection with live VHSV and SVCV regulated 12 and 35 genes, respectively; far less than the 120 significantly regulated by UV-VHSV at 36 h post-exposure. This was expected as virus infection shuts off cell transcription via reduced translation of ISGs [46]. Reduced gene modulation could also be due to the use of total RNA rather than messenger RNA, allowing hybridization of viral RNA to the microarray. In a separate microarray study in olive flounder, total RNA was similarly used to detect responses to VHSV. This reduction in gene expression, however, is also consistent with the immunosuppressive role of VHSV NV protein that downregulates RIG-I, Toll-like receptors, type II interferon, NF-kappa B signaling and others [47, 48]. SVCV appears to induce a similar early innate immunosuppression, for example in zebrafish the SVCV N protein was able to inhibit IFN1 by degrading the mitochondrial antiviral signaling protein [49]. Therefore further research is needed to understand more about this observation in salmonids. Genetic differences between fish families, such as in turbot, may also impact the immune response modulation post VHSV infection [50].

There were, however, several genes modulated by VHSV and SVCV but not by UV-VHSV and these genes are of interest for their potential role in viral evasion of immune responses. For VHSV infection, these included MRPL27, ENOA, LIPG, C14orf1, TP11, IER5, ACTA2, PSMD3, RPS23, PPAP2A and two unknown transcripts. Those for SVCV included TAGLN, COTT1, C14orf1, ACTA1, AACS, FLNA, INSIG1, ACAT2, TXN, and DUSP6. The protein products of genes like FLNA and ENOA are involved in viral entry with fibronectin receptors. In curcumin-treated fathead minnow cells, FLNA protein was increased following VHSV infection but was decreased in pretreated cells that had lower viral loads. However, the reverse was the case for ENOA [30]. In the present study, we reported an upregulation for FLNA unlike the downregulation noted in EPC cells in previous studies [51]. Another possibly important gene is PSMD3, which is a part of the 26 proteasome complex that is involved in several pathways including MHC class I-mediated antigen processing and presentation, and regulation of apoptosis [52].

**Pretreatment of RTgill-W1 with FliC**

Pretreatment of RTgill-W1 by FliC also led to a significant reduction in VHSV load at 3 and 6 dpi, respectively, but not as dramatically as that induced by UV-VHSV. Pretreatment with FliC, did regulate the genes such as HSPA4 and others mentioned above, like FLNA, IL-11, and MALT1, for UV-VHSV or SVCV in the same direction. The majority of transcripts regulated by FliC, however, were not regulated by NusA alone, nor by any other treatment. The large number of regulated genes following exposure to FliC supports the potentially crucial role of gill epithelium in the innate immune response to bacterial or viral infection. However, only the role of peroxiredoxin (TDX) is well understood. Peroxiredoxin enhances the cytotoxicity of natural killer cells and it is a member of an antioxidant family of proteins, which is present in organisms from all kingdoms [53]. Peroxiredoxins are involved in innate immunity against several viral diseases, including infectious hematopoietic necrosis caused by IHNV in Atlantic salmon [54], white spot

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**Fig. 5.** The differential gene expression by qPCR in 25 selected genes between UV-VHSV-pretreated and untreated RTgill-W1 cells following infection with VHSV at 1, 3 and 6 dpi. Non-infected (NI) cells were included as a negative control for the expression. Genes that were significantly regulated are marked with a red triangle or blue circle to demonstrate significant higher expression in un-treated or UV-VHSV-treated cells, respectively.
syndrome virus in shrimp *Fenneropenaeus indicus* [55], SVCV in common carp [56] and VSHV in rainbow trout [57]. In this study TDX, was upregulated in both the FlIC complex and NusA control pretreatments. The role of TDX merits further investigation since it was also regulated after *Neoparamoeba perurans* infection (Amoebic gill disease) in Atlantic salmon [58]. Further investigation is also needed on the immunogenicity of NusA for gill epithelium. NusA resulted in the regulation of 51 probes and reduced viral load equivalent to FlIC at 6 dpi. The NusA protein is immunogenic when used as a protein carrier in other systems [58].

Regulation of known innate immune genes

In the present study, melanoma differentiation associated gene 5 (*mda5*) and laboratory of genetics and physiology 2a (*lgp2a*) was significantly upregulated more in UV-VHSV pretreated infected cells over non-pretreated infected cells at Day 6. Both encode important RIG-like receptors that recognize viral RNA in the cytoplasm. Both genes are associated with resistance to VHSV infection in rainbow trout kidney and in RTG-2 cells [59]. In Hirame natural embryo cells overexpressing *mda5*, VHSV-infected cells had reduced CPE and reduced viral loads [60]. The genes *ifn1*, *ifn2*, *mx1*, and *mx3* were more significantly expressed in the untreated RTgill-W1 after VHSV infection for at least one or more timepoints post-infection. However, this increased expression in untreated cells corresponded with higher viral load than in the UV-VHSV treated cells. Previously, HA-VHSV did not modulate *ifn1*, *ifn2* and *mx1* expression in RTgill-W1 cells but reduced VHSV load and CPE after infection [12]. UV-irradiation targets the viral RNA, which can have a significant role in viral evasion. For example, the VSHV NV gene led to down-regulation of *ifn1*, *ifn2*, many IRFs, *mx1*, *mx2*, and *mx3* [47]. While *ifn* and *mx* proteins are known to contribute to immunity against VHSV partly by inducing micro-(mi)RNAs [61], the experimental results reported here also demonstrate that these are not the only contributors to the protection of RTgill-W1 cells against VHSV infection.

Teleost epithelium, in this case, RTgill-W1 epithelial cells, will play a pivotal role in innate immunity to both bacterial and viral infection. There are relatively few studies that have evaluated the potential role of RTgill-W1 cells in innate immunity. Diverse molecules described in the present study are deserving of further investigation to increase our understanding of the pathogenesis and host-pathogen interaction during VSHV or SVCV infection and molecules that might be stimulated to reduce viral replication.

Declaration of Competing Interest

None.

Acknowledgments

Misk was supported by the Egyptian Government. Research funding was from a Natural Sciences and Engineering Research Discovery Grant (Lumsden) and the Ontario Ministry of Agriculture, Food and Rural Affairs.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsiirep.2021.100043.

References

[1] S. Laputra, E. Misk, L. Al-Hussineen, J.S. Lumsden, F.S. Kibenge, M. Godoy, Rhabdoviruses of fish (Eds.). Aquaculture Virology, Academic Press, 2016.
partial growth, carcass traits, meat quality and immune traits, Can. J. Anim. Sci. 85 (4) (2005) 475–480.

[53] S.G. Rhee, H.Z. Chae, K. Kim, Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling, Free Radic. Biol. Med. 38 (12) (2005) 1543–1552.

[54] A. Booy, J. Haddow, L. Ohlund, D. Hardie, R. Olafson, Application of isotope coded affinity tag (ICAT) analysis for the identification of differentially expressed proteins following infection of Atlantic salmon (Salmo salar) with infectious hematopoietic necrosis virus (IHNV) or renibacterium s almoninarum (BKD), J. Proteom. Res. 4 (2) (2005) 325–334.

[55] C. Kiruthiga, S. Rajesh, V. Rashika, R. Priya, R. Narayanan, Molecular cloning, expression analysis and characterization of peroxiredoxin during WSSV infection in shrimp Fenneropenaeus indicus, J. Invertebr. Pathol. 109 (1) (2012) 52–58.

[56] R. Huang, L.-Y. Gao, Y.-P. Wang, W. Hu, Q.-L. Guo, Structure, organization and expression of common carp (Cyprinus carpio L.) NKEF-B gene, Fish Shellfish Immunol. 26 (2) (2009) 220–229.

[57] A. Cuesta, C. Tafalla, Transcription of immune genes upon challenge with viral hemorrhagic septicemia virus (VHSV) in DNA vaccinated rainbow trout (Oncorhynchus mykiss), Vaccine 27 (2) (2009) 280–289.

[58] G.H. Loo, D.L. Sutton, K.A. Schuller, Cloning and functional characterisation of a peroxiredoxin 1 (NKEF A) cDNA from Atlantic salmon (Salmo salar) and its expression in fish infected with neoparamoeba perurans, Fish Shellfish Immunol. 32 (6) (2012) 1074–1082.

[59] M. Chang, B. Collet, P. Nie, K. Lester, S. Campbell, C.J. Secombes, J. Zou, Expression and functional characterization of the RIG-I-like receptor MDA5 and LGP2 in Rainbow trout (Oncorhynchus mykiss), J. Virol. 85 (16) (2011) 8403–8412.

[60] M. Ostani, J. Hikima, H. Kondo, I. Hirono, T.S. Jung, T. Aoki, Characterization and antiviral function of a cytosolic sensor gene, MDA5, in Japanese flounder, Paralichthys olivaceus, Dev. Comp. Immunol. 35 (5) (2011) 554–562.

[61] D.B. Belay-ong, B.D. Schyth, J. Zou, C.J. Secombes, N. Lorenzen, Involvement of two microRNAs in the early immune response to DNA vaccination against a fish rhabdovirus, Vaccine 33 (28) (2015) 3215–3222.