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► **To cite this version:**

Laura Marroquí, Amparo Estepa, Luis Perez. Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus vhsv by real-time reverse-transcription PCR. *Veterinary Microbiology*, 2007, 122 (1-2), pp.52. 10.1016/j.vetmic.2007.01.002 . hal-00532191

HAL Id: hal-00532191

<https://hal.science/hal-00532191v1>

Submitted on 4 Nov 2010

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Accepted Manuscript

Title: Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus vhsv by real-time reverse-transcription PCR

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PII: S0378-1135(07)00010-7
DOI: doi:10.1016/j.vetmic.2007.01.002
Reference: VETMIC 3556

To appear in: *VETMIC*

Received date: 17-10-2006
Revised date: 2-1-2007
Accepted date: 5-1-2007

Please cite this article as: Marroquí, L., Estepa, A., Perez, L., Assessment of the inhibitory effect of ribavirin on the rainbow trout rhabdovirus vhsv by real-time reverse-transcription PCR, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.01.002

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5 ASSESSMENT OF THE INHIBITORY EFFECT OF RIBAVIRIN ON THE RAINBOW

6 TROUT RHABDOVIRUS VHSV BY REAL-TIME REVERSE-TRANSCRIPTION PCR

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9 Running title: Q-RT-PCR antiviral assay for trout virus

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23 **Abstract**

24 *Viral hemorrhagic septicemia virus* (VHSV) is one of the most ubiquitous viruses in
25 salmonid aquaculture in Europe. This infectious disease results in significant losses in the
26 farming industry and therefore effective therapeutic agents are needed to control outbreaks
27 caused by this pathogen. Thus, accurate methods to test new antiviral compounds need to
28 be developed. Our goal was to establish a model system for testing novel antivirals with
29 potential applications to aquaculture. In a previous study a TaqMan® real-time RT-PCR
30 assay was designed to detect and quantitate VHSV in rainbow trout tissues (Chico et
31 al.,2006) . In this report, we applied the real-time RT-PCR assay to the evaluation of the
32 inhibitory effect of ribavirin, a well-known broad spectrum antiviral drug, in a cell culture
33 system. When added from the beginning of the infection, ribavirin caused a dose-dependent
34 reduction of VHSV RNA accumulation. Real-time RT-PCR measurements showed 99.8%
35 inhibition at 25µg/ml ribavirin, with an IC₅₀ of 0.43µg/ml. Ribavirin maintained its
36 inhibitory activity against VHSV when added at 6 hours post-infection. Quantitation of N
37 protein messenger RNA and plus-stranded RNA showed a substantial decrease of viral
38 transcription in ribavirin-treated cells. Partial reversion of the effect of ribavirin by addition
39 of GTP was observed, confirming that ribavirin targets the synthesis of guanine
40 nucleotides in the cells. This is the first report of a real-time PCR-based assay for
41 addressing the efficacy and mechanism of action of an antiviral agent for rainbow trout.

42

43

44 **Keywords:** *viral hemorrhagic septicemia virus*, real-time PCR, antiviral

45 **1. Introduction**

46 *Viral hemorrhagic septicemia virus* (VHSV) is the agent of an important viral
47 disease of rainbow trout (*Oncorhynchus mykiss*). Infection with this virus may lead to high
48 mortality rates, occurring usually among juvenile fish. VHSV belongs to the
49 *Novirhabdovirus* genus of the *Rhabdoviridae* family, a group of bullet-shaped enveloped
50 viruses containing one molecule of negative-sense single-stranded RNA, coding for the
51 nucleoprotein (N), the glycoprotein (G) and four other viral genes (3'-N-P-M-G-NV-L-5')
52 which are expressed as individual transcripts (Schütze et al., 1999).

53 Search for specific and efficacious antiviral compounds is a matter of concern in the
54 control of fish virus infections. This search should be done in combination with an
55 experimental technique capable of providing rapid and reliable evaluation of the efficacy of
56 candidate molecules. We have recently developed a quantitative real-time RT-PCR (Q-RT-
57 PCR) to measure VHSV loads both *in vitro* and *in vivo* (Chico et al., 2006). Earlier,
58 successful detection of another rainbow trout rhabdovirus, *infectious hematopoietic*
59 *necrosis virus* (IHNV), in brain and kidney by Q-RT-PCR was described (Overturf et al.,
60 2001). In both studies, Q-RT-PCR was shown to be a fast, sensitive and reliable method to
61 measure viral RNA accumulation in cultured cells as well as in fish tissues. Other authors
62 have provided evidence that Q-RT-PCR can be a suitable technique to test the activity of
63 antiviral compounds by assessing the inhibitory effect of a series of chemicals on
64 herpesviruses (Fryer et al., 2004; Macé et al., 2003; Stránská et al., 2002) and retroviruses
65 (Haffar et al., 2005; Pesce et al., 2005). Efficacy evaluation of antiviral molecules *in vitro*
66 (on cell culture) by Q-RT-PCR has been reported for a number of RNA viruses (Garcia et
67 al., 2001; Günther et al., 2004; Wright et al., 2004).

68 To test the performance of Q-RT-PCR in our VHSV/carp cell system, the
69 nucleoside analogue ribavirin (1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide) was
70 chosen for this study for its well-known broad spectrum antiviral activity. Ribavirin was
71 discovered to have an inhibitory effect on the fish pathogen *infectious pancreatic necrosis*
72 *virus* (IPNV) *in vitro* (Hudson et al., 1988; Migus and Dobos., 1980). Later it was shown
73 that the ribavirin analogues EICAR and pyrazofurin were able to inhibit IPNV replication
74 *in vitro* as well as in trout fry (Jashes et al., 1996; Moya et al., 2000). With respect to
75 rhabdoviruses, ribavirin was found to block *vesicular stomatitis virus* (VSV) replication in
76 hamster cells (Toltzis and Huang, 1986; Toltzis et al., 1988). For fish rhabdoviruses, *in*
77 *vitro* inhibition of IHNV by ribavirin has been reported (Hudson et al., 1988). Due to its
78 broad spectrum effectiveness, ribavirin has been used in recent years as a model antibiotic
79 in studies where the inhibition of viral RNA synthesis was assessed by Q-RT-PCR
80 (Castelain et al., 2004; Friedrichs et al., 2004; Garcia et al., 2001; Günther et al., 2004;
81 Takhampunya et al., 2006; Wright et al., 2004).

82 We report in this paper the employment of a Q-RT-PCR assay with the fluorogenic
83 TaqMan® probe to study the effect of ribavirin on VHSV replication. We characterized the
84 effect of ribavirin on viral total RNA, plus-stranded RNA and messenger RNA
85 accumulation, establishing a model system to test the efficacy of VHSV inhibitors *in vitro*,
86 as well as a valuable tool to investigate the mechanism of action of novel antiviral drugs.

87

88 **2. Materials and methods**

89 *2.1. Cell lines and virus infection assay*

90 The carp *Epithelioma papulosum cyprini* (EPC) cells were purchased from the
91 European Collection of Cell cultures (ECACC#93120820, Salisbury, UK). *Viral*
92 *hemorrhagic septicemia virus* (VHSV, 07-71 European strain) was grown on EPC cells.

93 EPC cells were seeded on 24-well plates in RPMI 1640 medium (Gibco) containing
94 10% fetal calf serum (FCS). VHSV was added to the EPC cells at a multiplicity of infection
95 (m.o.i.) of 0.05 and incubated at 14°C in RPMI 1640 plus 2% FCS. For virus titration
96 purposes, VHSV culture supernatants were collected at 3 days p.i.

97

98 *2.2. Cytotoxicity assay*

99 Ribavirin (Sigma-Aldrich) stocks were prepared in sterile water and stored at -20°C
100 until use. Toxicity of ribavirin on EPC cells was evaluated by means of the colorimetric
101 MTS method (CellTiter 96® AQueous Cell Proliferation Assay, Promega Corporation).
102 Briefly, cells were seeded in 96 well plates and allowed to reach confluence. Ribavirin in
103 concentrations up to 100µg/ml was added and the cells were incubated in the presence of
104 the compound for 14 hours. The experiment was performed in triplicate wells following the
105 protocol provided by the manufacturer. Cell viability was determined by measuring
106 absorbance at 492nm.

107

108 *2.3. End-point dilution assay*

109 Briefly, 10-fold serial dilutions of supernatants from VHSV-infected cells were
110 added to 96-well plates with EPC cells and incubated at 14°C for 3 days. Wells with
111 cytopathic effect were counted after staining with a 1% crystal violet solution.

112 Determination of virus titer (expressed as TCID₅₀/ml) was done by the Reed-Münch end-
113 point calculation method.

114

115 *2.4. Isolation of RNA*

116 Total RNA was extracted from EPC cells cultivated in 24 well plates by using the
117 RNAagents® Total RNA Isolation System extraction kit (Promega Corporation), following
118 the manufacturer's instructions. Each RNA pellet was resuspended in 25µl RNase-free
119 water and the quantity of RNA was checked by measuring absorbance at 260 nm.

120

121 *2.5. TaqMan® PCR primer and probe design*

122 Amplicons in the VHSV N and VHSV G genes suitable for real-time PCR were
123 identified with the Primer Express™ 2.0 software (Applied Biosystems). Primers and 5'-
124 FAM-labeled probes were designed and standard curves were performed to check the
125 efficiency of the amplification reactions (Chico et al., 2006).

126 For β-actin, sequences were selected from a exon region of common carp β-actin
127 sequence (GenBank M24113): primer forward (5'-GCTGACAGGATGCAGAAAGAGA-
128 3'), and reverse (5'-GGGCAATGATCTTGATTTTCATT-3') generated a 67bp amplicon;
129 the FAM-TAMRA probe was (5'-ACATCCCTGGCCCCCAGCA-3').

130

131 *2.6. Real-time RT-PCR assay*

132 Unless otherwise indicated, 1µg total RNA was reverse-transcribed with 90ng
133 random hexamers, 0.5mM deoxynucleoside triphosphates (dNTPs) mix. After denaturing
134 for 5 min. at 65°C, 10 mM DTT, 20 units ribonuclease inhibitor, and 100 units MMLV-RT

135 enzyme (Invitrogen) were added in a final reaction volume of 20 μ l. The RT reaction profile
136 was: 10 min at 25°C, 50 min at 37°C and 15 min at 70°C.

137 Quantitative PCR assays were performed using an ABI PRISM® 7700 Sequence
138 Detector System (Applied Biosystems). Reactions were carried out in a final volume of
139 25 μ l, containing 300nM of each primer, 100nM probe, 2 μ l cDNA (unknown samples) and
140 1X Absolute™ QPCR Rox Mix (ABGene). Samples were subjected to the following
141 thermal cycler conditions: 2min at 50°C, 15min at 95°C, and 40 cycles (15s 90°C/1min.
142 60°C). Endogenous control for quantitation was the 18S ribosomal RNA gene. 18S rRNA
143 levels were determined with the TaqMan® Ribosomal RNA Control Reagents kit (Applied
144 Biosystems) following the manufacturer's guidelines.

145 For messenger RNA (mRNA) quantitation, the RT reaction was performed as
146 described above, but using 25 pmol oligo dT's instead of random hexamers.

147

148 *2.7. High temperature primer-specific RT reaction*

149 For quantitation of positive-sense RNA, reverse transcription was initiated by a
150 reverse primer, N996rev (5'-CGTAGCGCTCTTGGATGGAC-3'; nucleotide position 977-
151 996, N-gene sequence, antisense). Plus-strand specific cDNA synthesis was performed
152 using the high temperature reverse transcription capability of the Tth DNA polymerase.
153 cDNA complementary to plus-stranded VHSV (N gene) RNA was synthesized in a 40 μ l
154 RT reaction mix containing 5 μ l RNA (0.5 - 1 μ g), 25pmol *N996rev* primer, 200 μ M dNTPs,
155 1 mM MnCl₂, 10 units RNAGuard™ RNase inhibitor (Amersham Biosciences) and 2.5
156 units Tth DNApol (Promega Corporation). RT mixes were incubated 2 min at 60°C and 20
157 min at 70°C. An additional incubation of 30 min at 98°C with a Mn chelating buffer is
158 carried out to inactivate reverse transcriptase.

159

160 **3. Results**161 *3.1. Determination of VHSV susceptibility to ribavirin by real-time RT-PCR*

162 In the initial evaluation of the toxicity of ribavirin on the EPC carp cell line we
163 determined that it did not exert a cytotoxic effect at concentrations $\leq 100 \mu\text{g/ml}$. Numbers
164 of viable living cells in cultures treated with $100\mu\text{g/ml}$ ribavirin for 14 hours were $127.4 \pm$
165 17.1% compared to the untreated controls ($100 \pm 9.8\%$). Past reports had shown a $\approx 60\%$
166 suppression of cellular RNA synthesis by $50 \mu\text{g/ml}$ ribavirin in a hamster cell line (Toltzis
167 and Huang, 1986). Thus, the dose-effect study of ribavirin was carried out at concentrations
168 ranging from 1 to $25 \mu\text{g/ml}$, examining cytopathic effect and measuring virus yield. A very
169 strong inhibition of virus production was observed at 5, 10 and $25 \mu\text{g/ml}$ ribavirin (Fig. 1,
170 table). Virus titers in the infected cells supernatants were 4 to 5 logs lower in those cells
171 where ribavirin was added at the beginning of the infection (0 hours p.i.). No toxic effect
172 was observed up to $25 \mu\text{g/ml}$ ribavirin.

173 To investigate whether our Q-RT-PCR protocol could be suitable to measure
174 the inhibitory effect of ribavirin on virus replication, VHSV-infected EPC cells were
175 incubated with concentrations of ribavirin ranging from $0.1 \mu\text{g/ml}$ to $25 \mu\text{g/ml}$ and VHSV
176 RNA levels at 9 hours p.i. were determined by Q-RT-PCR (Fig. 1). A dose-dependent
177 inhibition of VHSV RNA accumulation was found in the cells treated with ribavirin: 83.3%
178 inhibition at $1 \mu\text{g/ml}$ ribavirin, 93.9% inhibition at $10 \mu\text{g/ml}$, and 98.5% inhibition at 25
179 $\mu\text{g/ml}$ (the latter point is not shown in Fig. 1). Ribavirin inhibitory concentration 50%
180 (IC₅₀) was calculated after fitting data points to a curve by using the Origin™ 7.0 software:
181 IC₅₀ = $0.43 \mu\text{g/ml}$ ($1.76 \mu\text{M}$). The closest concentration to the IC₅₀ tested in the
182 experiment was $0.5 \mu\text{g/ml}$ ribavirin, resulting in 54.9% inhibition of VHSV RNA synthesis.

183

184 *3.2. Kinetics of VHSV RNA accumulation. Effect of ribavirin*

185 To further examine the effect of ribavirin on VHSV, total RNA was extracted from
186 infected EPC cells from 2 to 10 hours p.i. Q-RT-PCR measurements of viral RNA
187 accumulation reflect the progress of infection (Fig. 2), with levels of VHSV RNA
188 increasing with time: ≈ 3000 -fold more viral RNA at 10 h p.i. than at 2 h p.i., indicating an
189 active replication of the virus within the cells from hour 6 post-infection. When 25 $\mu\text{g/ml}$
190 ribavirin was added at 0 h p.i. a high reduction of viral RNA accumulation occurred: at 10
191 hours p.i. 99.8% inhibition of VHSV RNA was achieved by treatment with ribavirin.

192

193 *3.3. Effect of ribavirin on VHSV transcription*

194 According to previous reports on VSV (Toltzis et al., 1988) ribavirin was expected
195 to target viral RNA synthesis: genome replication, mRNA synthesis, or both. Firstly, to
196 assess whether ribavirin was blocking an early and/or late step of the VHSV life cycle we
197 carried out a simple time-of-addition experiment (Fig 3A) where ribavirin was added to the
198 infected cells at 0 or at 6 hours p.i. and total RNA was extracted at 9 h p.i. and analysed by
199 Q-RT-PCR. As expected from our previous findings ribavirin almost completely inhibited
200 VHSV RNA accumulation when added at time 0 of infection (99.5% inhibition). When
201 ribavirin was added at 6 h p.i. it still retained its inhibitory activity (81.9% inhibition). In an
202 independent experiment where ribavirin was added at a later time (9 h p.i.) and VHSV
203 RNA analyzed at 24 h p.i., 81% inhibition of viral RNA accumulation was observed. These
204 results suggested that ribavirin exerts its action also at a late stage of the virus life cycle.

205 To measure messenger RNA levels and get an insight on the effect of ribavirin on
206 VHSV transcription, RNA extracted from infected cells was converted to cDNA by using

207 oligo dT's. Subsequently real-time PCR analysis was carried out with primers for the
208 VHSV G protein and N protein genes, and the relative amount of mRNA in each sample
209 was determined (Fig. 3B). A strong inhibition of 99.7% in VHSV G mRNA and 99.3% in
210 VHSV N mRNA levels was found when ribavirin was present since the beginning of the
211 infection. When added at 6 h p.i. inhibition of 59.9% and 54.1% for VHSV G and VHSV N
212 mRNAs levels were found, respectively. Ribavirin did not have a negative effect on the
213 transcription of the cellular β -actin gene at the concentration used in the experiment
214 (25 μ g/ml). The 3' - 5' attenuation of transcription is a well known feature of rhabdovirus
215 gene expression. According to our Q-RT-PCR analysis VHSV N mRNA levels were 3.3-
216 fold higher than VHSV G mRNA, confirming the validity of the Q-RT-PCR assay as a
217 quantitative technique.

218

219 3.4. Analysis of VHSV plus-stranded RNA in ribavirin-treated cells.

220 To further examine the effect of ribavirin on VHSV RNA accumulation minimizing
221 potential problems with secondary structures in RNA templates during cDNA synthesis, a
222 Q-RT-PCR protocol using a sequence-specific primer and high temperature reverse
223 transcription was developed. EPC cells were infected with VHSV and 25 μ g/ml ribavirin
224 was added at 6 hours post-infection. RNA was extracted at 7, 8 and 9 h p.i. and the N gene
225 sequence specific primer *N996rev* was used to convert plus-stranded VHSV RNA to cDNA
226 in a RT reaction with Tth DNA polymerase at 70°C prior to TaqMan® real-time PCR
227 analysis. Total cDNA was also synthesized with random hexamers and MMLV reverse
228 transcriptase (at 37°C) for β -actin RNA quantitative analysis (Fig. 4). Positive-sense VHSV
229 N RNA accumulates in the infected cells from 4 hours p.i. with a sharp increase from hour
230 7p.i., likely indicating the bulk of viral transcription. When ribavirin is added VHSV RNA

231 dropped to undetectable levels in two hours. VHSV infection does not seem to affect β -
232 actin RNA levels in EPC cells. In accordance with a previous result (Fig. 3B) the synthesis
233 of carp β -actin RNA did not appear to be sensitive to ribavirin treatment at the
234 concentration used.

235

236 3.5. Reversibility of ribavirin inhibitory effect by GTP

237 Ribavirin has been described as an inhibitor of the inosine monophosphate
238 dehydrogenase (IMPDH) enzyme, causing a depletion of the guanidine nucleotides pool
239 within the cell (Graci and Cameron, 2002; Graci and Cameron, 2006; Parker, 2005).
240 Therefore, some reversion of ribavirin action should be achieved by replenishing GTP
241 levels, as it has been shown in VSV-infected cells (Toltzis and Huang, 1986). In VHSV-
242 infected EPC cells viral RNA levels raised from $2.46 \pm 0.24\%$ to $27.79 \pm 3.07\%$ when GTP
243 was added to the ribavirin-treated cells (Fig. 5). Thus, an approximately 10-fold increase in
244 VHSV RNA synthesis is induced by the addition of GTP to ribavirin-treated cells. GTP
245 alone did not cause an augmentation of VHSV RNA levels; rather, they dropped to $69.54 \pm$
246 0.68% compared to control VHSV-infected cells. These results support the notion that one
247 of the targets of ribavirin is the guanidine nucleotide synthesis pathway.

248

249 4. Discussion

250 Viral diseases pose a significant threat to aquaculture production. With respect to
251 salmonid species, *viral hemorrhagic septicemia virus* (VHSV) and *infectious pancreatic*
252 *necrosis virus* (IPNV) cause the two major diseases in Europe with outbreaks that may lead
253 to mass mortalities. Research efforts are aimed to develop effective virus-specific agents
254 against fish pathogens. One of the most promising studies on this topic described the *in vivo*

255 inhibition of IPNV by ribavirin analogues (Jashes et al., 1996; Moya et al., 2000). This was
256 in accordance with earlier findings on the anti-IPNV activity of ribavirin *in vitro* (Migus
257 and Dobos, 1980). Ribavirin, a nucleoside-like molecule is a broad spectrum antiviral
258 whose inhibitory activity against rhabdoviruses, both mammalian (vesicular stomatitis
259 virus, VSV) and piscine (infectious haematopoietic necrosis virus, IHNV), has been
260 reported (Hudson et al., 1988; Toltzis and Huang, 1986). Taking all of the above in
261 consideration, we pursued to develop an assay to test the efficacy of nucleoside analogues
262 and other compounds against fish viruses *in vitro*, as a previous step to the *in vivo* trials in
263 experimental aquaria. This was achieved in combination with an accurate method for viral
264 RNA quantitation: real-time RT-PCR with TaqMan® probes.

265 In a previous work by our group a Q-RT-PCR assay was designed to run a reliable
266 diagnostic test for VHSV in experimentally challenged rainbow trout (Chico et al., 2006).
267 During the past years VHSV has been proven to be a good experimental model: it grows
268 well in cell culture, it is not hazardous for humans, and animal tests with large numbers of
269 individuals can be performed in experimental aquaria. Thus, a Q-RT-PCR assay for VHSV
270 was set up on laboratory grown virus and subsequently tested on different tissue samples
271 from VHSV-infected fish with success. The Q-RT-PCR analysis had a sensitivity of 1.6
272 TCID₅₀, and it was capable of identifying more VHSV-positive fish than the standard cell
273 culture assay (Chico et al., 2006).

274 Quantitative real-time PCR has been successfully employed to test the efficacy of
275 antiviral compounds in cell culture (Garcia et al., 2001; Günther et al., 2004). In this work,
276 the inhibition of VHSV RNA synthesis *in vitro* by ribavirin was determined by Q-RT-PCR.
277 At 10 hours post-infection 99.8% inhibition of viral RNA accumulation was determined in
278 VHSV-infected cells treated with 25µg/ml ribavirin. The IC₅₀ was calculated as 0.43µg/ml.

279 This value is about 2 logs lower than those reported for ribavirin inhibition of VSV, where
280 the IC₅₀ was determined by measuring radioactive-labelled viral RNA (Toltzis and Huang,
281 1986). For Lassa virus (*Arenaviridae*) a concentration of 9 µg/ml ribavirin caused 50%
282 inhibition of viral RNA accumulation as determined by Q-RT-PCR (Günther et al., 2004).
283 It is known that the Q-RT-PCR assay leads to lower IC₅₀ values than standard (i.e. plaque
284 assay) methods for virus quantitation (Macé et al., 2003; Stránská et al., 2002), likely due to
285 the fact that viral RNA species exist which do not correspond to infectious virus particles.

286 Ribavirin was effective at reducing VHSV total RNA as well as mRNA synthesis.
287 Early studies suggested a direct inhibitory effect on the VSV RNA polymerase (Toltzis et
288 al., 1988). For the IPNV birnavirus, the inhibition of both viral transcription and replication
289 by ribavirin has been speculated (Migus and Dobos, 1980). Our data from the late addition
290 of ribavirin to the infected cells also support this notion. Interference with both replication
291 and transcription may explain the difference between ribavirin effect on VHSV total RNA
292 (81.9% inhibition) and the effect on VHSV mRNAs (54 – 59% inhibition) when the drug is
293 added at 6 h p.i.: by detecting only mRNA molecules the inhibitory effect of ribavirin can
294 be underestimated, in contrast to the analysis of total RNA.

295 Regarding the Q-RT-PCR assay, it has been pointed out by a number of authors
296 (Purcell et al., 2006) that performing the RT reaction at a relatively low temperature (i.e.
297 37°C) may lead to inaccurate quantitation of the target RNA due to false priming, self-
298 priming and random priming events, resulting in synthesis of contaminating cDNA. To
299 check this issue we set up a RT protocol using a VHSV N sequence specific primer (instead
300 of random hexamers) and Tth DNApol, a polymerase capable of functioning up to 70°C.
301 This method allowed us to analyze plus-stranded VHSV RNA (N gene mRNAs as well as
302 whole length anti-genomes) and reassess the inhibitory effect of ribavirin. Consistently with

303 our previous experiments, a high reduction of viral plus-stranded RNA levels (81.6%
304 inhibition after one hour of treatment) was found.

305 One of the proposed mechanisms of action of ribavirin is the inhibition of the
306 enzyme inosine monophosphate dehydrogenase (IMPDH), causing the depletion of the
307 intracellular guanine nucleotides pool (Graci and Cameron, 2002; Graci and Cameron,
308 2006; Parker, 2005). We examined this possibility, finding a 10-fold increase of VHSV
309 RNA levels when GTP was added to the ribavirin-treated infected cells compared to
310 ribavirin alone, strongly supporting the hypothesis of ribavirin targeting the metabolic
311 pathway leading to GTP formation. We observed a $\approx 30\%$ decrease in VHSV RNA
312 accumulation due to GTP treatment. Although we did not pursue the investigation of this
313 phenomenon, we can speculate that the imbalance in the cellular NTPs pool caused by
314 addition of GTP might lead to a lesser efficiency of the viral RNA polymerase.

315 We are aware that by monitoring viral load by a PCR-based assay other antiviral
316 activities that lead to production of non-infectious virus can be overlooked. At the
317 concentration tested ($25 \mu\text{g/ml} \equiv 102 \mu\text{M}$) ribavirin has a lethal mutagen activity against a
318 number of RNA viruses (Graci and Cameron, 2002). The combination of two or more
319 mechanisms of action could explain the 10^5 -fold reduction in infectious virus production
320 caused by ribavirin.

321 In conclusion, Q-RT-PCR can be used, in combination with the measurements of
322 viral titers by classical methods, to study the progression of infection and the kinetics of
323 virus replication. By providing accurate quantitation of viral RNA accumulation, Q-RT-
324 PCR is a particularly suitable technique to assess the inhibitory activity of antiviral
325 molecules. Bearing in mind practical applications we should stress that there is not always a
326 direct correlation between *in vitro* results and protection of fish, so *in vivo* testing is

327 necessary. A previous work where the ribavirin analogue EICAR was administered daily by
328 immersion to IPNV-infected fish during a 20 day period, resulted in a statistically
329 significant reduction of mortality rates (Moya et al., 2000). Further research needs to be
330 conducted to determine the most cost-effective way to deliver antiviral compounds to trout
331 fry.

332 Finally, we believe that the real-time PCR technique will be increasingly
333 implemented to study the pathogenesis of fish virus infections, assess the efficacy of new
334 antiviral drugs, and address their mechanism of action. The findings obtained in the present
335 study may contribute to the further development of antiviral agents for VHSV and other
336 fish viruses.

337

338 **Acknowledgements**

339 This study was supported by grant GV04B-657 from Generalitat Valenciana, the Spanish
340 M.E.C. AGLZ004-07404-C02-01/ACU grant and by Spanish M.E.C. training fellowship
341 (Beca de Colaboración) to L. Marroquí. The expert technical assistance of Beatriz Bonmatí
342 is acknowledged.

343

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433 **Figure Legends**

434 Fig.1. Dose-dependent effect of ribavirin on VHSV replication. Left: VHSV-infected EPC
435 cell monolayers (m.o.i. = 0.05) were incubated with 0.1, 0.5, 1, 2.5 and 10 $\mu\text{g/ml}$ ribavirin.
436 Cells were harvested at 9 hours p.i. and total RNA was prepared. VHSV RNA levels were
437 measured by real-time RT-PCR by amplifying a sequence of the VHSV N gene. Each PCR
438 reaction was done in triplicate. The percent values represent the inhibition of VHSV RNA
439 accumulation compared to the non-treated VHSV control. Right: effect of ribavirin on virus
440 yield. Aliquots of VHSV-infected cells supernatants were sampled at 9 h p.i. and virus
441 titers were determined by the end-point dilution method. Ribavirin was added at 0 h p.i.
442

443 Fig.2. Time course of VHSV RNA accumulation in EPC cells infected with a m.o.i. of
444 0.05. Cell monolayers were harvested at the indicated times and samples were subjected to
445 the Q-RT-PCR assay as in figure 1 (filled squares). 25 $\mu\text{g/ml}$ ribavirin was added to the
446 cells (open circles) at the same time as the virus (0 h p.i.). Arbitrary units: Y axis values
447 represent the ratio between the amount of VHSV RNA in a given sample and the amount of
448 VHSV RNA at 2 h p.i. TaqMan® PCR reactions were run in triplicate. Error bars indicate
449 the standard deviations of those replicates.

450
451 Fig.3. A: Effect of ribavirin time of addition on VHSV RNA accumulation: 25 $\mu\text{g/ml}$
452 ribavirin was added at 0 h p.i. and at 6 h p.i. EPC cell monolayers were harvested at 9 hp.i.
453 and VHSV (N gene) RNA levels were determined by Q-RT-PCR (random hexamers RT).
454 B: Effect of ribavirin on VHSV transcription. RNA from VHSV-infected cells was
455 converted to cDNA by oligo dT's RT reaction prior to Q-PCR analysis of VHSV G and
456 VHSV N sequences. A carp β -actin gene sequence was also amplified as a control of

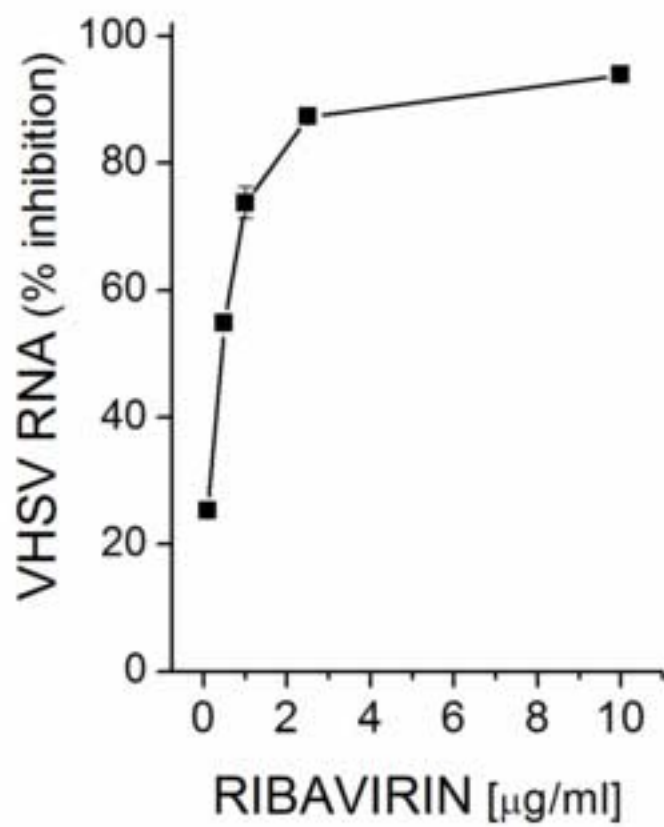
457 cellular messenger RNA. The internal control to normalize β -actin and VHSV RNA data
458 was the 18S rRNA (random hexamers RT). Results in panel B are expressed as the ratio
459 between the expression of mRNA in a given sample and the expression of VHSV G mRNA
460 after treatment with ribavirin from time 0 (lowest value of the experiment). Data are mean
461 \pm SD for one experiment performed in triplicate.

462

463 Fig.4. Time course of plus-stranded VHSV N gene RNA (squares) and β -actin RNA
464 (triangles) in EPC cells infected with VHSV (m.o.i. = 0.05). Infected cells were treated
465 with 25 μ g/ml ribavirin from 6 hours post-infection (arrows; open symbols) and harvested
466 at the indicated times for RNA extraction and real-time RT-PCR analysis. The sample with
467 the lowest target gene expression (VHSV N at 2 h p.i.) is given value = 1 and used as the
468 reference. VHSV N RNA at 8 h p.i. and 9 h p.i. in cells treated with ribavirin were below
469 detection limit (assigned value = 0). Data are mean \pm SD for one representative experiment
470 performed in triplicate.

471

472 Fig.5. Reversibility of ribavirin effect by GTP. EPC cells were infected with VHSV and
473 incubated in the presence of 25 μ g/ml (102 μ M) ribavirin (RIB) and/or 500 μ M GTP for 9
474 hours. Total RNA was prepared and subjected to Q-RT-PCR analysis as in figure 1.
475 Relative percentages of inhibition were calculated in comparison to the untreated VHSV
476 infection control. TaqMan® PCR reactions were run in triplicate. Error bars indicate the
477 standard deviations of those replicates.



<u>Ribavirin [μg/ml]</u>	<u>TCID50/ml</u>
0	$>10^9$
1	8×10^8
5	1×10^5
10	4×10^4
25	1.6×10^4

