Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Virological Methods 132 (2006) 154–159
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Rapid detection and quantitation of viral hemorrhagic septicemia virus in experimentally challenged rainbow trout by real-time RT-PCR

Veronica Chico, Noemi Gomez, Amparo Estepa, Luis Perez *

Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, 03202 Elche, Spain

Received 23 June 2005; received in revised form 15 September 2005; accepted 3 October 2005
Available online 4 November 2005

Abstract

A quantitative real-time RT-PCR (Q-RT-PCR) was developed to detect and determine the amount of viral hemorrhagic septicemia virus (VHSV) in organs of experimentally infected rainbow trout. Primers and TaqMan probes targeting the glycoprotein (G) and the nucleoprotein (N) genes of the virus were designed. The efficiency, linear range and detection limit of the Q-RT-PCR were assessed on cell cultured virus samples. VHSV N gene amplification was more efficient and more sensitive than the VHSV G amplicon. On cell culture grown virus, samples could be accurately assayed over a range of seven logs of infectious particles per reaction. To demonstrate the utility of Q-RT-PCR in vivo, bath infection trials were carried out and samples from fish spleen, kidney, liver and blood were harvested and tested for VHSV. Q-RT-PCR was a more reliable method than either conventional RT-PCR or the cell culture assay for virus diagnosis. Results of VHSV RNA detection in fish shortly after infection as well as on asymptomatic fish several weeks after experimental challenge are presented here. This is the first report showing the utility of Q-RT-PCR for VHSV detection and quantitation both in vitro and in vivo. The suitability of this method to test the efficacy of antiviral treatments is also discussed.

Keywords: VHSV; Real-time PCR; Fish diseases; Virus detection

1. Introduction

Viral hemorrhagic septicemia is a very contagious disease affecting predominantly rainbow trout (*Onchorrynchus mykiss*). It was formerly confined to continental Europe but in the last decade it has been found in North America (Meyers and Winton, 1995) and Asia (Isshiki et al., 2001). The agent of the disease (VHSV) is a virus belonging to the *Rhabdoviridae* family, *Novirhabdovirus* genus. VHSV has a negative-sense ssRNA genome containing six open reading frames: 3′-N-P-M-G-NV-L-5′. Due to the characteristic transcriptional attenuation of rhabdoviruses, the relative abundance of each gene mRNA depends on how close the gene is to the 3′end of the genome. VHSV causes high mortality and significant losses in aquaculture facilities. Therefore, rapid and reliable methods for VHSV detection are much needed to avoid contamination and spread of the disease. With the emergence of molecular biology techniques, new methods for virus detection have been developed to improve the standard determination of cytopathic effect on cell culture and additional virus-specific antibody confirmation (King et al., 2001). With respect to viruses infecting salmonids there are several techniques based on antibody detection of viral proteins (Estepa et al., 1995; Lorenzo et al., 1996; Mourton et al., 1992; Perez et al., 2002), as well as a number of protocols based on amplification of viral RNA by RT-PCR (Rodriguez Saint-Jean et al., 2001; Williams et al., 1999). In recent years real-time PCR has emerged as a powerful technique for the diagnosis of viral diseases, providing a mean to detect and quantify virus in tissue samples from the infected animal. In the field of aquaculture, real-time PCR have been applied to assess viral loads in shrimp (de la Vega et al., 2004; Dhar et al., 2002; Nunam et al., 2004; Tang et al., 2003). With respect to fish viruses, real-time PCR has been used to detect koi carp herpesvirus (Gilad et al., 2004), fish iridovirus (Goldberg et al., 2003) as well as viruses predominantly infecting salmonids (Munir and Kibenge, 2004; Overturf et al., 2001). Real-time RT-PCR using TaqMan probes allowed detection of 100 genome copies per reaction in samples from brain and kidney of trout infected with infectious hematopoietic necrosis virus (IHNV), a virus closely related to VHSV (Overturf et al., 2001). The low detection limit of

* Corresponding author. Tel.: +34 96 665 84 35; fax: +34 96 665 87 58.
E-mail address: luis.perez@umh.es (L. Perez).

0166-0934/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2005.10.005

this technique has proven useful dealing with asymptomatic fish which may contain minute amounts of virus and therefore being a potential a threat to healthy fish, as it has been reported for infectious pancreatic necrosis and infectious salmon anaemia viruses (Hirayama et al., 2005; Munir and Kibenge, 2004).

In the present study, detection of VHSV in samples from experimentally infected rainbow trout, is achieved by real-time RT-PCR with increased reliability when compared with other virus detection methods. Sequence of the N gene was chosen for PCR amplification based on the fact that N mRNA is the most abundant viral transcript within the infected cells. Both symptomatic and asymptomatic fish were collected, and samples from spleen, kidney, liver, and blood were analysed. In addition, real-time RT-PCR was utilized to determine whether those fish surviving a VHSV challenge become virus carriers.

2. Materials and methods

2.1. Cells and viruses

Epithelioma papulosum cyprini (EPC) cells were purchased from the European Collection of Cell cultures (ECACC#93120820, Salisbury, UK). Virus used was the viral hemorrhagic septicemia virus (VHSV), 07.71 European strain. VHSV stocks were prepared by infecting EPC cell monolayers with a multiplicity of infection (m.o.i.) of 0.001 pfu/cell. Virus from infected cell supernatants were recovered at day 7 post-infection by centrifugation at 4000 rpm for 25 min. Virus was titrated by a immunostaining assay (Lorenzo et al., 1996) on 96-well plates containing EPC cells. VHSV stock titer was 6.7×10^7 focus forming units (f.f.u.)/ml.

2.2. Animals and in vivo trials

Rainbow trout (*Oncorrynchus mykiss*) juveniles were obtained from Centro de Acuicultura *El Molino* (Madrid, Spain). Fish were held in dechlorinated water at a constant temperature of 11 °C and fed with a commercial diet (Trouw, Spain). Groups of 40 trouts (5–6 cm, 4–5 g) were experimentally challenged by immersion with 10^9 TCID50 VHSV for 1 h in 5 l water and then transferred to 240 l tanks for the rest of the experiment. Cumulative mortality was monitored daily over a 4-week period.

Fish were collected at the indicated days post-infection and the spleen and anterior kidney from a given animal were harvested and pooled together for RNA extraction. In some instances fish blood was also harvested, allowed to clot and spun down to collect the sera. An aliquot of serum was stored at −80 °C for RNA extraction, while the remaining serum was kept at −20 °C for ELISA testing.

2.3. RNA extraction

Total RNA was extracted from small pieces (5–10 mg) of fish organs using the RNAgents® Total RNA Isolation System extraction kit (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. RNA samples were dissolved in 100 μl DEPC-water and RNA concentration was determined by O.D. 260 nm and subsequently adjusted to 0.1 μg/μl for every sample. Aliquots were stored at −80 °C until RT-PCR reactions were carried out.

Table 1
Quantitative real-time PCR primers, probes and amplicon size

Name	5′ → 3′ sequence	PCR product size (bp)
VHSV-G-for	GGGCCTTCCTTCTACTGGTACTC	71
VHSV-G-rev	CGGAATCCCGTAATTTGGAAT	
VHSV-G-probe	CTGTTGCTGCAAGGCGTCCCCT	
VHSV-N-for	GACTCAACGGGACAGGAATGA	69
VHSV-N-rev	GGGCAATGCCCAAGTTGTT	
VHSV-N-probe	TGGGTTGTTCACCCAGGCCGC	

2.4. Quantitative RT-PCR assay

2.4.1. Primers and TaqMan® probes

Primers and probes for the VHSV G and VHSV N genes (Table 1) were designed by using the Primer Express™ software (Applied Biosystems). VHSV 07-71 pathogenic strain sequences were retrieved from the GeneBank database (Acc. numbers A10182 and D00687). TaqMan probes (Genotek, Spain) were labelled at the 5′ end with the reporter molecule 6-carboxy fluorescein (FAM) and at the 3′ end with the quencher 6-carboxytetramethyl-rhodamine (TAM).

2.4.2. Preparation of the VHSV RNA standards

Viral RNA was extracted from 20 μl of a VHSV-infected cell supernatant collected and titrated by an immunostaining focus forming assay previously described (Lorenzo et al., 1996; Perez et al., 2002). The RNA was resuspended in water and 10-fold serial dilutions were prepared before quantitation by the real-time RT-PCR assay.

To obtain a concentrated stock of virus, 300 ml of a VHSV-infected cell supernatant were centrifuged at 60,000 rpm for 45 min at 4 °C. VHSV pellet was resuspended in sterile water and stored at −70 °C. Concentrated VHSV titer was 1.1×10^{11} f.f.u/ml.

2.4.3. TaqMan real-time RT-PCR conditions

Reverse transcriptase (RT) reactions were performed by using 1 μg total RNA, 90 ng random hexamers and 0.5 mM dNTPs mix. After denaturing for 5 min at 65 °C, 10 mM DTT, 20 units ribonuclease inhibitor, and 200 units MMLV-RT enzyme were added in a final reaction volume of 20 μl. The RT reaction profile was: 10 min at 25 °C, 50 min at 37 °C and 15 min at 70 °C.

Quantitative PCR assays were performed using an ABI PRISM® 7700 Sequence Detector System. Reactions were carried out in a final volume of 25 μl, containing 300 nM of each primer, 100 nM probe, 2 μl RNA (unknown samples) and 1× TaqMan® Universal Master Mix (Applied Biosystems). Thermal cycler conditions were the standard default protocol of the instrument. Endogenous control for quantitation was the 18S ribosomal RNA gene. 18S rRNA levels were determined with

the TaqMan® Ribosomal RNA Control Reagents kit (Applied Biosystems) following the manufacturer's guidelines.

For absolute quantitation VHSV infectious units were calculated from the sample C_t and the standard curve regression equation, followed by normalization of the data to the 18S rRNA values. The efficiencies of the PCR reactions were calculated by using the formula $E = [10^{(-1/S)}] - 1$, with S being the slope of the standard curve.

2.5. VHSV cell culture assay

Briefly, 5–10 mg pieces of infected rainbow trout spleen and kidney were pooled together in eppendorf tubes containing PBS + gentamicin and were sonicated for 15 sec on ice. Samples were subsequently centrifuged at 3000 rpm for 20 min at 4 °C. Lysate supernantants were diluted 1:50 in culture medium prior to infection of EPC cell monolayers. At 7 days post-infection the cells were stained with crystal violet and the cytopathic effect observed.

2.6. ELISA test of rainbow trout sera

ELISA plates were coated with $\sim 2 \times 10^7$ TCID50 VHSV in 100 μl distilled water per well and incubated overnight at 37 °C. Wells were blocked with 0.3% rabbit serum in ELISA dilution buffer (0.24 mM merthiolate, 0.1% Tween 20, 50 mg/ml phenol red, 5 g/l BSA). Rainbow trout sera were tested at four dilutions (1/100, 1/200, 1/400, 1/800) in ELISA buffer. Serum dilutions were added to the VHSV coated wells and incubated at room temperature for 1 h. The plates were washed with water and a mouse anti-trout-IgM monoclonal antibody was added for 30 min followed by washing and incubation with a rabbit anti-mouse peroxidase antibody at 1:1000 dilution for 30 min. Then plates were thoroughly washed before adding the substrate (one tablet 1,2-phenylenediamine in 2 ml substrate developing buffer). Color reaction was allowed to develop at room temperature and stopped by adding 2 M H_2SO_4. O.D. at 492 nm was measured in an ELISA plate reader.

3. Results

3.1. Linearity, amplification efficiency and sensitivity of the real-time RT-PCR assay

To determine the linear range and sensitivity of the TaqMan RT-PCR assay with the VHSV-G and VHSV-N primers and probes (Table 1) an aliquot of a VHSV-infected cell culture supernatant (titer 6.7×10^7 f.f.u./ml) was used to perform total RNA extraction and RT reactions. Subsequently, serial 10-fold dilutions of the cDNA were amplified in triplicate by quantitative real-time PCR TaqMan reactions. Fig. 1A presents the standard curves plotting focus forming units versus the cycle threshold (C_t). A linear range over five orders of magnitude was observed for both the VHSV-N and VHSV-G amplicons. The slope of the standard curve indicates the efficiency of the PCR reaction. The optimal efficiency of 100% corresponds to a slope of −3.32, but in practice, efficiencies between 90 and 110% are considered acceptable. Calculation of the efficiency for each one of the amplification reactions resulted in $E_G = 120\%$ for VHSV-G and $E_N = 108\%$ for VHSV-N. Therefore, amplification of the VHSV N gene sequence was chosen for further analysis. Moreover, the sensitivity of the assay was better with VHSV-N as indicated by the lower C_t values. The lowest amount of VHSV that could be reliably quantified corresponded to 0.5 f.f.u.

The specificity of the Q-RT-PCR assay was assessed by testing RNA extracted from a cell culture infected with a related salmonid rhabdovirus: infectious haematopoietic necrosis virus (IHNV). No amplification with VHSV-N primers was observed for the IHNV samples, up to 10^4 TCID50 units of IHNV per reaction (data not shown).

By using a virus supernatant as a starting material the detection range of the Q-RT-PCR test had a top limit of 10^4 infectious units per reaction. In order to extend the detection range, a concentrated VHSV stock was prepared by ultracentrifugation. Likewise, 10-fold serial dilutions from the concentrated VHSV were analysed by Q-RT-PCR with the more efficient VHSV N gene primers. As a result (Fig. 1B), the quantitation limit

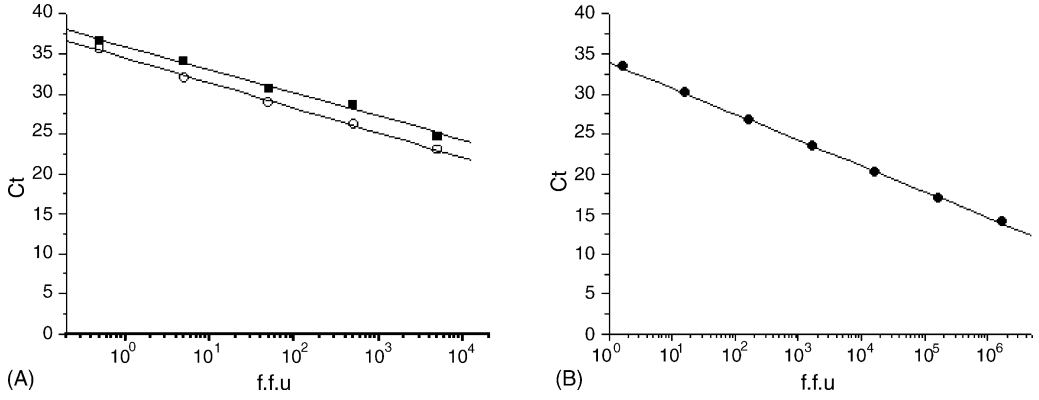

Fig. 1. Standard curves of VHSV focus forming units vs. C_t values. Standard curves were obtained from 10-fold dilutions of VHSV (titer = 6.7×10^7 f.f.u./ml) cDNA. C_t values are plotted against the log of the initial starting quantity of infectious virus (focus forming units). (A) Total RNA from VHSV cell culture supernatant. Amplification with VHSV G gene primers and probe (■); slope = −2.92, $R^2 = 0.977$. Amplification with VHSV N sequence primers and probe (◯); slope = −3.13, $R^2 = 0.999$. (B) Total RNA was extracted from a concentrated stock of VHSV (titer = 1.1×10^{11}) and after RT reaction, TaqMan PCR reactions targeting the VHSV N sequence was performed on 10-fold serial dilutions of VHSV cDNA; slope = −3.23, $R^2 = 0.999$.

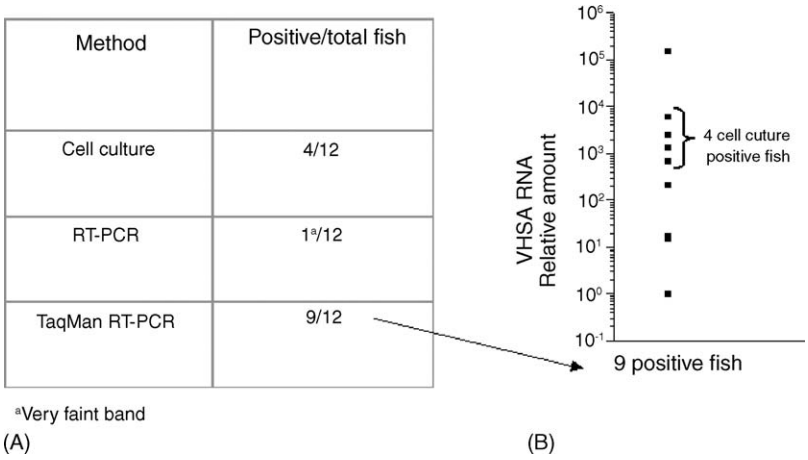

Fig. 2. (A) Comparison of cell culture, conventional RT-PCR and TaqMan RT-PCR assays performance on spleen + kidney samples from VHSV infected rainbow trout. (B) Relative quantitation of VHSV RNA in nine VHSV positive fish by TaqMan RT-PCR. The internal reference to normalize the data was the cellular 18S rRNA. The fish specimen with the lowest amount of VHSV RNA was assigned a value of 1.

was increased up to 10^6 f.f.u. per reaction, extending the linear range to seven orders of magnitude. In addition, the efficiency of the PCR amplification was improved ($E = 104\%$). The lowest amount of VHSV tested was ≈1.6 f.f.u. per reaction. The following sample dilution (that is, ≈0.16 f.f.u./reaction) did not provide reliable and accurate measurements.

3.2. Detection of VHSV RNA in experimentally infected fish

To assess the utility of the TaqMan RT-PCR assay on field specimens, spleen plus kidney tissue samples from rainbow trout challenged with VHSV in aquaria were collected during the first 10 days post-infection and analysed as described earlier. The real-time RT-PCR assay yielded more VHSV-positive fish than either conventional RT-PCR or the standard cell culture assay (cytopathic effect) as shown in Fig. 2A. Therefore, Q-RT-PCR was in our hands the most reliable method for VHSV detection in vivo. The amount of virus capable of inducing a visible cytopathic effect after a 7-day incubation appeared to be smaller than the amount of virus required to obtain a detectable product by RT-PCR. The lack of sensitivity of classical RT-PCR is not surprising: usually, nested PCR on the first-round PCR product is necessary to get a positive detection, as it has been reported by a number of groups (de la Vega et al., 2004; Hirayama et al., 2005).

Liver samples from the same fish specimens were also tested in a separate experiment, with the result of five VHSV positive fish. These five fish had already been shown to have VHSV RNA by Q-RT-PCR analysis of the spleen and kidney samples. This suggests that spleen and kidney are good sources of material for VHSV diagnosis in rainbow trout.

To get an preliminary insight on the variability of the amount of virus within the infected fish population, a second assay was carried out on the nine VHSV positive fish, normalizing C_t values with respect to an endogenous cellular standard (18S rRNA). This approach leads to a relative quantitation where the arbitrary amount of "1" is assigned to the positive sample with the smallest quantity of VHSV RNA. A difference of four orders of magnitude between the fish with the highest and the lowest viral loads was observed (Fig. 2B). It was also noticed that, with only one exception, there seemed to be a fairly good correlation between the cell culture test results and the amount of VHSV RNA found in the fish tissues.

3.3. Detection of VHSV in fish surviving experimental challenge

To demonstrate the value of the TaqMan Q-RT-PCR method for the detection of VHSV in apparently healthy fish, spleen and kidney samples were harvested from survivor fish, 9 weeks post-challenge. The overall mortality in that particular experimental trial was 49%, with fish dying between days 5 and 33 post-infection. Nine weeks after the first challenge neither infectious particles (tissue culture assay) or VHSV RNA were detected in four fish analysed (Table 2), suggesting that those fish surviving the infection were able to eliminate the virus from their bodies.

We observed in previous trials that those fish surviving VHSV infection appeared to be refractory to a second infection with the

Table 2
Detection of VHSV in spleen + kidney samples from fish surviving experimental challenge

Group	Weeks post-second challenge	VHSV+ cell culture	VHSV+ Q-RT-PCR
I	[a]	0/4	0/4
II	1	2/4	4/4
III	4	1/4	2/4[b]
IV	7	0/4	0/4[b]
Total		3/16	6/16

Rainbow trout fingerlings (≈5 g) were challenged by immersion with VHSV and maintained in aquaria for 9 weeks. After the 9-week period, the surviving fish were subjected to a second VHSV bath infection. Four fish were sampled at the indicated times. Cell culture: supernatants from spleen + kidney homogenates tested for cytopathic effect on EPC cell monolayers. Q-RT-PCR: real-time TaqMan RT-PCR targeting the VHSV N gene sequence.

[a] Nine weeks after first challenge (not subjected to a second challenge).

[b] Same result when RNA from blood samples was tested.

same virus. To further prove that those survivors were resistant to VHSV and to determine whether they had become carriers or, on the contrary, no virus remained in their bodies, 32 fish survivors of a previous experimental VHSV infection were subjected to a second challenge with VHSV (Table 2). Four fish were collected at weeks 1, 4 and 7 after this second challenge, and spleen/kidney samples were tested by cell culture and Q-RT-PCR assays. As shown in Table 2, no virus was detected at the last time point (7 weeks post-challenge). VHSV positive fish were only found at 1 and 4 weeks post-challenge: three out of eight by cell culture assay compared to six out of eight by Q-RT-PCR. Therefore, the real-time RT-PCR analysis was again a more reliable method for VHSV diagnosis.

To determine whether blood samples could be used for VHSV detection and quantitation, serum was harvested from the 4 and 7 weeks post-challenge fish for RNA extraction and Q-RT-PCR analysis. The results exactly matched those obtained from the spleen and kidney samples. The fish sera were also checked for anti-VHSV antibodies by ELISA assay. Only three out of the eight fish analysed were found to have anti-VHSV antibodies. Thus, from our results it was not possible to establish a correlation between antibody response and virus clearance.

A final remark. The fish employed in this experiment (survivors of a VHSV challenge) were actually resistant to the virus, since those fish left in the tanks (not sacrificed for testing) did not experience mortality and did not show symptoms of viral disease after the second VHSV infection.

3.4. Determination of VHSV infectious units in rainbow trout tissues

To estimate the actual number of infectious VHSV particles in a given fish sample, a standard curve was derived from a VHSV stock grown in cell culture and titrated by the immunostaining focus forming assay. Two positive fish from four time points along the experimental trial were analysed by Q-RT-PCR. Focus forming units per sample were calculated from the standard curve linear regression equation and normalized to the 18S rRNA endogenous control C_t values (Fig. 3). VHSV infectious units values ranged from 3 f.f.u./μg RNA to 0.98×10^4 f.f.u./μg RNA. When compared to the cell culture assay it appeared that $\approx 10^2$ f.f.u. was the lowest amount capable of inducing a visible cytopathic effect in vitro. As mentioned earlier, one exception to this was found at 3 d.p.i.: $a \approx 10^4$ f.f.u./μg RNA fish yielded a negative result by cell culture testing. We may speculate that VHSV defective particles were produced in that fish.

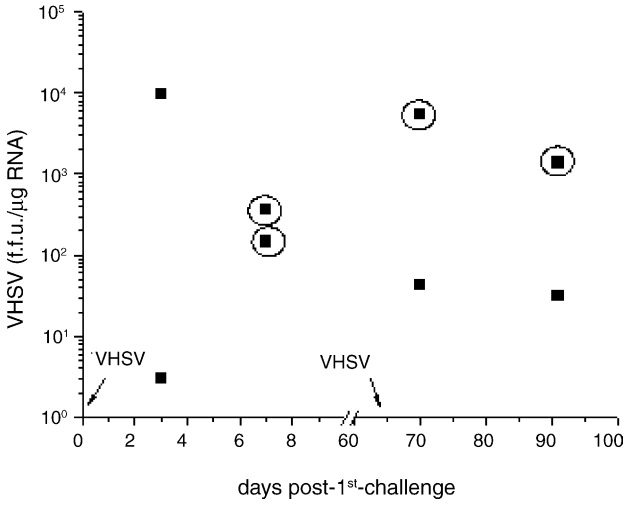

Fig. 3. Determination of VHSV infectious particles in spleen + kidney samples from experimentally infected rainbow trout by real-time RT-PCR targeting VHSV N sequence. Fish collected at 70 and 91 days had been subjected to a second VHSV infection at day 63 post-first-challenge. A standard curve with RNA from a titrated VHSV stock was run in parallel. The equation $y(C_t) = 37.75 - 3.43 \times (\log \text{f.f.u.})$ was used to calculate focus forming units (f.f.u.). Data were normalized to total RNA per reaction with the 18S rRNA C_t values. Surrounded by circles: fish diagnosed as VHSV positive by the cell culture assay.

4. Discussion

Improvement of the currently available methods for virus detection is a key issue in virology. In the fish farming industry, rapid and reliable virus diagnosis is greatly demanded. In this study, we explored the potential of the real-time RT-PCR technique as a tool for VHSV detection and quantitation. TaqMan probes and primers targeting the N gene sequence of VHSV were designed since N RNA transcripts are the most abundant in rhabdovirus infections. In fact, better sensitivity and efficiency of the PCR amplification was achieved by using VHSV N primers and probe as compared to the VHSV glycoprotein (G) amplicon. The results from our in vivo trials support the validity of the TaqMan PCR assay: low detection limit (0.5 infectious units) and a linear range spanning seven orders of magnitude. Although the detection limit of the Q-RT-PCR assay in vitro is roughly equal to the theoretical cell culture assay detection limit (1 f.f.u.), the former is still a preferable method since it is faster (2.5 days total compared to ≥7 days), it is virus specific, and above all, Q-RT-PCR has shown to be more reliable for in vivo testing: when tissue samples from infected fish were analysed, Q-RT-PCR yielded a higher number of VHSV positive fish than the cell culture assay. In principle, one may expect to have higher sensitivity with the PCR-based techniques since it is assumed that the number of viral genome and mRNA molecules is larger than the number of infectious particles in an infected cell (Bae et al., 2003). Due to the different ways authors refer to detection limits (i.e. sample dilution, plasmid/genome copies, infectious particles) a comparison with our data is not straightforward. Those who refer to infectious units (i.e. TCID50) have reported a 10^1 to 10^2 TCID50/ml detection limit by Q-RT-PCR (Boxus et al., 2005; El Mubarak et al., 2005; Orrú et al., 2004). We have roughly estimated as 50 TCID50/ml the detection limit of our assay. Moreover, the TaqMan probe Q-RT-PCR was in our hands the most efficient method to detect VHSV in apparently healthy fish. Other groups have also reported detection of a fish virus by real-time PCR in experimental trials where no mortality was observed (Gilad et al., 2004). In addition, Q-PCR is also suitable to confirm virus clearance from the body after infection, as it has been shown in here.

Another obvious benefit of real-time PCR is its application as a quantitative method. On this regard we believe that Q-PCR

may become the method of choice for testing prophylactic and therapeutic treatments against viruses. In this preliminary work we have found a high degree of variability in fish belonging to the same experimental group: up to 10,000-fold difference in spleen/kidney samples, as well as in liver samples. Over 2 log differences have been reported in fish infected with koi herpes virus as measured by real-time PCR (Gilad et al., 2004).

In this study, we explored the possibility of performing Q-RT-PCR on RNA extracted from blood samples, bearing in mind the utility of a diagnosis method which would not require to sacrifice the animal. This is not a matter of concern in many cases but it might be worthy when dealing with particularly valuable fish specimens. Furthermore, it can be useful for research purposes to be able to analyse blood samples from the same animal at different time points to learn about the spread of the infection following experimental challenge. The potential of Q-PCR in this field had already been demonstrated for bovine respiratory syncytial virus (Boxus et al., 2005). Our results indicate that fish blood is a suitable material for RNA extraction and PCR amplification, with a virus detection performance equivalent to the analysis of spleen and kidney samples.

Finally, we may conclude that Q-RT-PCR is a convenient method for diagnosis and quantitation of VHSV, a pathogen with severe impact on salmonid aquaculture. Q-RT-PCR is sensitive, reliable and suitable for studying viral replication and progression of the disease following experimental infection. Further research is currently conducted to assess the potential of this technique to determine the efficacy of antiviral treatments in vivo.

Acknowledgements

This study was supported by Generalitat Valenciana Grant GV04B/657 and GV-Grupos-03/039, and by the Spanish MEC AGL2004-07404-C02-01/ACU grant. We thank B. Bonmati for excellent technical assistance.

References

Bae, H.-G., Nitsche, A., Teichmann, A., Biel, S.S., Niedrig, M., 2003. Detection of yellow fever virus: a comparison of quantitative real-time PCR and plaque assay. J. Virol. Meth. 110, 185–191.

Boxus, M., Letellier, C., Kerkhofs, P., 2005. Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. J. Virol. Meth. 125, 125–130.

de la Vega, E., Degnan, B.M., Hall, M.R., Cowley, J.A., Wilson, K.J., 2004. Quantitative real-time RT-PCR demonstrates that handling stress can lead to rapid increases in gill-associated virus (GAV) infection levels in *Penaeus monodon*. Dis. Aquat. Org. 59, 195–203.

Dhar, A.K., Roux, M.M., Klimpel, K.R., 2002. Quantitative assay for measuring the Taura syndrome virus and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry. J. Virol. Meth. 104, 69–82.

El Mubarak, H.S., De Swart, R.L., Osterhaus, A.D.M.E., Schutten, M., 2005. Development of a semi-quantitative real-time RT-PCR for the detection of measles virus. J. Clin. Virol. 32, 313–317.

Estepa, A., De Blas, C., Ponz, F., Coll., J.M., 1995. Detection of trout haemorrhagic septicaemia rhabdovirus by capture with monoclonal antibodies and amplification with PCR. Bull. Eur. Ass. Fish. Pathol. 15, 49–53.

Gilad, O., Yun, S., Zagmutt-Vergara, F.J., Leutenegger, C.M., Bercovier, H., Hedrick, R.P., 2004. Concentrations of Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. Dis. Aquat. Org. 60, 179–187.

Goldberg, T.L., Coleman, D.A., Grant, E.C., Inendino, K.R., Philipp, D.P., 2003. Strain variation in an emerging iridovirus of warm-water fishes. J. Virol. 77, 8812–8818.

Hirayama, T., Imajoh, M., Oshima, S., 2005. Quantification of aquabirnaviruses isolated from different host species by real-time RT-PCR. Microbiol. Immunol. 49, 361–371.

Isshiki, T., Nishizawa, T., kobayashi, T., Nagano, T., Miyazaki, T., 2001. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. Dis. Aquat. Org. 47, 87–99.

King, J.A., Snow, M., Smail, D.A., Raynard, R.S., 2001. Distribution of viral haemorrhagic septicaemia virus in wild fish species of the North Sea, north east Atlantic Ocean and Irish Sea. J. Virol. Meth. 47, 81–86.

Lorenzo, G., Estepa, A., Coll, J.M., 1996. Fast neutralization immunoperoxidase assay for viral haemorrhagic septicaemia with anti-nucleoprotein monoclonal antibody. J. Virol. Meth. 58, 1–6.

Meyers, T.R., Winton, J., 1995. Viral hemorrhagic septicemia virus in North America. Ann. Rev. Fish Dis. 5, 3–24.

Mourton, C., Romestand, B., DeKinkelin, P., Jeffroy, J., LeGouvello, R., Pau, B., 1992. Highly sensitive immunoassay for direct diagnosis of viral hemorrhagic septicemia which uses antinucleocapsid monoclonal antibodies. J. Clin. Microbiol. 30, 2338–2345.

Munir, K., Kibenge, F.S.B., 2004. Detection of salmon anaemia virus by real-time RT-PCR. J. Virol. Meth. 117, 37–47.

Nunam, L.M., Tang-Nelson, K., Lightner, D.V., 2004. Real-time RT-PCR determination of viral copy number in *Penaeus vannamei* experimentally infected with Taura syndrome virus. Aquaculture 229, 1–10.

Orrú, G., De Santis, P., Solinas, F., Savini, G., Piras, V., Caporale, V., 2004. Differentiation of Italian field and South African vaccine strains of bluetongue virus serotype 2 using real-time PCR. J. Virol. Meth. 122, 37–43.

Overturf, K., LaPatra, S.E., Powell, M., 2001. Real-time PCR for the detection and quantitative analysis of IHNV in salmonids. J. Fish Dis. 24, 325–333.

Perez, L., Mas, V., Coll, J.M., Estepa, A., 2002. Enhanced detection of viral hemorrhagic septicemia virus (a salmonid rhabdovirus) by pretreatment of the virus with a combinatorial library-selected peptide. J. Virol. Meth. 106, 17–23.

Rodriguez Saint-Jean, S., Borrego, J.J., Perez-Prieto, S.S., 2001. Comparative evaluation of five serological methods and RT-PCR assay for the detection of IPNV in fish. J. Virol. Meth. 97, 23–31.

Tang, K.F.J., Durand, S.V., White, B.L., Redman, R.M., Mohney, L.L., Lightner, D.V., 2003. Induced resistance to white spot syndrome virus infection in *Penaeus stylirostris* through pre-infection with infectious hypodermal and hematopoietic necrosis virus—preliminary study. Aquaculture 216, 19–29.

Williams, K., Blake, S., Sweeney, A., Singer, J.T., Nicholson, B.L., 1999. Multiplex reverse trascriptase PCR assay for simultaneous detection of three fish viruses. J. Clin. Microbiol. 37, 4139–4141.