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

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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

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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