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Detection and quantification of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) by SYBR Green real-time reverse transcriptase-polymerase chain reaction

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Abstract

A real-time reverse transcriptase-polymerase chain reaction assay using the fluorogenic dye SYBR Green I was developed for the detection and quantification of infectious hematopoietic necrosis virus (IHNV) infecting rainbow trout (*Oncorhynchus mykiss*). Using primers designed for the IHNV nucleocapsid (N) and surface glycoprotein (G) genes, virus was detected in liver, kidney, spleen, adipose tissue, and pectoral fin samples from fish challenged in the laboratory via either injection or immersion and in fish collected from the field. The N- and G-gene amplicons provided melting curves with a single peak at 85.5 and 86.5 °C, respectively. Among different tissues tested, overall the N-gene was expressed in greater abundance than the G-gene in both laboratory-challenged and field samples. Kidney, liver, and spleen tissues had higher copies of the N- and G-genes compared to adipose tissue and pectoral fin. In samples from IHNV immersion challenge fish, the virus could be detected in the pectoral fin as early as 1 day post-challenge, and the viral load appears to decline by 6 days post-challenge. To evaluate the usefulness of non-invasive tissue sampling for IHNV detection, pectoral fin samples were collected from fish that were either apparently healthy or showing clinical signs of IHNV infection from commercial operations. Among the apparently healthy fish, using SYBR Green real-time RT-PCR the N-gene was detected in 2 out of 24 (8.3%), while the G-gene was detected in 8 of 24 (33%) fish. Among the fish showing clinical signs of IHNV infection, the N-gene was detected in 15 out of 36 (42%), while the G-gene was detected in 24 of 36 (67%) fish tested. Using a viral plaque assay, virus was detected in 4 of 24 (17%) apparently healthy fish and 33 of 36 (92%) fish showing clinical sign of IHNV infection. The higher level of IHNV detection by plaque assay compared to real-time RT-PCR might be due to the presence of more than one isolate in the field samples, and the inability to detect all the IHNV isolates using the current set of primers used in real-time RT-PCR. In conclusion, we developed a real-time RT-PCR assay for the detection and quantification of IHNV by SYBR Green real-time RT-PCR. This study demonstrates the potential of using fin clip as a non-invasive tissue source for detecting IHNV and possibly other viruses infecting salmonids in commercial aquaculture and in the field.

Keywords: Infectious hematopoietic necrosis virus; IHNV; Real-time RT-PCR; SYBR Green; Non-invasive virus detection; Rainbow trout

1. Introduction

Infectious hematopoietic necrosis virus (IHNV), the type species in the genus *Novirhabdovirus* within the family *Rhabdoviridae*, infects several species of wild and cultured salmonids. The virus causes severe losses in epizootics in young fish and infects adults that remain asymptomatic and potential carriers. The virus is endemic throughout the American Pacific Northwest from Alaska to California and inland to Idaho. The virus has been detected in Asia and Europe and it has been speculated that this occurred through movement of infected fish and contaminated eggs (Winton, 1991). The genome of IHNV contains a single-stranded, negative-sense RNA of approximately 11.1 kb (Morzunov et al., 1995; Schuetze et al., 1995). There are six genes in the IHNV genome that are in the 3′ to 5′ order: nucleocapsid (N), polymerase-associated phosphoprotein (P), matrix (M), surface glycoprotein (G), nonvirion protein (NV),

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and virus polymerase (L) (Morzunov et al., 1995; Schuetze et al., 1995). Among the IHNV encoded genes, the N-gene is expressed first and is the most abundant during viral infection (Bootland and Leong, 1999). Therefore, the N-gene is a good target for early detection of IHNV. Compared to the N-gene, the G-gene is expressed later in the infection process (9–10 h post-infection). However, the middle of the G-gene (also called mid-G) was found to be variable among different IHNV isolates making this section of the G-gene potentially a good marker for phylogenetic analysis of IHNV isolates (Troyer et al., 2000; Garver et al., 2003; Kurath et al., 2003).

Due to the extensive losses caused by IHNV in salmon and trout aquaculture facilities, several methods have been developed for detecting IHNV (Winton, 1991). These include isolating the virus from candidate fish by infection of established cell lines then confirming the identity by serum neutralization assay (OIE, 2000), enzyme-linked immunosorbent assay (ELISA), *in situ* hybridization using biotinylated probe (Deering et al., 1991), immunohistochemical and immunogold labeling (Drolet et al., 1995), reverse transcriptase-PCR (RT-PCR) (Arakawa et al., 1990; Chiou et al., 1995; Barlic-Maganja et al., 2002), and real-time RT-PCR (Overturf et al., 2001; Purcell et al., 2006). Currently, the plaque assay is considered the gold standard for IHNV detection but is very time consuming, usually taking 7–10 days to complete the assay. Among the different methods of IHNV detection, RT-PCR is rapid and highly sensitive but fails to distinguish between infectious *vs.* non-infectious virus. In addition, quantification of the target gene by conventional RT-PCR is laborious, time consuming, and relies on post-PCR analysis of the amplified product. The limitations of conventional RT-PCR have been overcome with the development of real-time PCR assays (reviewed in Bustin, 2000; Mackay et al., 2002). The real-time RT-PCR method has a greater sensitivity than conventional PCR, requires little initial RNA, and thus, has become useful when dealing with a limited amount of tissue. In addition, it has a wide dynamic detection range, does not require post-PCR analysis, and can be formatted for high throughput applications (Bustin, 2000). Different methods have been employed to detect amplicons generated by real-time RT-PCR. These include detection using DNA-binding fluorophores (such as SYBR Green I), linear oligoprobes, 5′ nuclease oligoprobes, molecular beacons, and auto-fluorescent amplicons (Mackay et al., 2002). Among them, detection by SYBR Green I is the simplest method and least expensive since it does not require the design of fluorogenic oligoprobes. The higher melting temperature of the expected amplicon allows discrimination of target amplicons from primer-dimer in SYBR Green real-time RT-PCR (Ririe et al., 1997). Real-time RT-PCR has proven valuable for the detection of viral pathogens in plants and animals including human (reviewed in Mackay et al., 2002; Niesters, 2002). In recent years, real-time PCR has increasingly been used for the detection and quantification of viruses infecting fish and shellfish. For example, real-time PCR has been used for the detection of IHNV in trout (Overturf et al., 2001; Purcell et al., 2006), infectious salmon anemia virus in salmon (Munir and Kibenge, 2004); koi herpes virus in koi (Gilad et al., 2004); and infectious hypodermal and hematopoietic necrosis virus, white spot syndrome virus, Taura syndrome virus, yellowhead virus, gill-associated virus, Mourilyan virus, infectious myonecrosis virus and *Vibrio penaeicida* in shrimp (de la Vega et al., 2004; Dhar et al., 2001, 2002; Rajendran et al., 2006; Tang and Lightner, 2001; Tang et al., 2004; Andrade et al., 2007; Goarant and Merien, 2006). The real-time detection methods published for IHNV in trout are based on the TaqMan assay (Overturf et al., 2001; Purcell et al., 2006). However, TaqMan real-time PCR is costly due to the need to make a probe, and therefore, a less costly alternative will be useful for some applications. The aim of the present study was to develop a real-time RT-PCR assay for the detection and quantification of IHNV using a minor groove DNA binding dye SYBR Green I. Using SYBR Green real-time RT-PCR, the virus was detected in IHNV-infected cell culture as well as in the kidney, liver, spleen, adipose tissue and pectoral fin samples of rainbow trout (*Oncorhynchus mykiss*) challenged in the laboratory or in the field. The ability to detect the virus in pectoral fin opens up the possibility of developing an IHNV detection method based on non-invasive tissue sampling.

2. Materials and methods

2.1. Cell culture and viral isolates

Epithelioma papulosum cyprinid (EPC) cells were inoculated with IHNV (Strain 220-90) using a virus inoculum that contained 2.5×10^7 pfu/mL (MOI of 0.526) in a 24-well microplate as previously described (LaPatra et al., 1994). Virus inoculated and control cell cultures were maintained at 17 °C in minimum essential medium supplemented with 2% fetal bovine serum. Four days post-inoculation, control and virus inoculated cells were harvested and 500 μL TRI Reagent™ (Molecular Research Center, Inc., Cincinnati, OH) was added before storing the cells at −75 °C until use.

2.2. Experimental infection of rainbow trout with IHNV and tissue sampling

Two methods of viral challenge were used in this study (injection and immersion) and are described below. Additionally, naturally infected fish were obtained in and around Idaho.

2.2.1. IHNV challenge by injection

Virus challenge was performed by injecting specific pathogen-free rainbow trout (*O. mykiss* Walbaum, average weight 50 g) intraperitoneally with approximately 100 μl of tissue culture fluid that contained ~10^7 pfu/mL of IHNV (IHNV Strain 220-90; LaPatra et al., 1994). Seventy-two hours post-injection, virus injected fish appeared moribund. Moribund fish were sacrificed; tissue samples (approximately 100 mg) from the liver, kidney, spleen, adipose tissue, and pectoral fin were collected in TRI reagent and stored at −75 °C until RNA isolation was performed. Tissue samples from control (sham injected) fish were also collected in a similar manner. There were six virus injected and four control fish. Five different tissue samples were collected from each fish resulting in a total of 50 tissue samples.