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Sensitive and specific detection of *Salmonid alphavirus* using real-time PCR (TaqMan®)

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Abstract

Pancreas disease is responsible for major economic losses in the European salmonid farming industry. It was previously believed that a single subtype (salmon pancreas disease virus) of the virus species *Salmonid alphavirus* (SAV) was responsible for all outbreaks of pancreas disease in the UK and Norway. However, the recent discovery that pancreas disease in Norway is caused by a new and distinct subtype of salmonid alphavirus, exclusively found in Norway, has advocated the need for better diagnostic tools. In the present paper, three real-time PCR assays for all known subtypes of salmonid alphavirus have been developed; the Q_nsP1 assay is a broad-spectrum one that detects RNA from all subtypes, the Q_SPDV assay specifically detects the salmon pancreas disease virus subtype, and the Q_NSAV assay only detects the new Norwegian salmonid alphavirus subtype.

The results demonstrated the assays to be highly sensitive and specific, detecting <0.1 $TCID_{50}$ of virus stocks. Regression analysis and standard curves calculated from the C_t-values from 10-fold serial dilutions of virus stocks showed that the assays were highly reproducible over a wide range of RNA input. Thirty-nine field samples were tested in triplicate and compared with traditional RT-PCR. Overall, the real-time assays detected 35 positive compared to 29 positives in standard RT-PCR, and were thus shown to be more sensitive for detecting salmonid alphaviruses in field samples.

Keywords: *Salmonid alphavirus*; Real-time PCR; Virus detection

1. Introduction

Pancreas disease in farmed salmonids is commonly associated with infections by *Salmonid alphavirus* (SAV). The disease occurs mainly in Atlantic salmon in their first or second year at sea, and diseased fish are often lethargic, with abnormal swimming behaviour. Histopathological lesions in association with pancreas disease always include various degrees of heart and skeletal muscle myopathy. Acute and chronic pancreatic lesions in exocrine pancreatic tissue may also be present in diseased fish (McLoughlin et al., 2002). The virus responsible for pancreas disease in Ireland and Scotland have been isolated and identified as an alphavirus, and the name salmon pancreas disease virus (SPDV) was suggested (Nelson et al., 1995; Welsh et al., 2000; Weston et al., 1999). Because pancreas disease affected fish from Norway show similar clinical symptoms and gross pathology, it has been of the common opinion that pancreas disease in the British Isles and Norway is caused by the same virus. However, Hodneland et al. (2005) recently showed that pancreas disease from Atlantic salmon and rainbow trout in Norway is in fact caused by a different and distinct virus subtype, and named it Norwegian salmonid alphavirus (NSAV). Together with the sleeping disease virus (SDV) (Boucher and Baudin Laurencin, 1994; Branson, 2002; Castric et al., 1997; Graham et al., 2003b; Villoing et al., 2000a) and salmon pancreas disease virus, the Norwegian salmonid alphavirus is included in the species *Salmonid alphavirus* in the genus Alphavirus of the family Togaviridae. All three salmonid alphavirus subtypes have a genomic organization characteristic to the Alphaviruses; with a positive-sense, single stranded genome of approximately 11.8 kb size. The 5′-terminal end codes for the four non-structural proteins (nsP1–nsP4) essential for virus replication, whereas the 3′-terminal comprises the genes for the structural proteins E1–E3,

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capsid and 6K. Nucleotide sequence comparisons have shown that Norwegian salmonid alphavirus, salmon pancreas disease virus and sleeping disease virus are approximately equal in evolutionary distance to each other, with differences ranging from 91.6 to 92.9% (Hodneland et al., 2005).

Previously, the diagnosis of pancreas disease and sleeping disease was based on clinical sign in combination with histopathological findings. Detection of antibodies or virus isolation in fish cells may also be used to verify the aetiology of the disease (Graham et al., 2003a; Jewhurst et al., 2004). However, the presence of virus-specific antibodies does not provide any information about the viraemic status of an infected fish, and considering the high percentage similarity of the structural proteins among the salmonid alphaviruses, the potential for cross-reactivity of serological assays is high. Furthermore, it is not possible to unequivocally distinguish subtypes of salmonid alphavirus in cell-cultures. As a result of the relative insensitivity of non-molecular detection methods, molecular methods such as RT-PCR based techniques have been developed for a number of fish RNA viruses, and have been demonstrated successfully to increase the detection rate. Villoing et al. (2000b) presented a two-step RT-PCR assay for detection of sleeping disease virus RNA in naturally infected salmonids, which also proved useful for amplification of salmon pancreas disease virus in experimentally infected fish. However, the RT-PCR could not discriminate between the two subtypes without further sequencing studies.

With the discovery of the third salmonid alphavirus subtype and the distinct geographical distribution of at least salmon pancreas disease virus and Norwegian salmonid alphavirus, the ability to distinguish between types or strains of virus that may have distinct biological properties is important for both national and international management and control of the disease. At present, existing methods are not sufficient to rapidly distinguish between the different pheno-/genotypes, and the development of a more powerful diagnostic assay for direct identification of salmonid alphavirus subtypes, with respect to sensitivity, specificity and speed will be useful. The real-time PCR technology is now used commonly for detection and quantification of many viruses (Mackay et al., 2002; Niesters, 2001; Niesters, 2002), however the only piscine viruses where real-time PCR assays have been developed are piscine nodavirus (Starkey et al., 2004), infectious salmon anemia virus (ISAV) (Munir and Kibenge, 2004) and infectious haematopoietic necrosis virus (IHNV) (Overturf et al., 2001).

The present paper describes the development and validation of real-time PCR assays for the sensitive detection and differentiation of three subtypes of salmonid alphavirus (salmon pancreas disease virus, sleeping disease virus and Norwegian salmonid alphavirus) using the TaqMan® probe chemistry. By developing these assays it is now possible to screen rapidly for all known salmonid alphavirus subtypes without the need for prior isolation and culture, or time-consuming post-PCR steps. The advantage of using real-time PCR for detection of salmonid alphaviruses not only saves time and labor, but also has the potential to differentiate and quantitate any subtype of salmonid alphavirus within the host.

2. Material and methods

2.1. Virus stocks and clinical samples

The specificity of the real-time PCR assay was determined using control strains as a RNA source. Cultured virus stocks from three different subtypes of salmonid alphavirus were used as reference templates in the real-time assays: Norwegian salmonid alphavirus isolated from Norwegian salmon suffering from pancreas disease (SavH10/02, Genebank accession no. AY604237), salmon pancreas disease virus from pancreas disease affected salmon from Ireland (F93-125, Genebank accession no: AJ316244), and a sleeping disease virus isolate originating from rainbow trout from France (kindly supplied by Dr. K.E. Christie, Intervet Norbio AS, Bergen, Norway). Virus titers ($TCID_{50}$) was $10^{5.8}$/ml for Norwegian salmonid alphavirus, $10^{7.6}$/ml for salmon pancreas disease virus, and $10^{6.25}$/ml for the sleeping disease virus cell culture. Heart tissues from 39 salmon from various fish farms were collected and tested to evaluate the performance of the real-time PCR assays from field samples. The fish in the salmon farms were diagnosed, or suspected to suffer from pancreatic disease.

2.2. RNA extraction

RNA extraction from both infected cell cultures and tissues was performed as described by Devold et al. (2000). The purity of the RNA was evaluated by measuring the absorbance ratio at 260/280 nm (optimal 1.8–2.0), and RNA quality was checked on ethidium bromide-stained agarose (1%) gel using UV illumination. RNA from tissue samples was dissolved in RNAse free water at a working concentration of 100 ng/ul.

2.3. Standard RT-PCR

Standard RT-PCR assays were performed by incubating 2 ul of dissolved total RNA with 1.0 ul (1 ug/ul) random hexamer pd(N)6 primer and 7.0 ul ddH_2O at 70 °C for 5 min and placed on ice. The RT-reaction was carried out at 37 °C for 60 min with 10 U Rnasin, 5.0 μl 5 × RT-buffer, 3.0 U M-MLV-reverse transcriptase, 1.25 μl DTT (200 mM), 2.5 μl dNTP (10 mM). The PCR was performed in a 25 ul reaction volume containing 2.0 μl cDNA template, 2.5 μl 10 × Taq buffer, 1.0 μl (10uM) of each PCR primer (Table 1), 2.0 μl (10 mM) dNTP mix, 0.1 μl (5 U/ul) Taq DNA polymerase and 16.4 μl ddH_2O. The PCR profile was as follows: one cycle at 95 °C in 3 min; then 40 cycles at 94 °C for 30 s; 55 °C for 45 s; and 72 °C for 90 s; followed by one cycle at 72 °C for 10 min. The amplification and cDNA synthesis were performed in GeneAmp PCR System 9700 (Perkin-Elmer). PCR products were visualized on an ethidium bromide-stained agarose (1%) gel using UV illumination.

2.4. Primers and probes

TaqMan PCR primers and probes were designed according to standard cycling conditions using the PrimerExpress software package (PE Applied Biosystems), and were derived from an