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TaqMan® real-time RT-PCR detection of infectious salmon anaemia virus (ISAV) from formalin-fixed paraffin-embedded Atlantic salmon *Salmo salar* tissues

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ABSTRACT: The objective of this study was to evaluate the application of a TaqMan® real-time reverse transcriptase PCR (RT-PCR) assay for the detection of infectious salmon anaemia virus (ISAV) in formalin-fixed paraffin-embedded (FFPE) fish tissues from Atlantic salmon *Salmo salar* with and without clinical signs of infection, and to compare it with histological and immunohistochemical (IHC) techniques. Sixteen fish samples obtained in 2007 and 2008 from 4 different farms in Chile were examined. The real-time RT-PCR allowed the detection of ISAV in FFPE samples from 9 of 16 fish, regardless of the organs analyzed, whereas 4 of the real-time RT-PCR negative fish were positive as indicated by histological examination and 3 of the real-time RT-PCR positive fish were negative as indicated by immunohistochemistry evaluation. The presence of ISAV in RT-PCR positive samples was confirmed by amplicon sequencing. This work constitutes the first report on the use of real-time RT-PCR for the detection of ISAV in FFPE sections. The assay is very useful for the examination of archival wax-embedded tissues, and allows for both prospective and retrospective evaluation of tissue samples for the presence of ISAV. However, the method only confirms the presence of the pathogen and should be used in combination with histopathology, which is a more precise tool. The combination of both techniques would be invaluable for confirmatory diagnosis of infectious salmon anaemia (ISA), which is essential for solving salmon farm problems.

KEY WORDS: Detection · Infectious salmon anaemia virus · Fixed tissue · Real-time RT-PCR

INTRODUCTION

Infectious salmon anaemia (ISA), a serious viral disease of marine-farmed Atlantic salmon *Salmo salar*, was first recognized in Norway (Thorud & Djupvik 1988). The disease is caused by infectious salmon anaemia virus (ISAV), which belongs to the genus *Isavirus*, family *Orthomyxoviridae* (Falk et al. 1997, Mjaaland et al. 1997). Since its discovery in Norway, the disease has been identified in different countries in the northern hemisphere such as Canada (Mullins et al. 1998), Scotland (Rodger et al. 1998), USA (Bouchard et al. 2001) and the Faroe Islands (Lyngøy 2003). In the southern hemisphere, ISAV was first detected in Chile in 1999 in marine-farmed coho salmon *Oncorhynchus kisutch* (Kibenge et al. 2001). However, the involvement of ISAV in a disease outbreak was officially verified in Atlantic salmon in mid-June 2007 (Godoy et al.

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2008), and to date 62.3% of Chilean marine salmon farm sites are affected by the disease. Until now, the infection has been confined to salmon held in seawater or exposed to seawater, but the presence of ISAV in fish in freshwater cannot be ruled out. In fact, there is a previous report of an outbreak in first-feeding salmon fry in a Norwegian hatchery that did not use seawater (Nylund et al. 1999).

The disease is characterized by severe anaemia and leucopoenia, pale gills, exophthalmia and haemorrhages in the eyes, congestion of the liver, spleen and foregut, haemorrhagic hepatic necrosis, renal interstitial haemorrhage and acute tubular necrosis, petechiae in the viscera and ascites (Thorud & Djupvik 1988, Evensen et al. 1991). Current methods for diagnosing ISAV includes epidemiological and clinical observation (Jarp & Karlsen 1997), gross pathology and histopathology (Evensen et al. 1991, Speilberg et al. 1995, Byrne et al. 1998), virus isolation in Chinook salmon *Oncorhynchus tshawytscha* embryo (CHSE-214) cells (Bouchard et al. 1999) and Atlantic salmon head kidney (SHK–1) cells (Dannevig et al. 1995), immunohistochemistry (IHC) (Falk & Dannevig 1995, Falk et al. 1998) and reverse transcriptase polymerase chain reaction (RT-PCR) (Mjaaland et al. 1997, Snow et al. 2006, Workenhe et al. 2008). Although desirable, it is not always possible to apply all currently available diagnostic methods to a single case. RT-PCR has proven to be very useful in diagnosing subclinical infections in Atlantic salmon (Mjaaland et al. 1997, Opitz et al. 2000, Mikalsen et al. 2001, Snow et al. 2003).

In Chile, the ability to diagnose infections is limited and this is primarily related to the fact that the fish farms are too far from the diagnostic laboratories; transportation of samples is often slow, which can influence the quality of diagnostic test results (Mikalsen et al. 2001). Consequently, the lack of availability of fresh tissue specimens requires health professionals to collect and fix whole fish and/or several fish organs (i.e. kidney, gill, heart, pancreas, spleen, pyloric caecae and liver) in formalin before transportation to the laboratory, particularly for fish mortalities that occur early in the disease process in the absence of clinical signs. Diagnostic methods such as histopathology are often used on formalin-fixed paraffin-embedded (FFPE) tissues, which are routinely stored as FFPE blocks. To date, the application of RT-PCR assays for the detection of ISAV in fixed tissues has remained largely unexplored, yet there is a real need for the development of a primary ISA diagnostic method based on histopathological examination combined with a confirmatory test.

We present the first report on the use of real-time RT-PCR with TaqMan® (Roche Diagnostics) probe chemistry for the detection of ISAV in FFPE sections that have histopathological changes consistent with ISA. In addition, the performance of the real-time RT-PCR protocol was compared with histological and IHC techniques.

MATERIALS AND METHODS

Samples. From 2007 to 2008 total of 16 Atlantic salmon samples, weighing between 1.5 and 2.0 kg, were received at the Biovac S.A. laboratory in Puerto Montt, Chile, from 4 fish farms (Farms A to D) located in southern Chile. ISAV had never been detected in these farms, but anecdotal information from each farm suggested that the fish in these populations seemed to be affected by ISAV. Sampled fish were killed by an overdose of anaesthetic (BZ-20®) in a saltwater solution and immediately subjected to a full necropsy. Tissue samples were aseptically collected from each fish for histological and IHC evaluation.

Histology. Portions of organs (gill, liver, pyloric caecae, pancreas, spleen and kidney) from each fish were fixed in 10% buffered formalin, dehydrated and embedded in paraffin following standard procedures. Tissue blocks were sectioned at 5 µm and 3 sections per tissue per fish were stained with haematoxylin and eosin (H&E) to describe histopathological alterations. The cells were viewed at 200× or 400× magnification under a Leica DM2000 microscope.

IHC. The IHC assay was performed on a single section of tissue per fish, as described by Godoy et al. 2008. Briefly, tissues were tested using anti-ISAV monoclonal antibody P10 (Aquatic Diagnostics). Antigen–antibody complexes were detected with the IHC kit from Vector Laboratories. Negative control tissue sections were obtained from Atlantic salmon cultured at a local farm that was registered free from ISAV and were analyzed by histological and real-time RT-PCR techniques to confirm the absence of ISAV. Positive controls for IHC were obtained from fish experimentally inoculated with ISAV. Appropriate positive and negative controls were run in parallel.

Total RNA extraction. FFPE tissues chosen for this study were the same as those that had been analyzed by histological and IHC techniques, including 3 fish from Farm C that were negative as indicated by these techniques. A positive ISAV diagnosis was based on the previous findings by histological examination and a positive reaction between the virus and monoclonal antibody against ISAV by IHC examination.

Total RNA was extracted from FFPE tissues (3 extractions per block) using the MagNa Pure LC RNA Isolation kit III (tissue) (Roche Diagnostics) according to the manufacturer's instructions. RNA purification was carried out automatically by the MagNa Pure L instrument. Real time RT-PCR reactions were carried out with RNA obtained from 288 samples (equivalent of 16 fish × 6 different organs × 3 extractions per organ). The purified RNA was quantified on a NanoPhotometer™ (Implen), diluted with ultra-pure water when necessary to adjust to a concentration of 8 to 20 ng μl^{-1} and stored at –20°C. A volume of 1 μl of each RNA solution was used in the amplification reaction.

Real-time RT-PCR and nucleic acid sequencing. Real-time one-step RT-PCR assays were performed on a LightCycler® 480 (Roche Diagnostics) using the ISAV specific primer pair-targeting genomic segment 8 described by Snow et al. (2006), which gives an amplification product of 104 bp. In the 1-step RT-PCR assay, reverse transcription and PCR were performed using the LightCycler® 480 RNA Master Hydrolysis Probes (Roche Diagnostics) according to the manufacturer's instructions. The PCR thermal profile consisted of an initial reverse transcription step of 63°C for 3 min, followed by 30 s at 95°C and 45 cycles of 95°C for 15 s each, 60°C for 1 min and a final extension at 72°C for 10 s. Each run included 2 positive controls (one RNA extracted from FFPE tissue experimentally infected with ISAV and the other one infected with nonfixed tissue seeded with ISAV), at least 1 FFPE negative control tissue containing no target and a RNA-free negative control. The reproducibility of the results was assessed by performing 3 independent PCR assays and samples were considered to be positive when all 3 replicate PCR assays were ISAV positive. Each amplification cycle was analyzed and the average crossing point (CP) without baseline adjustments were recorded in all samples.

Amplified products were separated on a 1% agarose gel containing 0.5 μg ml^{-1} of ethidium bromide (Bio-Rad). Bands of 104 bp in size were extracted from the gel using the Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence analysis used the Basis Local Alignment Search Tool (BLAST) program.

RESULTS

Histology

The Atlantic salmon included in this study were from 4 seawater farms that were part of the ISA surveillance programme in Chile. None of these fish showed any external clinical signs that indicated the presence of ISA or any other health problems. However, upon necropsy, enteritis and signs of hemorrhaging in the pyloric caecae were evident. The results of the histopathological examination together with the IHC and real-time RT-PCR analyses on these samples are summarized in Table 1. Of the 16 fish examined, 13 showed histological alterations; in other words, all 3 sections for each organ sampled showed ISA pathology in the 13 fish. In the gill samples, detachment of the epithelium from the basal membrane was observed. The kidney showed some moderate degeneration and necrosis of the renal tubular epithelium (Fig. 1A). In this organ, a mild diffuse sinusoidal congestion was also observed in the same 13 fish. The liver had sinusoidal congestion and peliosis (Fig. 1B), and in some samples the hepatocytes displayed pyknotic nuclei and intravascular erythrophagia. The pyloric caecae were the most severely affected, showing significant pancreatic acinar cell loss and pyknotic nuclei typical of ISA microscopic lesions.

IHC

When single sections of the gill, liver, pyloric caecae, pancreas and kidney were subjected to IHC, ISAV protein was detected in all organs from all 6 salmon obtained from Farm A (Table 1). Positive staining of

Table 1. *Salmo salar*. Detection of infectious salmon anaemia virus (ISAV) in 16 Atlantic salmon by means of histological and immunohistochemistry evaluations, and real-time RT-PCR from paraffin-embedded blocks of samples of gill, liver, pyloric caecae, pancreas, spleen and kidney from each fish. Total number of samples tested for each technique is given in parentheses (16 fish × 6 organs sampled × number of replicates per organ). Fish were considered to be positive when all replicates from at least one organ was ISAV positive, regardless of the diagnostic technique employed

Source	No. of fish	Year of sampling	Method of ISAV detection					
			Histological observation (n = 288)		Immunohistochemistry (n = 96)		Real-time RT-PCR (n = 288)	
			Positive	Negative	Positive	Negative	Positive	Negative
Farm A	6	2007	6	0	6	0	6	0
Farm B	3	2008	3	0	0	3	3	0
Farm C	3	2008	0	3	0	3	0	3
Farm D	4	2008	4	0	0	4	0	4

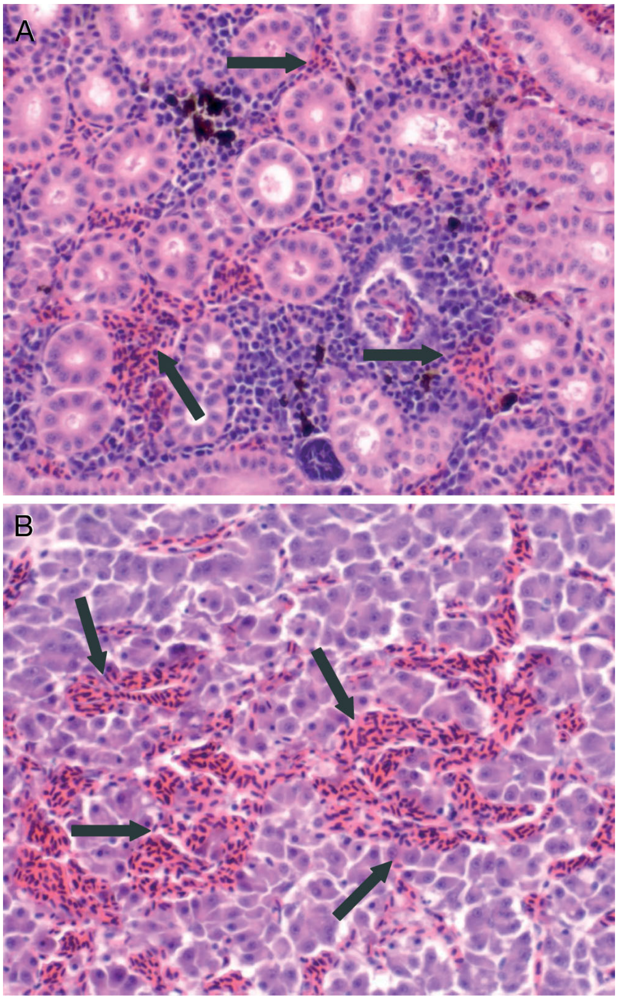

Fig. 1. Infectious salmon anaemia virus (ISAV) in *Salmo salar*. A representative selection of H&E-stained sections of Atlantic salmon tissues. (A) Kidney tissue showing diffuse sinusoidal congestion (200×). (B) Liver tissue with multifocal to coalescing of sinusoidal congestion and peliosis (200×). Arrows indicate cellular alterations

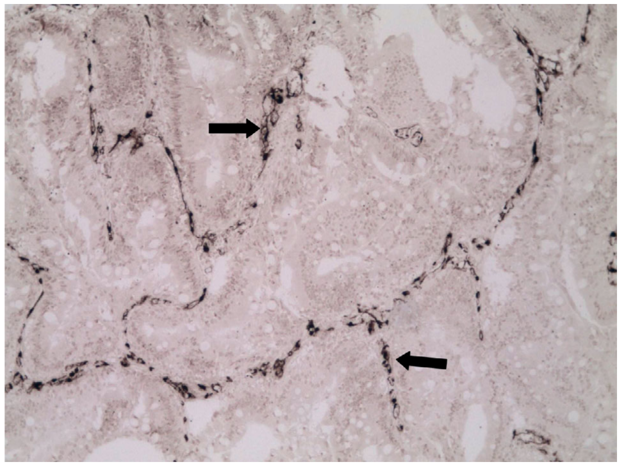

Fig. 2. Infectious salmon anaemia virus (ISAV) in *Salmo salar*. Photomicrograph of an immunohistochemical assay using a monoclonal anti-ISAV antibody showing positively stained (dark brown colour, arrows) cells of the pyloric caecae of Atlantic salmon (magnification 100×)

cells of the pyloric caecae, which together with the liver samples showed the strongest IHC staining relative to control tissue, is shown in Fig. 2. Samples from Farms B, C and D and negative controls were negative for stain.

RT-PCR assays

Real-time RT-PCR detected ISAV in 9 of 16 fish, with all 3 replicate FFPE sections of kidney, gill, liver, pyloric caecae, pancreas and spleen giving positive results (Table 1). Samples were considered positive with a CP of 33 or less and when all 3 replicate real-time RT-PCR reactions per sample were positive. The mean (±SD) CP value of positive samples was 24.08 ± 2.33 and in these cases the RNA concentration from FFPE ranged from 8.5 to 16.5 ng μl^{-1}. All IHC positive cases were also positive in real-time RT-PCR, whereas all IHC negative samples from Farm B were positive by real-time RT-PCR. All FFPE blocks prepared with fish samples from Farms C and D were negative by real-time RT-PCR regardless of the organs analyzed. According to histological analysis, all specimens from Farm D were positive; however, they were negative by real-time RT-PCR. All negative control blocks were negative.

Real-time RT-PCR positives generated products of the expected size (data not shown), and sequence analysis of amplicons confirmed the presence of ISAV sequences.

DISCUSSION

Methods for the diagnostic confirmation of ISAV in natural and experimentally infected Atlantic salmon include histological observation, IHC, virus isolation and PCR-based techniques such as real-time RT-PCR. Recently, an ISAV TaqMan® RT-PCR assay was developed to detect all known variants of ISA (Snow et al. 2006). This procedure has been extensively used by our working group on fresh tissues, showing high specificity and sensitivity as denoted also by Snow et al. (2006, 2009), but its potential application on FFPE fish tissues was untested. The evaluation of FFPE tissues and other archival material has been successfully employed for both prospective and retrospective assessment within a number of fish bacterial and viral pathogens as well as the mammalian and human viral pathogens (Bhudevi & Weinstock 2003, Dixon et al. 2004, Bhatnagar et al. 2007, Crumlish et al. 2007, Karatas et al. 2008, Cano et al. 2009). The fixation of fish tissues in formalin and long-term storage in paraffin blocks are common laboratory practices for histological evaluation of tissues for disease diagnosis. The traditional diagnostic method of virus isolation plus identification usually takes 8 to 12 d for definitive identification of ISAV.

The results obtained in the present work clearly demonstrate the presence of ISAV in different FFPE organs from Atlantic salmon using real-time RT-PCR, which was supported by nucleotide sequencing of the expected 104 bp amplicon. This suggests that all the tissue types tested here could be used to detect the virus by this real-time RT-PCR. Tissues testing positive had a mean CP value of 24.03, but no data on CP versus viral load from fixed tissues have been actually analyzed.

All 4 fish samples from Farm D rendered histopathological alterations due to ISAV, but, even after a few days when these fish were examined and showed signs of disease followed by mortality, the real-time RT-PCR failed to identify these fish, regardless of the FFPE-fixed organs analyzed. There could be a variety of reasons why FFPE samples can give false negative readings by real-time RT-PCR. In fact, in most studies that examine FFPE samples, the effects of temperature and time of tissue handling, processing and storage of tissues in formalin may reduce the sensitivity of RT-PCR as well as have a tendency to degrade RNA or DNA, which can result in short fragments and low copies of genetic material (Fiallo et al. 1992, Foss et al. 1994, Bhudevi & Weinstock 2003, Bhatnagar et al. 2007, Pourahmad et al. 2009).

Our findings also suggest that the results of some tissues submitted for IHC evaluation were false negatives (Farm B), since these same 3 fish (i.e. 18 samples) showed positive with histological examination and real-time PCR. These false negative findings are in agreement with the observations by Opitz et al. (2000), who used experimentally infected salmon. Opitz et al. (2000) also found a high number of false negatives with IHC and were able to detect ISAV in only 4 of 22 infected fish, and that the nested RT-PCR method was significantly more sensitive than IHC. Similar results have been reported for the detection of *Piscirickettsia salmonis* (Karatas et al. 2008).

Reproducibility of PCR results was demonstrated by obtaining the same results in 3 independent real-time RT-PCR assays. Wilson (1997) denotes that in some tissues, such as the ones used in this study, it is common to observe the presence of undefined inhibitors of PCR; however, the presence of fish DNA did not interfere with the real-time RT-PCR assay.

Specific detection using TaqMan® chemistry is significantly improved when compared with either conventional RT-PCR or real-time RT-PCR based on SYBR green chemistry (Snow et al. 2006, Workenhe et al. 2008). Further investigation must be done to establish the detection limits of the procedure used in the present study for FFPE samples. It is generally known that some fixation methods, including type of fixative, fixation time and RNA extraction procedure, can damage the RNA and deleteriously affect subsequent PCR analysis (Greer et al. 1991). In the case of compromised RNA, it is recommended that the best sensitivity for RT-PCR on fixed tissue RNA can be obtained with amplicon sizes less than 200 bp (Greer et al. 1991, Godfrey et al. 2000). Based on this, an optimized commercial RNA extraction kit for FFPE tissues and a real-time RT-PCR assay (Snow et al. 2006) were used.

Real-time RT-PCR assay is useful for examination of archival wax-embedded material and allows for both prospective and retrospective evaluation of tissue samples for the presence of ISAV. However, this method only confirms the presence of the pathogen and should be used in combination with histopathological examination of the fixed tissue section, which is a more precise tool, and the combination of both techniques would be invaluable for rapid confirmatory diagnosis of this disease, which is essential for solving fish farm problems.

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