Aquaculture 485 (2018) 220–224

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

The first instance of HPR-deleted ISAV detection in eviscerated, fresh salmon at a Chinese entry-exit port

Lu Xiao[b], Hua Lin[a,1], Miao Yang[a], Shijie Chen[a,*], Wei An[a], Xulong Wu[c], Hong Liu[d], Dandan Li[e], Yubao Yan[a], Juan Hu[a], Jun Wang[a], Yingjie Sun[a]

[a] Sichuan Entry–Exit Inspection and Quarantine Bureau, Chengdu, China
[b] Sichuan Animal Science Academy, Chengdu 610066, China
[c] College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China
[d] Shenzhen Entry–Exit Inspection and Quarantine Bureau, Shenzhen, China
[e] Hainan Entry–Exit Inspection and Quarantine Bureau, Haikou, China

ARTICLE INFO

Keywords:
Infectious salmon anemia virus
Highly polymorphic region
Deletion
Fresh salmon
Chinese entry-exit port

ABSTRACT

Infectious salmon anemia virus (ISAV) is a serious epidemic in fish that is characterized by severe anemia and variable hemorrhaging, and is one of the most important pathogens that China tests for in imported salmon and salmon products. The Sichuan Entry-Exit Inspection and Quarantine Bureau (SCCIQ) tested a total of 79 batches of salmonid products from Norway for ISAV during 2015, using the TaqMan RT-qPCR targeting segment 7 and conventional RT-PCR with segment 6 HPR (highly polymorphic region) primers, and by virus isolation attempts using Atlantic salmon kidney (ASK) cell line monolayers. Only one batch of eviscerated, fresh salmon (imported random sample 2015CD0652) was shown to be suspected positive for HPR-deleted ISAV. The sequence from the conventional PCR product was analyzed by phylogenetic alignment, and the results show that 2015CD0652 has a 51 bp deletion in the HPR, and a close relationship with ISAV9 and 9/93 HPR 3 isolates from Norway; however, the virus isolation attempts on the sample were negative. This is the first published report of the detection of HPR-deleted ISAV sequences in salmon flesh rather than salmon viscera in a Chinese entry-exit port, which suggests the need for heightened vigilance regarding imported fresh salmon.

1. Introduction

Infectious salmon anemia virus (ISAV) is a member of the family *Orthomyxoviridae* and the genus *Isavirus* (Thorud and Djupvik, 1988). The first reported outbreak of ISAV occurred in Norway in 1984 (Dannevig et al., 2008), and was subsequently listed in the first edition of the International Aquatic Animal Health Code of 1995. Since then, ISAV has inflicted great economic losses to Atlantic salmon (*Salmo salar*)-producing countries such as Norway, Chile, Scotland, the USA and Canada (Bouchard et al., 2001; Godoy et al., 2008; Krossoy et al., 2001a; Mullins et al., 1998; Plarre et al., 2012). Natural outbreaks of ISAV have only been recorded in farmed Atlantic salmon (Kibenge et al., 2001), and brown trout and sea trout show sub-clinical symptoms after infection with ISAV (Kibenge et al., 2004); infected pollock (*Pollachius virens*) and cod (*Gadus morhua*) have also been identified (King et al., 2011), and ISAV has been found to be pathogenic to ayu (*Plecoglossus altivelis*) and sockeye salmon (*Oncorhynchus nerka*) by intraperitoneal injection (Ito et al., 2015). When fish have developed ISA, the endothelial cells in all of their organs become infected, including their gills, heart, liver, kidney, spleen, etc. (Aamelfot et al., 2012). As the gills come into direct contact with water, they are more likely to contain impurities; thus, tissue from the heart and mid-kidney is preferred for PCR or virus isolation to diagnose ISAV.

ISAV has a genome consisting of eight single-stranded RNA segments, and the Haemagglutininesterase (HE) encoded by segment six of ISAV, which is an indicator of the pathogenicity of ISAV (Godoy et al., 2013; Krossoy et al., 2001b). Virulent ISAV isolates have a deletion in the highly polymorphic region (HPR) spanning residues ^{337}Val to ^{372}Met in HE (HPR-deleted); ISAV without a deletion in HPR (HPR 0-ISAV) has low or no pathogenicity (Godoy et al., 2014). According to sequence differences in segment six, ISAV isolates have been divided into two major groups: European and North American. Various HPR-deleted ISAV isolates show large differences in HE, but there are few differences between the HPR 0-ISAV isolates in HE (Fourrier et al., 2014; Vanderstichel et al., 2015).

ISAV is predominantly spread horizontally by water-borne

* Corresponding author.
E-mail address: cworld@126.com (S. Chen).
[1] The author contributed equally.

https://doi.org/10.1016/j.aquaculture.2017.12.001
Received 15 May 2017; Received in revised form 8 November 2017; Accepted 3 December 2017
Available online 05 December 2017
0044-8486/

transmission, and natural or anthropogenic vectors may also spread the virus. Shipping is one of the major contributors to such transport, but the investigation of virus spread by shipping is usually impractical (Murray et al., 2002). In 1998–1999, ISA was detected in Scotland, and it was later confirmed that the movement of well boats, via the shipment of live fish, and visits for harvesting had the greatest impact (Murray et al., 2002; Murray et al., 2010). From 2007 to 2009, the outbreak of ISAV in southern Chile was also shown to be associated with the movement of live or harvested fish, and shipping increased the risk of virus spread (Mardones et al., 2014). In Norway, it has also been suggested that the transportation of infected salmon in well boats may play an important role in the spread of ISAV (Nylund et al., 2003; Nylund et al., 2007). Thus far, there has been no recorded occurrence of ISA in China, but with the development of salmon farming and the growing trade of import shipments of Atlantic salmon products, ISAV may soon reach China; its arrival poses a great threat to fish farming and aquaculture. The Chinese Entry-Exit Inspection and Quarantine Bureaus play a key role in preventing ISAV from spreading to China, and are crucial for stopping the cross-border spread of this virus. The study describes the laboratory detection of HPR-deleted ISAV detection at a Chinese entry-exit port, and this is the first instance of ISAV in a Chinese port, which warns us to be alert to ISAV and strengthen surveillance.

2. Materials and methods

2.1. Samples

Samples were randomly collected from the 79 batches of salmon products imported from Norway to Chengdu, Sichuan province, during 2015. One piece of specimen was random collected and every batch was inspected. The test specimen was randomly sampled in November 2015, under the sampling number 2015CD0652.

Segment 6 (Gen Bank accession no. EU118820.1) and segment 7 (Gen Bank accession no. EU118821.1) of HPR0-ISAV were synthesized by Shanghai Genecore Bio Technologies Co, Ltd. (Shanghai, China), and the recombinant plasmids pMD19T-HPR0-ISAV-S6 and pMD19T-HPR0-ISAV-S7 were kept at − 70 °C until use as controls in the assays.

2.2. Primers and probes

Primer and probe sequences were obtained from the Manual of *Diagnostic Tests for Aquatic Animals* (2014) and synthesized by Invitrogen™ (Thermo Fisher Scientific, Waltham, MA, USA). The details are shown in Table 1.

2.3. RNA extraction

Specimens were pre-processed with a homogenizer (MP Biomedicals, Irvine, CA, USA) in Lysing Matrix Tubes (MP Biomedicals, Irvine, CA, USA). Total RNA was extracted with an automated nucleic acid extraction apparatus (Thermo Fisher Scientific, Waltham, MA, USA) using the Magnetic Viral DNA/RNA Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions, and were stored at 4 °C.

Table 1
Primers and probe.

Name	Sequence (5′–3′)	Product size (bp)	Genomic segment
S7-F	CAGGGTTGTATCCATGGTTGAAAT	155	7
S7-R	GTCCAGCCCTAAGCTCAACTC		
S7-Probe	FAM-CTCTCTCATTGTGATCCC-*MGBNFQ*		
S6-F	GACCAGACAAGCTTAGGTAACACAGA	304	6
S6-R	GATGGTGGAATTCTACCTCTAGACTTGTA		

2.4. TaqMan RT-qPCR

The TaqMan RT-qPCR was targeted to segment 7, and the protocol was carried out in a 25 μL reaction using the PrimeScript™ One Step RT-PCR Kit (Perfect Real Time) (Takara, Dalian, China). The reaction contained 12.5 μL 2 × One Step RT-PCR Buffer III, 1 μL Takara Ex *Taq* HS (5 U/μL), 1 μL PrimeScript RT Enzyme Mix II, 1 μL S7-F/R (10 μmol/L), 1 μL S7-Probe (10 μmol/L), 4 μL RNA template, and 3.5 μL ddH_2O. The amplification was performed as follows: 95 °C for 10 s, 60 °C for 45 s and 42 °C for 5 min (40 cycles). A duplication of the sample was run at the same time. Meanwhile, positive and negative controls were included. DdH_2O was used as a negative control in this assay, and pMD19T-HPR0-ISAV-S7 was used as a positive control. The experiment was re-checked using the fresh nucleic acid extracts as a template.

2.5. Conventional RT-PCR and sequence analysis

Conventional RT-PCR was targeted towards segment 6, and carried out in a 25 μL reaction using the PrimeScript™ One Step RT-PCR Kit Ver.2 (Takara, Dalian, China). Reactions contained 12.5 μL of 2 × 1 Step Buffer, 2 μL of PrimeScript 1 Step Enzyme Mix, 2 μL of 10 μmol S6-F/R, 2 μL of template, and 4.5 μL ddH_2O. The mixture was incubated at 50 °C for 30 min then at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. A negative and positive control were performed simultaneously using ddH_2O or a pMD19T-HPR0-ISAV-S6, respectively. Amplified products were visualized by gel electrophoresis on a 1.5% agarose gel. The RT-PCR product was reverse-transcribed into cDNA, then the cDNA was purified and cloned into pMD19T. The sequencing of the positive plasmids containing the cloned segment 6 was performed by Sangon Biotech (Shanghai, China). The obtained 2015CD0652 clone and 17 existing nucleotides and predicted amino acid sequences for segment 6 of different groups of ISAV (HPR 1–15) published in the GenBank database were cleaned, aligned and analyzed using DNASTAR software. The re-testing was checked using the fresh nucleic acid extracts as the template.

2.6. Virus culture

The Atlantic salmon kidney (ASK) cell line was used for the propagation of ISAV. The ASK cell monolayers in 24-well tissue culture plates were inoculated with 100 μL per well of the suspected positive tissue homogenate supernatant filtered by 0.45 μm syringe filters. The plates were sealed and incubated at 15 °C for 2 h to allow virus adsorption. Maintenance medium was then added and the plates were further incubated at 15 °C with microscopic examination at regular intervals for the occurrence of CPE. RNAs of the cell cultures were extracted and tested by RT-qPCR.

3. Results

3.1. Results of the TaqMan RT-qPCR assay

The TaqMan RT-qPCR was implemented in accordance with OIE (Office International Des Epizooties), and the detection steps were all implemented in the BSL-2 laboratory. Only one specimen (2015CD0652) tested positive for ISAV from the 79 batches of imported salmon products during 2015. As the result shows (only the result of 2015CD0652 is presented) in Fig. 1, both amplification curves have the typical “S” shape; the Ct of the positive control is 18.92 and the Ct of the average of the two 2015CD0652 duplicates is 22.51. The No Template Control (NTC) appeared to have no amplification. The results of the RT-qPCR assay confirmed that 2015CD0652 was infected with ISAV, although more work was needed to identify its group or subgroup. Similar results were obtained in the re-check assays.