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Outbreak of viral haemorrhagic septicaemia (VHS) in lumpfish (*Cyclopterus lumpus*) in Iceland caused by VHS virus genotype IV

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Abstract

A novel viral haemorrhagic septicaemia virus (VHSV) of genotype IV was isolated from wild lumpfish (*Cyclopterus lumpus*), brought to a land-based farm in Iceland, to serve as broodfish. Two groups of lumpfish juveniles, kept in tanks in the same facility, got infected. The virus isolated was identified as VHSV by ELISA and real-time RT-PCR. Phylogenetic analysis, based on the glycoprotein (G) gene sequences, may indicate a novel subgroup of VHSV genotype IV. In controlled laboratory exposure studies with this new isolate, there was 3% survival in the I.P. injection challenged group while there was 90% survival in the immersion group. VHSV was not re-isolated from fish challenged by immersion. In a cohabitation trial, lumpfish infected I.P. (shedders) were placed in tanks with naïve lumpfish as well as naïve Atlantic salmon (*Salmo salar* L.). 10% of the lumpfish shedders and 43%–50% of the cohabiting lumpfish survived after 4 weeks. 80%–92% of the Atlantic salmon survived, but no viral RNA was detected by real-time RT-PCR nor VHSV was isolated from Atlantic salmon. This is the first isolation of a notifiable virus in Iceland and the first report of VHSV of genotype IV in European waters.

KEYWORDS
challenge models, genotype IV, Iceland, lumpfish, viral haemorrhagic septicaemia virus (VHSV)

1 | INTRODUCTION

Viral haemorrhagic septicaemia (VHS) is a severe infectious disease affecting wild and farmed fish stocks in the northern hemisphere. In its acute phase, the disease is characterized by haemorrhagic septicaemia. Affected fish display darkening of the skin, exophthalmia and abnormal swimming behaviour. The most common necropsy findings are petechial bleedings in internal organs and severe anaemia of the gills. The disease may also appear in a chronic phase characterized by nervous signs (OIE 2017).

Historically, the disease affected rainbow trout production in Europe (Wolf, 1988) and was first described in Germany in the 30s (Schäperclaus, 1938) The aetiological agent was isolated for the first time in 1962, in Denmark (Jensen, 1965). Since its first detection, the known host range has been expanded to include over 80 fish species from fresh and saltwater (reviewed in (OIE 2017). The virus has solely been isolated in the northern hemisphere (Elsayed et al., 2006; Meyers & Winton, 1995;

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Skall, Olesen, & Mellergaard, 2005). The aetiological agent, viral haemorrhagic septicaemia virus (VHSV, species Piscine novirhabdovirus, ICTV 2017), is a member of the genus *Novirhabdovirus* of the family *Rhabdoviridae* (Walker et al., 2000). Based on sequence analysis of the glycoprotein (G), nucleocapsid (N) protein and non-virion (NV) protein genes, four genotypes (I, II, III and IV) have been described. The molecular classification represents geographical differentiation (Einer-Jensen, Ahrens, Forsberg, & Lorenzen, 2004; Snow et al., 2004) and host specificity to varying degrees (Emmenegger, Moon, Hershberger, & Kurath, 2013; Skall et al., 2005). Currently, genotype IV is divided into three subgroups, that is genotype IVa that occurs in marine environment of the North-Eastern Pacific Ocean, Japan and Korea (Bernard, Bremont, & Winton, 1992; Garver et al., 2013; Hedrick et al., 2003; Traxler, Kieser, & Richard, 1999), genotype IVb, a freshwater isolate found in the Laurentian Great Lakes region (Elsayed et al., 2006; Thompson et al., 2011), and genotype IVc that occurs in estuarine environment on the east coast of Canada (Gagné et al., 2007; Pierce & Stepien, 2012).

The first report of VHVS IVa on the Atlantic Ocean side of N-America was isolation from Atlantic herring (*Clupea harengus* L.) caught in the marine coastal waters of Maine in 2003 (Ammayappan & Vakharia, 2009). Recently, the identification of genotype IVa from the east coast of Canada was reported, and currently, areas of Newfoundland, Labrador and Atlantic Ocean North are declared as infected with VHSV genotype IVa (Notice to Industry—Viral Haemorrhagic Septicaemia Virus detected in Atlantic herring in Newfoundland and Labrador - Canadian Food Inspection Agency; Notice to Industry—Viral Haemorrhagic Septicemia Virus detected in Atlantic herring—Canadian Food Inspection Agency).

Salmon louse (*Lepeophtheirus salmonis*) is one of the biggest concerns in the salmon industry today (Torrissen et al., 2013). Measures applied to fight the louse have included the use of various chemicals but since the louse often becomes resistant and given that long time use of chemicals is unacceptable for environmental reasons (Burridge, Weis, Cabello, Pizarro, & Bostick, 2010), other methods such as the use of cleaner fish have been adopted (Aaen, Helgesen, Bakke, Kaur, & Horsberg, 2015). Promising results obtained in sea lice control using lumpfish as cleaner fish have led to rapid increase in the number of farms producing lumpfish juveniles in countries around the North Atlantic Ocean. In Norway, the estimated production of lumpfish juveniles in 2015 was 12–14 million and 24–25 millions in 2016 (Fish health report 2016). In Iceland, lumpfish juveniles have been farmed for export since 2014.

In the current paper, we describe the occurrence, isolation and characterization of new VHSV isolates originating from wild lumpfish caught in shallow waters on the south coast of Breiðafjörður Bay in West Iceland (approximately 65.0754°N–22.7298°W). Further, results of phylogenetic analysis, based on full G-gene sequences, may indicate that the Icelandic isolates are part of a new subgroup of VHSV genotype IV. This is the first isolation of VHSV in Iceland and the first time that genotype IV is found in Europe. In addition, as a first step to a comprehensive evaluation of the risk of using lumpfish as cleaner fish in the salmon industry, we report the results of challenge trials conducted in lumpfish and salmon with the newly discovered VHSV isolates.

2 | MATERIAL AND METHODS

2.1 | Case description

In July 2015, brood fish was caught in Breidafjördur Bay and transferred to a land-based farm. The temperature in the bay in July was 9°C and salinity 34.8‰. The land-based facility uses borehole sea water with a constant temperature of 9°C and 32‰ salinity. Part of the catch was sampled in mid-July, a few days after arrival and another part 2 weeks later. Remaining brood fish was kept in the facility for some weeks. On both sampling occasions, 80 fish were dissected for kidney, heart and spleen samples for analysis in five fish pools. When sampled, the brood fish did neither show external nor internal macroscopic signs of disease. Due to time constraint, the samples were frozen at −80°C and processed for cell culture in September. When these samples were being processed, two groups of juvenile lumpfish on the farm that had been in tanks adjacent to the wild brood fish for 4–6 weeks showed clinical signs of a disease and some mortalities occurred. One group originated from a batch of eggs obtained in May 2014 and was intended to become F1 brood fish while the other group consisted of fingerlings obtained from spawning in April 2015. Some of the fish in these groups had greyish nodules on the spikes, erosion and superficial ulcers were present on the skin and the gills were pale. Necropsy revealed pale organs, especially kidneys, spleen, liver and hearth. Three individuals from the older group and five from the younger group were sampled for histopathology and virological examination.

2.2 | Histopathology

Tissue samples fixed in 10% phosphate-buffered formalin from the two groups of juveniles were processed and embedded in paraffin wax according to standard procedures. Sections (4–6 μm) were stained with Giemsa and examined by light microscopy.

2.3 | Virological examination

Pooled samples consisting of heart, spleen and kidney from lumpfish were processed according to procedures given in Commission Implementing Decision 2015/1554/EC. Briefly, samples were homogenized (according to guidelines diluted 1:10 w/v) and the samples cleared by low-speed centrifugation. 150 μl of the supernatants was inoculated in 10-fold dilutions onto subconfluent monolayer of BF-2 (Wolf, Gravell, & Malsberger, 1966) and EPC (Fijan et al., 1983) cell cultures in 24-well tissue culture plates. Inoculated cultures were incubated at 15°C and inspected regularly for the occurrence of cytopathic effect (CPE) at 40× magnification. When CPE was evident, the cell

culture medium was collected for virus identification. The virus was identified as VHSV by ELISA using Monoclonal Antibody (Mab) IP5B11 (Skall, Slierendrecht, King, & Olesen, 2004). Two isolates, one from the wild brood fish and one from the juvenile group, spawned in April 2015 (named IS_15_19852_1 and IS_15_19852_3) were sent to the EURL for fish disease for confirmation and further characterization.

2.4 | PCR and sequencing

RNA was extracted from cell culture supernatant with the RNeasy (Qiagen) according to manufacturer's instructions. For diagnostic purposes, real-time RT-PCR for the detection of VHSV RNA was performed according to Jonstrup, Kahns, Skall, Boutrup, and Olesen (2013). For further characterization of the Icelandic isolates, the full G-genes were amplified and sequenced using the GB ±primer pair (Einer-Jensen et al., 2004). RT-PCR was performed using the One-Step RT-PCR kit (Qiagen) following the protocol recommended by the manufacturer but in a 25 µl reaction, final primer concentration of 0.6 µM each and 5 µl of RNA template. The cycling conditions were as follows: 50°C for 30 min, 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s and 68°C for 1 min, and finally 68°C for 7 min. The PCR products were purified with QIAquick PCR Purification Kit according to manufacturer's instructions. Sequencing was done using internal primers (Einer-Jensen et al., 2004). Nucleotide sequences have been submitted to the GenBank database and designated accession numbers MH836523 (IS_15_19852_1) and MH836522 (IS_15_19852_3), Table 2.

2.5 | Phylogenetic analysis

A data set including sequence of the full G-gene (1,542 nt) for 100 VHSV isolates was aligned using Muscle 3.8.425 (Edgar, 2004) as implemented in the Geneious ® 11.0.2 (Biomatters Ltd.). Besides the two Icelandic isolates, the alignment includes all publicly available sequences of VHSV genotype IV, as well as selected isolates all other VHSV genotypes (Table 1). A maximum-likelihood tree was generated using PyML 3.2.2 (Guindon et al., 2010) using the GTR+G substitution model. Branch support values were calculated with 1000 non-parametric bootstrap replicates in the program RAxML v. 8.2.11 (Stamatakis, 2014).

2.6 | Bacteriology

Kidney samples were streaked directly onto blood agar with 2% NaCl (BA-NaCl), incubated at 16°C and observed every other day for a week.

2.7 | Infection trials

Two infection trials were conducted in the high contained experimental facility at DTU-AQUA (comparable to biosafety level BSL 3 for terrestrial animal). Experimental procedures were in accordance with the recommendations in the current animal welfare regulations under licence 2013-15-2934-00976. The protocols were approved by the Danish Animal Research Authority. Experimental fish were monitored on a daily basis regarding the state of health and environment.

2.7.1 | Fish

A batch of approximately 500 lumpfish juveniles, mean weight 5 g, were imported from a commercial company in Iceland. The facility is declared free from listed diseases. Screening for bacteria was performed by streaking kidney samples from 10 fish onto Blood Agar, TYES and Marine Agar. One pool of three fish was screened for virus as described in the paragraph of virological examination. Atlantic salmon juveniles, mean weight 14 g, were imported from a commercial farm and brought into the quarantine facility of DTU-AQUA. The farm is certified free from listed diseases and from IPNV and BKD as well. Screening for bacteria was performed as described above.

2.7.2 | Virus isolate

The Icelandic VHSV isolate IS_15_19852_1 from broodfish was propagated in BF-2 cells and titrated by TCID 50 (Reed & Muench, 1938).

2.7.3 | Trial 1

This experiment aimed to assess the pathogenicity of the new VHSV isolate in lumpfish. Naive juveniles were infected by immersion or by I.P. injection. Both infection routes were performed in triplicate tanks containing 30 fish each. Fish were maintained in 8 L tanks run with 1 L/h flow-through renewal of UV-treated marine sea water (salinity 30‰). The aerated water temperature was maintained at 12 ± 1°C, photoperiod was L:D 12:12, stocking density below 60 kg/m^3 and feeding 1.5% of biomass. The tanks were kept closed with a transparent lid to prevent contamination by aerosols and prevent fish from jumping out. Negative controls included two tanks containing 30 lumpfish sham-injected with EMEM (50 µl) and two tanks with 30 fish exposed by sham immersion for the same time 30 sham-injected lumpfish with Eagle's minimal essential medium (EMEM) (50 µl) as infected fish with filter sterilized cell culture supernatant from uninfected cells, BF-2 cells.

Immersion challenge: To obtain a concentration of approximately 10^5 $TCID_{50}$ ml^{-1} water, the virus isolate was added to a vial containing 10 ml dilution of EMEM (pH 7) according to the titre. Water supply was stopped when the viral solution was added and the immersion exposure time was 7 hr, after which continuous flow was resumed.

Intra-peritoneal challenge: Virus isolate was mixed in a vial with 10 ml dilution medium to obtain a dose of approximately 10^5 $TCID_{50}$ per fish in an injection volume of 50 µl. Fish were anaesthetized by bathing in benzocaine solution (80 mg/L), injected intraperitoneally with a new needle (29 G) and syringe for each tank group and transferred to tanks with running salt water.

TABLE 1 Viral haemorrhagic septicaemia virus (VHSV) isolates used in this study arranged by genotype, location and species

Accession Nr.	Isolate name	Genotype	Year of isolation	Geographic origin	Host	Reference
AY546619	GE-1.2	Ie	1981	Georgia	Rainbow trout	Einer-Jensen et al. (2004)
U28798	Hededam	I	1970	Denmark	Brown trout	Benmansour et al. (1997)
AY546579	DK-1p86	Ib	1996	Baltic Sea	Sprat	Einer-Jensen et al. (2004)
AY546631	UK-MLA98/6HE1	Ib	1998	North Sea	Herring	Einer-Jensen et al. (2004)
Z93414.2	Cod Ulcus	Ib	1979	Baltic sea	Atlantic cod	Einer-Jensen et al. (2004)
AF143863.1	FR-14-58	Ia	1990	France	Rainbow trout	Einer-Jensen et al. (2004)
AY546603.1	DK-9895174	Ia	1999	Denmark	Rainbow trout	Einer-Jensen et al. (2004)
EU708812.1	Dvgeig	Ia	2009	Germany	Rainbow trout	Unpublished
EU708733.1	Au917-04	Ia	2004	Germany	Rainbow trout	Unpublished
AY546602	DK-9995144	Ia	1999	Denmark	Rainbow trout	Einer-Jensen et al. (2004)
AY546588	DK-5123	Ic	1988	Denmark	Rainbow trout	Einer-Jensen et al. (2004)
AY546585	DK-2835	Ic	1982	Denmark	Rainbow trout	Einer-Jensen et al. (2004)
AM086357.1	FiP03.00	Id	2000	Finland	Rainbow trout	Raja-Halli et al. (2006)
AM086378.1	FiA17.02	Id	2002	Finland	Rainbow trout	Raja-Halli et al. (2006)
KM244767	DK-1p49	II	1996	Baltic Sea	Atlantic herring	Kai and Chi (2008)
AY546577.1	DK-1p53	II	1996	Baltic Sea	Herring	Einer-Jensen et al. (2004)
AY546576.1	DK-1p52	II	1996	Baltic Sea	Sprat	Einer-Jensen et al. (2004)
HQ112243.1	ka560_04	II	2004	Finland	Baltic herring	Gadd, Jakava-Viljanen, Tapiovaara, Koski, and Sihvonen (2011)
HQ112246.1	ka646_04	II	2004	Finland	Baltic herring	Gadd et al. (2011)
GQ504013.1	FI-lamprey-743.03	II	2003	Finland	Lamprey	Gadd et al. (2011)
HQ112242.1	ka494_05	II	2005	Finland	Baltic herring	Gadd et al. (2011)
HQ112240.1	ka427_04	II	2004	Finland	Baltic herring	Gadd et al. (2011)
HQ112248.1	ka664_04	II	2004	Finland	Baltic herring	Gadd et al. (2011)
HQ112241.1	ka436_04	II	2004	Finland	Baltic herring	Gadd et al. (2011)
AY546582	DK-4p168	III	1997	Skagerrak	Herring	Einer-Jensen et al. (2004)
AY546629	UK-H17/2/95	III	1995	North Sea	Haddock	Einer-Jensen et al. (2004)
AY546630	UK-H17/5/93	III	1993	North Sea	Cod	Einer-Jensen et al. (2004)
AY546618	FR-L59X	III	1987	France	Eel	Einer-Jensen et al. (2004)
EU481506.1	FA281107	III	2007	Norway	Rainbow trout	Duesund, Nylund, Watanabe, Ottem, and Nylund (2010)

(Continues)

TABLE 1 (Continued)

Accession Nr.	Isolate name	Genotype	Year of isolation	Geographic origin	Host	Reference
AY546632.1	UK-MLA98/6PT11	III	1998	North sea	Norway pout	Einer-Jensen et al. (2004)
AY546620.1	IR-F13.02.97	III	1997	Ireland	Turbot	Einer-Jensen et al. (2004)
AY546581.1	DK-4p101	III	1997	Denmark	Whiting	Einer-Jensen et al. (2004)
KM244768.1	GH40	III	?	Spain	Greenland halibut	Lopez-Vazquez, Bandín, and Dopazo (2015)
AY546628.1	UK-860/94	III	1994	UK	Turbot	Einer-Jensen et al. (2004)
KC117248.1	BC09-31-2	IVa	2009	Canada	Pacific herring	Garver et al. (2013)
KC117246	BC08-2816-1	IVa	2008	Canada	Atlantic salmon	Garver et al. (2013)
KC117247.1	BC09-31-8	IVa	2009	Canada	Pacific herring	Garver et al. (2013)
KC117242.1	BC07-286-10	IVa	2007	Canada	Atlantic salmon	Garver et al. (2013)
KC117231.1	BC04-040	IVa	2004	Canada	Atlantic salmon	Garver et al. (2013)
KC117221.1	BC00-397	IVa	2000	Canada	Pacific herring	Garver et al. (2013)
KC117219.1	BC98-249	IVa	1998	Canada	Pacific herring	Garver et al. (2013)
KC117237.1	BC313-1A	IVa	2005	Canada	Chinook Salmon	Garver et al. (2013)
KC117245.1	BC07-13-2	IVa	2007	Canada	Pacific herring	Garver et al. (2013)
KC117243.1	BC07-21-2	IVa	2007	Canada	Pacific Sardine	Garver et al. (2013)
KC117244.1	BC07-15-7	IVa	2007	Canada	Atlantic salmon	Garver et al. (2013)
KC117235.1	BC301-1A	IVa	2005	Canada	Chinook Salmon	Garver et al. (2013)
KC117238.1	BC05-197	IVa	2005	Canada	Pacific Sardine	Garver et al. (2013)
KC117236.1	BC283-1B	IVa	2005	Canada	Chinook Salmon	Garver et al. (2013)
KC117241.1	BC06-37-2	IVa	2006	Canada	Atlantic salmon	Garver et al. (2013)
KC117232.1	BC05-011	IVa	2005	Canada	Atlantic salmon	Garver et al. (2013)
KC117233.1	BC05-014-2	IVa	2005	Canada	Pacific herring	Garver et al. (2013)
KC117220	BC00-LF	IVa	2000	Canada	Pacific herring	Garver et al. (2013)
KC117228.1	BC02-232-1	IVa	2002	Canada	Pacific Sardine	Garver et al. (2013)
KC117223.1	BC02-03	IVa	2002	Canada	Atlantic salmon	Garver et al. (2013)
KC117230.1	BC04-028-1	IVa	2004	Canada	Atlantic salmon	Garver et al. (2013)
KC117215.1	BC95-297	IVa	1995	Canada	Atlantic salmon	Garver et al. (2013)
KC117218.1	BC96-265-6	IVa	1996	Canada	Pacific herring	Garver et al. (2013)
KC117217.1	BC96-265-3	IVa	1996	Canada	Pacific herring	Garver et al. (2013)
KC117216.1	BC95-225	IVa	1995	Canada	Pacific herring	Garver et al. (2013)
KC117214.1	BC93-390	IVa	1993	Canada	Pacific herring	Garver et al. (2013)
DQ401189.1	WA91Clearwater	IVa	1991	USA	Coho salmon	Elsayed et al. (2006)
KC117249.1	BC10-42-13	IVa	1995	Canada	Atlantic salmon	Garver et al. (2013)
KC117225.1	BC02-41-14	IVa	2014	Canada	Shiner Perch	Garver et al. (2013)
KC117224.1	BC02-41-9	IVa	2002	Canada	Pacific Sardine	Garver et al. (2013)
DQ401186.1	BC93-372	IVa	1993	Canada	Pacific herring	Elsayed et al. (2006)
KC117226.1	BC02-47-21	IVa	2002	Canada	Pacific herring	Garver et al. (2013)
KC117222.1	BC02-28-6	IVa	2002	Canada	Pacific Sardine	Garver et al. (2013)
KC117229.1	BC02-229	IVa	2002	Canada	Pacific Sardine	Garver et al. (2013)
KC117234.1	BC05-014-7	IVa	2005	Canada	Pacific herring	Garver et al. (2013)
DQ401194.1	BC99-010	IVa	1999	Canada	Pacific herring	Elsayed et al. (2006)

(Continues)

TABLE 1 (Continued)

Accession Nr.	Isolate name	Genotype	Year of isolation	Geographic origin	Host	Reference
JQ651388.1	KR-CJA	IVa	2010	South Korea	Olive flounder	Cho et al. (2012)
KM926343.1	JF-09	IVa	2009	South Korea	Olive flounder	Kim, Thu, Skall, Vendramin, and Evensen (2014)
KP334106.1	FP-VHS2010-1	IVa	2010	South Korea	Olive flounder	Hwang et al. (2016)
KF477302.1	FYeosu05	IVa	2005	South Korea	Olive flounder	Kim, Kim, Nishizawa, and Oh (2013)
JF792424.1	KJ2008	IVa	2008	South Korea	Olive flounder	Kim et al. (2013)
KC685626.1	No name	IVa	2005	China	Olive flounder	Zhu and Zhang (2014)
AY167587.1	No name	IVa	2009	South Korea	Olive flounder	Kim,S.M. and Park, S.I unpublished
AB490792.1	JF00Ehi1	IVa	2002	Japan	Olive flounder	Ito,T., Olesen,N.J., Sano,M., Kurita,J., Nakajima,K. and Iida,T.
KC117240.1	BC06-089-4	IVa	2006	Canada	Pacific Sardine	Garver et al. (2013)
KC117251.1	BC11-191	IVa	2011	Canada	Pacific hake	Garver et al. (2013)
U28747.1	makah	IVa	1988	USA	Coho Salmon	Benmansour et al. (1997)
DQ401192.1	ME03	IVa	2003	USA	Atlantic herring	Elsayed et al. (2006)
DQ401195.1	BC99-001	IVa	1999	Canada	Pacific Sardine	Elsayed et al. (2006)
DQ401187.1	BC98-250	IVa	1998	Canada	Atlantic salmon	Elsayed et al. (2006)
DQ401188.1	BC99-292	IVa	1999	Canada	Atlantic salmon	Elsayed et al. (2006)
DQ401191.1	JP99Obama25	IVa	1999	Japan	Japanese flounder	Elsayed et al. (2006)
AB179621.1	KRRV9822	IVa	1998	Japan	Japanese flounder	Byon, Ohira, Hirono, and Aoki (2006)
KC117239.1	BC06-089-1	IVa	2006	Canada	Pacific Sardine	Garver et al. (2013)
DQ401193.1	MI03GL	IVb	2003	USA	Muskellunge	Elsayed et al. (2006)
GQ385941.1	MI03GL	IVb	2003	USA	Muskellunge	Ammayappan and Vakharia (2009)
HQ453209	U13653	IVb	2005	Canada	Freshwater drum	Thompson et al. (2011)
AB672615.1	Goby1-5	IVb	2006	USA	Round goby	Ito et al. (2012)
KC117250	GL2010-098	IVb	2010	Canada	Round goby	Garver et al. (2013)
EF079897.2	CA-NB02-01	IVc	2002	Canada	Striped bass	Gagné et al. (2007)
EF079896.2	CA-NB00-01	IVc	2000	Canada	Mummychog	Gagné et al. (2007)
HQ168405.1	CA-NB00-02	IVc	2000	Canada	Three-spined stickleback	Gagné et al. (2007)
EF079899.2	CA-NS04-01	IVc	2004	Canada	Brown trout	Gagné et al. (2007)
HQ453208.1	CA-NB04-01b	IVc	2004	Canada	Striped bass	Thompson et al. (2011)
MH836523	IS_15_19852_1	IV	2015	Iceland	Lumpfish	This work
MH836522	IS_15_19852_3	IV	2015	Iceland	Lumpfish	This work

The numbers of clinically affected fish were recorded daily, and moribund fish were collected, killed, pooled and processed as described in virological examination paragraph. The trials were terminated after 28 days. Surviving fish were killed with an overdose of benzocaine (800 mg/L) and counted. Ten single fish from the each of the three tanks infected by immersion were collected and processed for virus isolation. Negative fish were also collected, obtaining two pools of five fish from each negative control tank that were processed for virus isolation.

2.7.4 | Trial 2

This experiment aimed to investigate viral transmission in a cohabitation setting.

The trial was set up in 150 L tanks, run with 15 L/h flow-through renewal UV-treated marine sea water (15‰ salinity) at the following conditions: 12 ± 1°C, L:D 12:12; stocking density below 60 kg/m^3; and feeding of 1.5% of biomass. The tanks were kept closed with a transparent lid to prevent contamination by aerosols and prevent fish

from jumping out. Lumpfish shedders were injected as in trial 1 (same viral batch production, same injection volume) and tagged by clipping the dorsal fin. The shedders were cohabited with naïve lumpfish and Atlantic salmon. This setting was performed in duplicate, and 30 lumpfish shedders were cohabited with 30 naïve lumpfish and 50 naïve Atlantic salmon. As a negative control, one tank was included where 30 lumpfish injected with media were cohabited with 30 naïve lumpfish and 30 naïve Atlantic salmon. In order to monitor the progress of infection in the at selected time points, that is 3, 5, 7, 10, 14, 21 and 28 days postinfection, a set of specimen consisting of three fish from each group (three lumpfish shedders, three lumpfish cohabitants and three salmon cohabitants) were collected, killed and sampled. In order to mitigate the impact of reduction in fish due to sampling at selected time points on the disease development in the tank challenged with VHSV, specimen was collected alternatively (i.e., one infected tank at 3, 7, 14 and 28 dpi,; the other tank at 5, 10 and 21 dpi). At the end of the trial, 10 salmon were sampled from each of the infected tanks, and two pools of five fish (one of salmon and one of lumpfish cohabitant) were collected from the negative control tank. At each sampling point, the spleen was aseptically dissected from single fish, placed in RNAlater and tested by real-time RT-PCR for VHSV as described above.

3 | RESULTS

3.1 | Disease outbreak

Increased mortality and signs of external infections were observed in two batches of juvenile lumpfish, reared in tanks in the same room as the broodfish, on the farm in question in September 2015. One batch originated from wild broodfish spawned in May 2014 and the other from broodfish spawned in April 2015. There were greyish nodules on the spikes, erosion and superficial ulcers on the skin and the gills were pale. Filamentous bacteria (*Flexibacter/Flavobacterium* spp.) were observed in fresh preparations from the ulcers. Necropsy revealed pale organs. Supernatants of EPC cultures, showing cytopathic effects 5 days after inoculation, were identified as VHSV-positive using RT-PCR and ELISA. Further, VHSV was isolated from six out of sixteen and eight out of sixteen samples from wild-caught broodfish that had been brought to and sampled at the facility in July 2015. OIE was notified of the outbreak in accordance with the European Community Council Directive 2006/88/EC, and a notification to member states was issued on 23 October 2015. Measures taken were stamping out, disinfection of the facilities and official disposal of carcasses, by-products and waste.

http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=18938 (retrieved: 20 August 2018).

http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?reportid=19020 (retrieved: 20 August 2018).

3.2 | Histopathology

Varying degrees of pathological changes were observed in most organs of the VHSV-infected juveniles. Haemorrhages were observed in skeletal muscles of some infected fish and commonly associated with thinning, loss of striation and necrosis of muscle fibres (Figure 1a). Pathological changes in the kidney were characterized by focal, and in some cases, disseminated, necrosis and haemorrhages in the hematopoietic tissue as well as vacuolar degeneration of kidney tubules (Figure 1b–d). In the liver, focal necrosis was observed and pyknosis and karyolysis observed in the affected areas (Figure 1e,f). Similarly, focal necrosis was commonly observed in the spleen and the pancreas (Figure 1g). The most prominent histopathological changes in the gastrointestinal tract were observed in the glandular part of the stomach, characterized by severe vacuolar degeneration and in the most severe cases a total necrosis of large areas (Figure 2a,b). Furthermore, focal vacuolar degeneration was observed in the muscular parts of the gastrointestinal tracts. In some cases, the epithelial lining of the intestines was necrotic and sloughed off. The heart was characterized by disseminated degeneration, especially of muscle fibres in the myocardium, but also to some extent in the epicardium. Many of the endocardial cells, covering the inner layers of the myocardium, were seemingly hypertrophic (Figure 2c). In some cases, these changes were associated with considerable haemorrhage and infiltration of inflammatory cells. In the gills, hypertrophy of epithelial cells, especially in the basal parts of the secondary lamellae, was commonly observed (Figure 2d).

3.3 | Virus characterization

To determine the genotype of the Icelandic isolates, the full G-gene was sequenced for two isolates, one from the wild broodfish (IS_15_19852_1) and one from the juveniles spawned in 2015 (IS_15_19852_3). The sequences retrieved show a single nucleotide of difference (C or T in position 987), which is a synonymous change. The phylogenetic analysis placed the Icelandic isolates as a member of genotype IV with strong support (BS = 100%) but not within any of the three recognized subgroups (Figure 3) (Pierce & Stepien, 2012). The Icelandic isolate is closest to genotype IVa (BS = 80%). The most similar sequences were BC93-372 isolated in 1993 from Pacific herring in Vancouver; and to ME03, isolated in 2003 from Atlantic herring in the United States (Elsayed et al., 2006), with a difference of 46–47 nucleotides and 11 aa. Table 2.

3.4 | Infection trials

3.4.1 | Infection trial 1

Intra peritoneal injection of the IS_15_19852_1 VHSV isolate decreased survival to 0; 3 and 7% in triplicate tanks during 28 days of the trial. Clinically affected fish showed haemorrhages and ascites. VHSV was isolated from every fish that succumbed to the infection. All I.P. sham-injected fish survived. In the immersion trial, 93%, 83% and 90% of challenged as well as control fish survived (Figure 4a). The virus was not isolated from any sample of non-survivors or from samples taken at the end of the infection trial 1. Bacteriological examinations, conducted on the fish which did not survive in the immersion trial, tested negative.

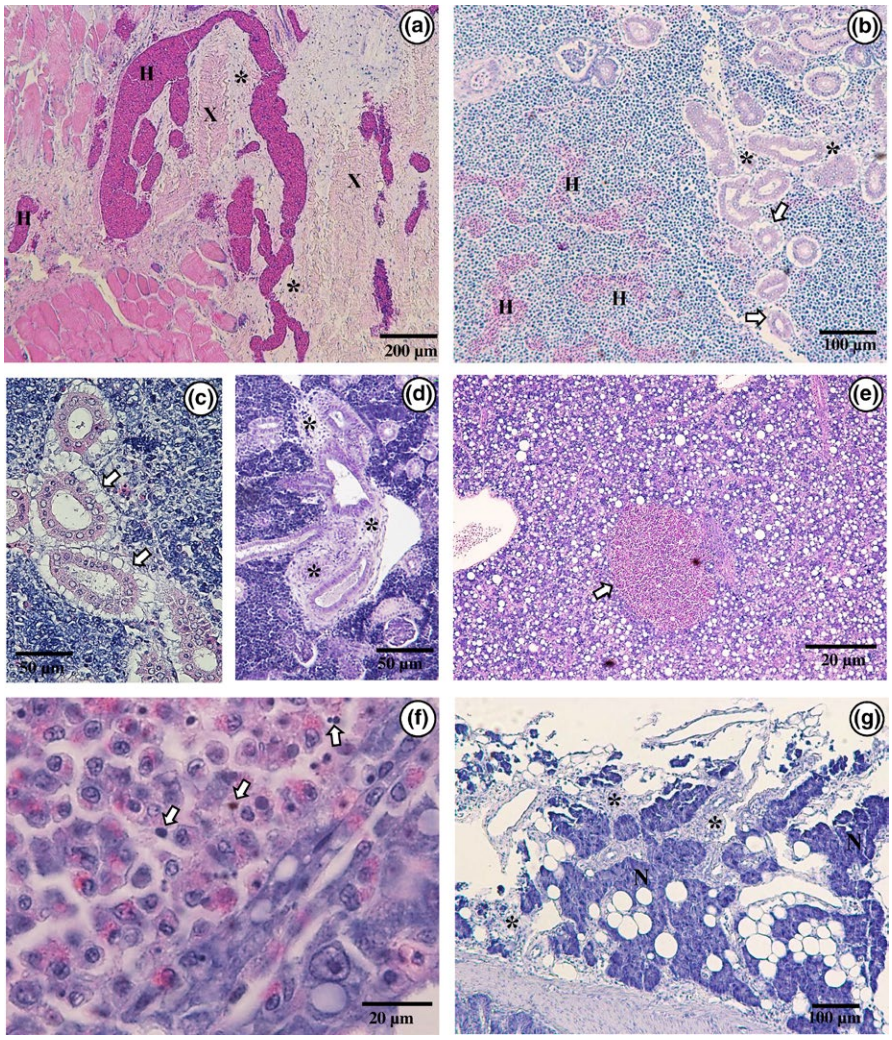


FIGURE 1 (a) Skeletal muscle of VHSV-infected lumpfish showing severe haemorrhage (H) associated with loss of striation and thinning of muscle fibres (X) and total muscular necrosis in some areas (*). (b) Haemorrhage in the renal haematopoietic tissue (H) necrotic areas surrounding the kidney tubules (*), some of which show vacuolar degeneration (arrows). (c,d) Higher magnification of a kidney showing severe vacuolar degeneration of the tubules (c) (arrows) and necrosis around the kidney tubules (d) (*). (e) Focal necrosis in the liver (arrow). (f) Higher magnification of the necrotic area in showing degenerating hepatocytes. Note the pyknotic nuclei (arrows). (g) A section through the pancreas showing necrotized (*) and normal cells (N) of the exocrine part [Colour figure can be viewed at wileyonlinelibrary.com]

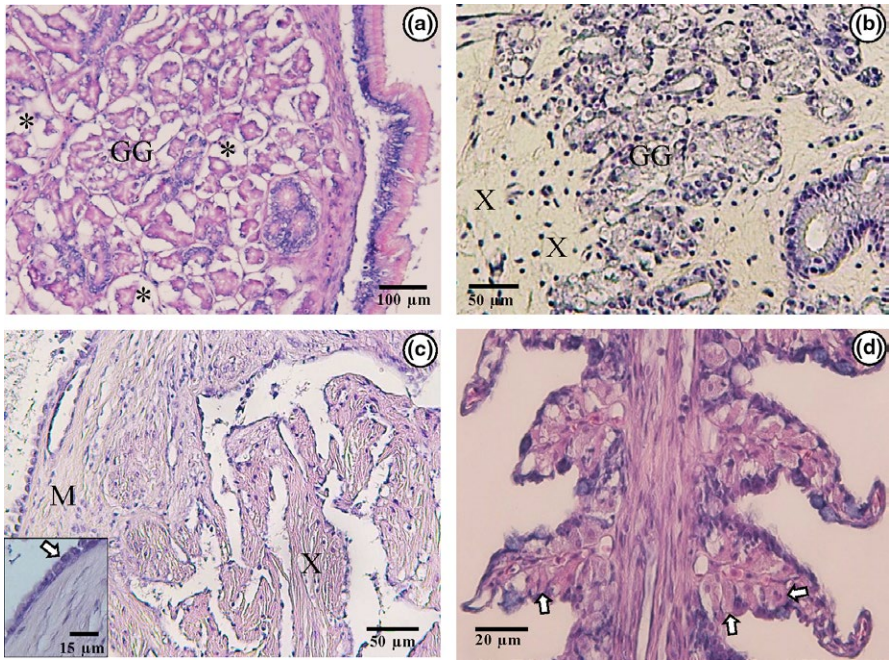


FIGURE 2 (a) Severe vacuolar degeneration (*) of the gastric glands (GG) of VHSV-infected lumpfish. (b) Severely affected gastric glands with large areas totally necrotized (X). (c) Sections through the heart of a VHSV-infected lumpfish. On the left side of the picture are relatively normal myocardial muscle fibres (M) while the right part has severely degenerated ones (X). The inserted picture shows hypertrophic endocardial cells (arrow). (d) Hypertrophic cells (arrows) in the interlamellar area and at the basal part of the secondary gill lamellae [Colour figure can be viewed at wileyonlinelibrary.com]

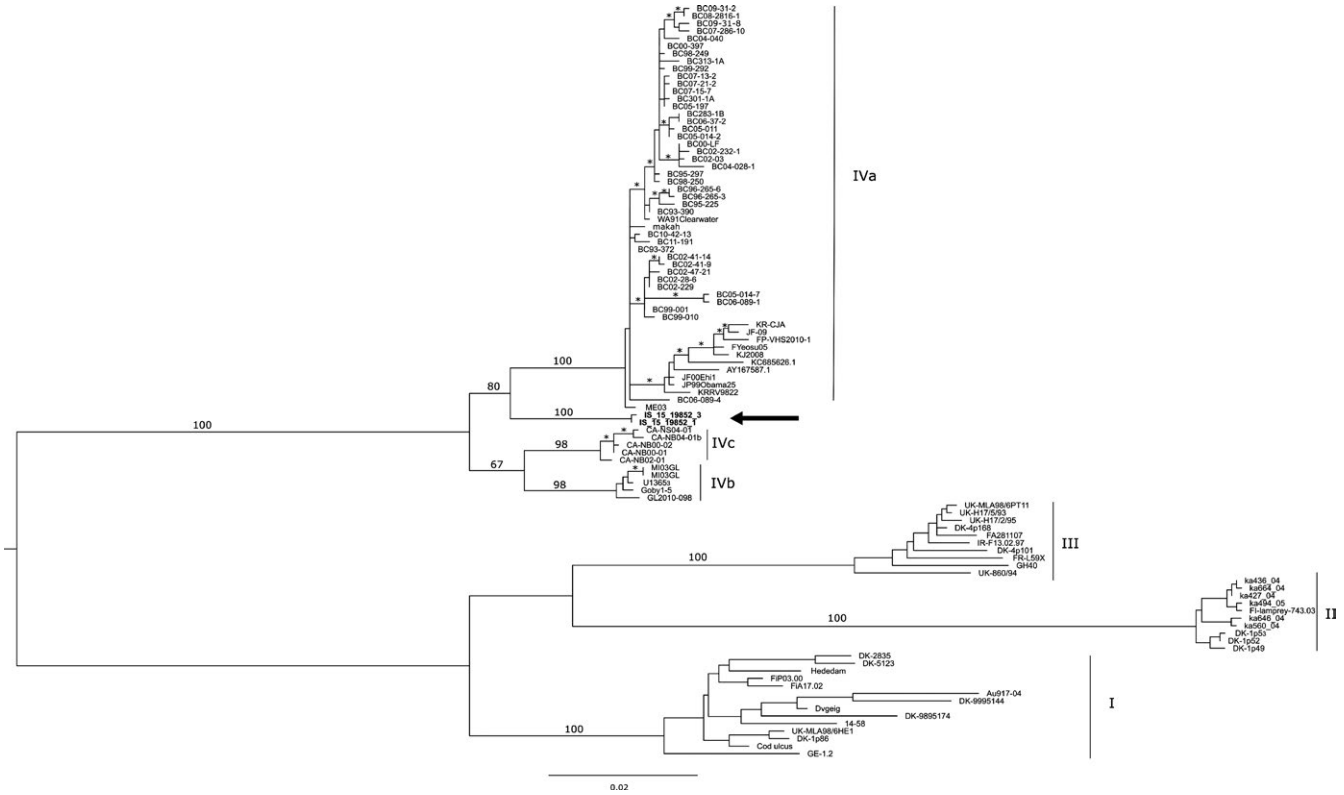


FIGURE 3 ML tree of the full G-gene of VHSV (-log likelihood = −1856.31). The two Icelandic isolates are marked by the arrow and written in bold. If not stated with numbers, bootstrap support values larger than 70% are indicated with an asterisk (only shown for genotype IV). On the right side, the roman numbers define the different VHSV genotypes

3.4.2 | Infectious trial 2

The VHSV cohabitation challenge was conducted in three tanks, including duplicate tanks for virus challenge and one negative control tank. No reduced survival, neither for lumpfish nor the salmon, was recorded in the negative control tank. In duplicate tanks exposed to VHSV, the survival of lumpfish was 7 and 17% for the shedders and 43 and 50% for the cohabitants, respectively (Figure 4). VHSV was re-isolated from the lumpfish that did not survive and therefore considered to be the cause of reduced survival. Regarding the Atlantic salmon included in trial 2, there were 80 and 92% survival in the groups of Atlantic salmon cohabitants and all fish in the control group survived. Specimen from every Atlantic salmon which did not survive was examined by virological examination on cell culture and real-time RT-PCR tested negative. Fish collected at selected time points were not included when calculating survival.

Sampling at selected time points was conducted as described above. An overview of the results is provided in Figure 5. It was possible to sample lumpfish shedders only at three time points (3, 5 and 7 dpi); afterwards, no survivor was available for this. Notably, lumpfish shedders tested positive at the first two time points (3 and 5 dpi) with low Ct values whereas at 7 dpi. fish tested negative, suggesting clearance of the virus after acute clinical disease. VHSV RNA was detected in the spleen of Lumpfish cohabitants 3, 5 and 15 dpi. The proportion of positive fish per time point was 1 out of 3 (at 3 dpi) and 2 out of 3 (at 5 and 15 dpi) indicating efficient transmission of the virus horizontally.

4 | DISCUSSION

In this paper, isolation and characterization of VHSV, originating in wild lumpfish on the west coast of Iceland, are described. This constitutes the first report of VHSV in Iceland and the first time that VHSV is isolated from lumpfish. Phylogenetic analyses possibly suggest a novel subgroup of genotype IV.

Sea lice, especially the salmon louse, cause increasing challenges to net pen salmon aquaculture worldwide. The increase in resistance to traditional therapeutic treatments and the fact that long time use of chemicals is unacceptable for environmental reasons (Burridge et al., 2010; Fallang et al., 2004; Lees, Gettinby, & Revie, 2008) have led to the adoption of alternative methods such as the use of cleaner fish (Aaen et al., 2015; Imsland et al., 2014; Rae, 2002). This cohabitation of species calls for thorough screening for pathogens, followed by risk assessments (Murray, 2016). Experiments have demonstrated that marine fish species can function as reservoirs and transmitters of trout-adapted VHSV isolates (Schönherz, Lorenzen, & Einer-Jensen, 2013). The cleaner fish industry still relies mostly on wild-caught fish, and the lumpfish production in Iceland uses wild-caught broodfish. So far, this has led to the isolation of two previously unknown viruses, that is

TABLE 2 Number of differences in G-gene between the Icelandic isolates IS_15_19852_1 and IS_15_19852_3 and other genotype IV isolates

GenBank accession	Isolate Name	Genotype	Place of origin	No of differences nucleotides	No of differences amino acids
DQ401186.1	BC93-372	IVa	Canada	**46–47**	11
DQ401192.1	ME03	IVa	USA	**46–47**	11
KC117249.1	BC10-42-13	IVa	Canada	48–49	11
DQ401195.1	BC99-001	IVa	Canada	49–50	12
U28747.1	makah	IVa	USA	49–50	12
DQ401194.1	BC99-010	IVa	Canada	49–50	12
KC117251.1	BC11-191	IVa	Canada	50–51	11
KC117222.1	BC02-28-6	IVa	Canada	50–51	12
KC117214.1	BC93-390	IVa	Canada	50–51	13
DQ401189.1	WA91Clearwater	IVa	USA	50–51	13
DQ401189		IVa	USA	50–51	13
KC117229.1	BC02-229	IVa	Canada	51–52	13
KC117216.1	BC95-225	IVa	Canada	51–52	14
DQ401188.1	BC99-292	IVa	Canada	51–52	14
KC117225.1	BC02-41-14	IVa	Canada	52–53	12
DQ401187.1	BC98-250	IVa	Canada	52–53	13
EF079896.2	CA-NB00-01	IVc	Canada	54–55	11
KC117224.1	BC02-41-9	IVa	Canada	54–55	12
KC117226.1	BC02-47-21	IVa	Canada	52–53	13
KC117221.1	BC00-397	IVa	Canada	52–53	14
KC117215.1	BC95-297	IVa	Canada	52–53	14
KC117219.1	BC98-249	IVa	Canada	53–54	14
KC117237.1	BC313-1A	IVa	Canada	53–54	14
KC117244.1	BC07-15-7	IVa	Canada	53–54	15
KC117218.1	BC96-265-6	IVa	Canada	54–55	14
KC117240.1	BC06-089-4	IVa	Canada	54–55	14
KC117245.1	BC07-13-2	IVa	Canada	54–55	15
KC117243.1	BC07-21-2	IVa	Canada	54–55	16
KC117235.1	BC301-1A	IVa	Canada	54–55	16
KC117238.1	BC05-197	IVa	Canada	54–55	16
KC117217.1	BC96-265-3	IVa	Canada	55–56	14

(Continues)

TABLE 2 (Continued)

GenBank accession	Isolate Name	Genotype	Place of origin	No of differences nucleotides	No of differences amino acids
KC117233.1	BC05-014-2	IVa	Canada	55–56	15
KC117232.1	BC05-011	IVa	Canada	55–56	16
AB490792.1	JF00Ehi1	IVa	Japan	55–56	16
DQ401191.1	JP99Obama25	IVa	Japan	55–56	16
KC117242.1	BC07-286-10	IVa	Canada	56–57	15
KC117231.1	BC04-040	IVa	Canada	56–57	15
KC117236.1	BC283-1B	IVa	Canada	56–57	15
KC117220	BC00-LF	IVa	Canada	56–57	16
KC117246	BC08-2816-1	IVa	Canada	57–58	15
KC117223.1	BC02-03	IVa	Canada	57–58	15
KC117247.1	BC09-31-8	IVa	Canada	58–59	15
KC117248.1	BC09-31-2	IVa	Canada	58–59	15
KC117241.1	BC06-37-2	IVa	Canada	58–59	15
KC117228.1	BC02-232-1	IVa	Canada	58–59	15
AB179621.1	KRRV9822	IVa	Japan	58–59	18
EF079897.2	CA-NB02-01	IVc	Canada	55–56	11
HQ168405.1	CA-NB00-02	IVc	Canada	55–56	12
HQ453208.1	CA-NB04-01b	IVc	Canada	56–57	**9**
EF079899.2	CA-NS04-01	IVc	Canada	57–58	**10**
KC117230.1	BC04-028-1	IVa	Canada	60–61	16
JF792424.1	KJ2008	IVa	South Korea	60–61	20
HQ453209	U13653	IVb	Canada	61–62	18
AB672615.1	Goby1-5	IVb	USA	61–62	18
KC117234.1	BC05-014-7	IVa	Canada	62–63	15
KC117239.1	BC06-089-1	IVa	Canada	62–63	16
DQ401193.1	MI03GL	IVb	USA	63–64	19
GQ385941.1	MI03GL	IVb	USA	63–64	19
KC117250	GL2010-098	IVb	Canada	63–64	19
KM926343.1	JF-09	IVa	South Korea	63–64	20
KF477302.1	FYeosu05	IVa	South Korea	63–64	21
AY167587.1	No name	IVa	South Korea	64–65	21

(Continues)

TABLE 2 (Continued)

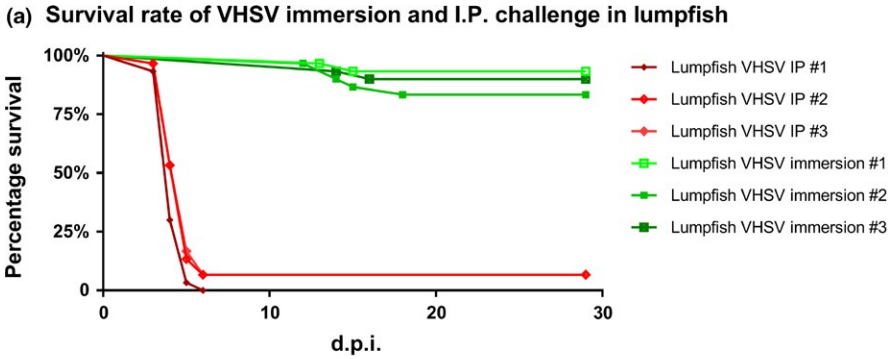
GenBank accession	Isolate Name	Genotype	Place of origin	No of differences nucleotides	No of differences amino acids
KP334106.1	FP-VHS2010-1	IVa	South Korea	66–67	22
KC685626.1	No name	IVa	China	66–67	21
JQ651388.1	KR-CJA	IVa	South Korea	67–68	21

References for the isolates are given in Table 1. Isolates are ordered by similitude (nt).

a ranavirus (Guðmundsdóttir 2016, Stagg et al. 2017) and the VHSV isolates reported in the present paper.

The production of lumpfish juveniles, offspring of wild brood fish, started in Iceland in 2014. Since then, samples from every brood fish (a total of 1.107 individuals 2015–2017) have been subjected to virological examination according to The European Commission 2015, in order to provide health certificate for the export of juveniles. After the VHSV outbreak in 2015, additional precaution that included testing all lumpfish brood stock fish individually for VHSV by qPCR (Jonstrup et al., 2013) was implemented. All of these fish tested negative (Annual reports KELDUR, accessible at http://keldur.is/arsskyrslur). In a survey for VHSV in wild marine fish in Finmark (not including lumpfish), various organs were tested using real-time RT-PCR. Gills from 12 fish (four species) were positive (subtype Ib), while five of these fish tested positive in internal organs. This indicates that testing gills might detect more positive samples than testing internal organs. However, it has to be acknowledged that positive gills may either reflect true infection or virus being passively carried in the gill mucus (Sandlund et al., 2014) and hence are not suitable for diagnostic purposes.

The VHSV isolate from lumpfish in Iceland belongs to genotype IV. So far, this genotype has never been detected in samples from European waters (Kim & Faisal, 2011; Skall et al., 2005). All previous isolates of genotype IV have originated not only from fish around the Pacific Ocean in North America, Japan and Korea, but also from the Atlantic coast of United States (IVa) (Bernard et al., 1992; Elsayed et al., 2006; Garver et al., 2013; Meyers & Winton, 1995; Traxler et al., 1999), in the great lakes on the border of United States and Canada (IVb) (Elsayed et al., 2006; Lumsden et al., 2007) or on Canadian East coast (IVc) (Gagné et al., 2007; Pierce & Stepien, 2012). Interestingly, the new Icelandic isolates are closest to genotype IVa BS = 80%). The closest available sequence belongs to a VHS isolate from Atlantic herring in the coastal waters of Maine and to isolates from Pacific herring in Canada. Although the full sequence of the G-gene is used here, our phylogenetic analysis shows only limited ability to resolve phylogenetic relationships within genotype IV. This is caused by a reduced taxon sampling belonging to genotypes IVb and IVc, as only a few full G-gene sequences are publicly available for both subgroups. In addition, isolation/identification of genotype IVa from the east coast of Canada was reported in spring 2017, and currently, areas of Newfoundland, Labrador and Atlantic Ocean North are declared as infected with VHSV genotype IVa (Notice to Industry—Viral Haemorrhagic Septicaemia Virus detected in Atlantic herring in Newfoundland and Labrador—Canadian Food Inspection Agency; Notice to Industry—Viral Haemorrhagic Septicemia Virus detected in Atlantic herring—Canadian Food Inspection Agency), but those isolates could not be included in our taxon sampling and phylogenetic analyses, as sequences are currently not publicly available. In this context, it is interesting to note that the stocks of lumpfish on the east coast of North America and around Iceland belong to distinct genetic groups, showing limited gene flow between lumpfish stocks in these areas (Pampoulie et al., 2014). In



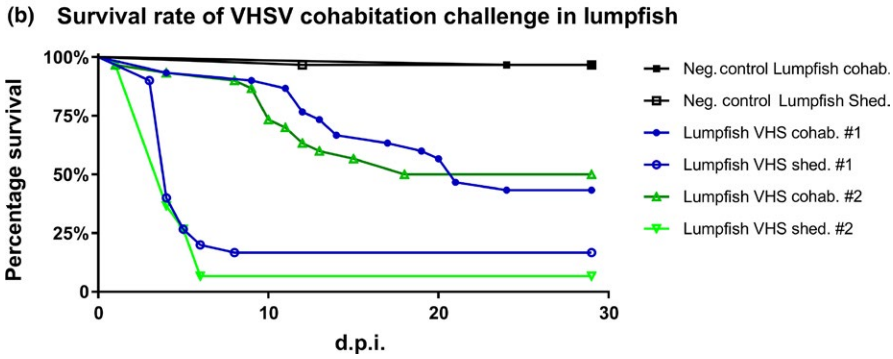
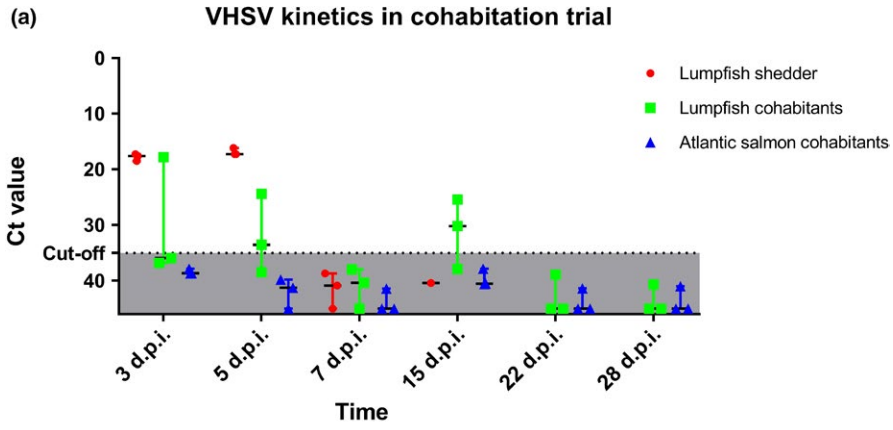


FIGURE 4 (a) Kaplan–Meier survival curves of lumpfish challenged by VHSV immersion and I.P. injection. Each treatment in triplicate tanks. (b) Kaplan–Meier survival curve of lumpfish challenged by cohabitation in duplicate tanks. Data of mock injected fish are not shown [Colour figure can be viewed at wileyonlinelibrary.com]



(b)

	Days postinfection					
	3 d.p.i	5 d.p.i.	7 d.p.i.	15 d.p.i.	22 d.p.i.	28 d.p.i
LF Shedder 1	18.45	16.11	NEG	N/A	N/A	N/A
LF Shedder 2	17.25	17.22	NEG	N/A	N/A	N/A
LF Shedder 3	17.59	17.25	NEG	NEG	N/A	N/A
qPCR +ve	3/3	3/3	0/3	0/1	N/A	N/A
LF Cohab.1	NEG	NEG	NEG	30.15	NEG	NEG
LF Cohab.2	NEG	24.36	NEG	25.41	NEG	NEG
LF Cohab.3	17.77	33.51	NEG	NEG	NEG	NEG
qPCR +ve	1/3	2/3	0/3	2/3	0/3	0/3
AS Cohab.1	NEG*	NEG*	NEG*	NEG*	NEG*	NEG*
AS Cohab.2	NEG*	NEG*	NEG*	NEG*	NEG*	NEG*
AS Cohab.3	NEG*	NEG*	NEG*	NEG*	NEG*	NEG*
qPCR +ve	0/3	0/3	0/3	0/3	0/3	0/3

FIGURE 5 Transmission kinetics of VHSV in a cohabitation challenge model. (a) graphical representation of mean value of the 3 ct values obtained per group per time point, below each mean value, the standard deviation is displayed. (b) Ct values obtained from sampling at different time points in the three different groups tested in cohabitation trial: Lumpfish Shedders—CF Shed, Lumpfish Cohabitants—CF Cohab; Atlantic salmon Cohabitants—AS. "N/A" specimen not available. Neg: sample tested negative (CT >35) [Colour figure can be viewed at wileyonlinelibrary.com]

future, comparison with VHSV genotype IVa sequences from East coast of North America, corroborated by migratory information of wild Atlantic herring stocks, might offer important insight into virus ecology. Increased availability of sequences from isolates belonging to VHS genotypes IVb and IVc will allow a better resolution of the phylogenetic clustering of genotype IV.

Viral haemorrhagic septicaemia virus (VHSV) genotype III recently caused an epidemic in wrasse used as cleaner fish in net pens in Scottish waters (Hall et al., 2013). The finding of VHSV genotype IV in Lumpfish in Iceland and VHS genotype III in wrasse on the Shetland Isles north of Scotland in 2012 (Munro et al., 2015) highlight how cleaner fish captured from the wild may harbour relevant pathogens for farmed stocks. The findings reported here and the ones described by Munro and colleagues (Munro et al., 2015) underline the need for targeted surveillance (Murray, 2016) and warrant an extension of the list of susceptible species for VHSV in European legislation and in the OIE aquatic code.

In order to investigate the virulence of this new isolate, infectious trial under experimental conditions were conducted. In the first trial, the isolate was shown to be highly pathogenic in I.P. injected lumpfish (0; 3 and 7% survival 5 days postchallenge). In the immersion challenge, neither clinical signs nor reduced survival that could be ascribed to VHSV virus was observed, despite long exposure time and high viral titre. In a second trial, using cohabitation, horizontal transmission of the virus was investigated (Figure 5). Lumpfish cohabitants experienced clinical signs and showed reduced survival (43 and 50%). Interestingly, all lumpfish shedders 7 dpi. and the only survivor sampled 15 dpi. tested negative possibly suggesting a rapid clearance of the virus. Lumpfish cohabitants were detected positive at three time points during the infectious trial, and prevalence of VHSV positive fish varied between 33 and 66% at each time point. In this trial, only spleen samples were examined, and future study using larger fish will allow a better description of tissue tropism during VHSV infection in lumpfish. This suggests that cohabitation is a well-suited model to reproduce natural infection in lumpfish under experimental condition.

The second aim of the cohabitation trial was to investigate the transmission from infected lumpfish to naïve salmon. Notably, no transfer was observed under our experimental conditions. A small amount of Atlantic salmon cohabiting lumpfish shedders was terminated during the experiment due to the appearance of clinical signs. These fish were thoroughly analysed to investigate the presence of VHSV; however, it was neither possible to re-isolate viable VHSV particle nor detect VHSV RNA by real-time RT-PCR in spleen samples of these fish. In order to better understand whether VHSV was transferred from lumpfish shedders to Atlantic salmon cohabitants, survivors were collected at the end of the experiment and analysed. Samples tested negative. Possibly, Atlantic salmon included in the present study suffered minor reduced survival due to lack of adaptation to increased salinity used in the experimental setting.

Future studies will address further characterization of the genome of this new isolate to better define the genotype and a refined risk assessment, assessing the virulence of this strain for farmed salmonids in Europe.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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