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Rapid sequence modification in the highly polymorphic region (HPR) of the hemagglutinin gene of the infectious salmon anaemia virus (ISAV) suggests intra-segmental template switching recombination

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Abstract

The ISAV has a genome composed of eight segments of (–)ssRNA, segment 6 codes for the hemagglutinin–esterase protein, and has the most variable region of the genome, the highly polymorphic region (HPR), which is unique among orthomyxoviruses. The HPR has been associated with virulence, infectivity and pathogenicity. The full length of the HPR is called HPR0 and the strain with this HPR is avirulent, in contrast to strains with deleted HPR that are virulent to varying degrees. The molecular mechanism that gives rise to the different HPRs remains unclear. Here, we studied in vitro the evolution of reassortant recombinant ISAV (rISAV) in Atlantic salmon head kidney (ASK) cells. To this end, we rescued and cultivated a set of rISAV with different segment 6-HPR genotypes using a reverse genetics system and then sequencing HPR regions of the viruses. Our results show rapid multiple recombination events in ISAV, with sequence insertions and deletions in the HPR, indicating a dynamic process. Inserted sequences can be found in four segments of the ISAV genome (segments 1, 5, 6, and 8). The results suggest intra-segmental heterologous recombination, probably by class I and class II template switching, similar to the proposed segment 5 recombination mechanism.

KEYWORDS

ISAV, *Orthomyxoviridae*, recombinant virus, salmonid pathogen

1 | INTRODUCTION

The aetiological agent of infectious salmon anaemia, which mainly affects Atlantic salmon (*Salmo salar*), is the infectious salmon anaemia virus (ISAV) (Rimstad, Falk, Mikalsenl, & Teigl, 1999). ISAV belongs to the *Orthomyxoviridae* family and is the only member of the genus Isavirus. Its genome is composed of eight negative-sense single-stranded RNA (vRNA) segments that code for at least 10 proteins (Kibenge et al., 2001; Kibenge, Xu, Kibenge, Qian, & Joseph, 2007; Mjaaland, Rimstad, Falk, & Dannevig, 1997). Segments 1, 2 and 4 code for the three polypeptide subunits of the polymerase heterotrimer, which in association with nucleoproteins (NP) coded in segment 3 and the viral segments, constitute the ribonucleoprotein complexes (vRNPs). Segments 5 and 6, respectively, code for the surface proteins, fusion (F) and hemagglutinin–esterase (HE). Segment 7 codes at least for two non-structural proteins, NS1 and NS2 interferon antagonist and nuclear exporting protein (NEP) (Cottet, 2010), respectively. Finally, segment 8 posses two ORFs that code for two polypeptides (Cottet et al., 2011).

ISAV segment 6 has a highly polymorphic region (HPR) that is the most variable region in the entire genome (Cottet et al., 2010). This region is unique for ISAV; in fact, this degree of genetic variability has not been observed in any other members of the *Orthomyxoviridae* family. Around European, North American and Chilean 30 variants with varying HPR lengths have been described based on the high degree of their polymorphism (Kibenge et al., 2016). ISAV HPR0 has the full HPR sequence (35 amino acids) and is found in avirulent ISAV isolates (Mcbeath, Bain, & Snow, 2009), with a prevalence of approximately 30% on fish farms in Chile and other countries (Christiansen, Ostergaard, Snow, Dale, & Falk, 2011), in contrast to the deleted HPR-isolated viruses, called HPRΔ, that have varying degrees of virulence (Griffiths, Cusack, Mcgeachy, & Ritchie, 2004). A characteristic genotype of Chilean HPRΔ is HPR7b, which has been characterized as highly virulent in marine cages (Cottet et al., 2010). Other genotypes that have caused health emergencies in Chilean salmon culture centres are HPR14 and HPR3, which are characterized as moderately virulent (Godoy, 2013). The HPR10 genotype is reported to have caused high mortality in Norway (Nylund, Devold, Plarre, Isdal, & Aarseth, 2003).

There are two main hypotheses to explain the high degree of HPR variation. The first is that the different virulent HPRΔ originated as a deletion from an ancestral avirulent HPR0 strain (Cunningham, 2002) that allows ISAV to infect and spread. The second hypothesis is that HPRΔ acquired sequences by insertions in the HPR region giving rise to the full-length HPR region strain, HPR0 (Kibenge, Godoy, Fast, Workenhe, & Kibenge, 2012), which evolved from a virulent to an avirulent virus. The hypothesis that deletions from an ISAV HPR0 precursor gave rise to ISAV HPRΔ is widely accepted (Cunningham, 2002).

It has been proposed that secondary structures are among the most important factors in RNA replicative recombination (Lai, 1992; Rowe et al., 1997; Worobey & Holmes, 1999) by stopping synthesis performed by the polymerase and release from the original template. Replicative recombination can be divided in three categories: Class I Essential Similarity, which involves base pairs between nascent and acceptor RNA used as primers in the new template and then extended by viral polymerase; Class II Non-essential Similarity, no base pair is required, but a secondary structure of acceptor RNA is the main recombination guide, probably by *cis-acting* properties; and Class III Assisted Similarity, which involves base pairs between nascent and acceptor RNA and a new secondary template structure (Balol, 2010).

It has been reported that the HPR in ISAV is in a complex stem-loop structure that allows recombination by stopping and releasing polymerase, the first steps in replicative recombination. Five insertions have also been reported in segment 5 whose sequences are found in other segments of the viral genome (Cárdenas, Ojeda, Labra, & Marshall, 2019). This strongly supports template switching recombination in ISAV (Markussen et al., 2008). An interesting case of template switching in *Orthomyxoviridae* family is the genome of defective interfering particles of influenza A, which is formed by short deleted RNAs (Marcus, Ngunjiri, & Sekellick, 2009; Nayak, Chambers, & Akkina, 1985). Fields and Winter (1982) reported that one of these short RNAs was composed of fragments from two segments (1 and 3) of the influenza genome. They proposed the mosaic model supported by template switching (Fields & Winter, 1982) and also found AU-rich sequences that probably act like junction sites for recombination.

In this work, we examine the genetic stability of HPR of recombinant ISAV (rISAV) generated by a reverse genetics system in six different genotypes of rISAV segment 6-HPR reassortants (HPR0, HPR7b, HPR3, HPR4c, HPR10 and HPR14). We found rapid genetic variation of viruses among cell culture passages in Atlantic salmon head kidney cells. The genotypes of most of the viruses changed within at least 7 days post-infection, inserting and/or deleting HPR sequences. We also found evidence of homologous intra-segment recombination like class I and II template switching, which allowed for recombination until the HPR0 genotype was obtained.

2 | MATERIALS AND METHODS

2.1 | Cell line and growth conditions

Atlantic salmon head kidney cells (ASK, ATCC CRL-247) were used as cell line. The ASK cells were cultured in Leibovitz medium (L-15, HyClone) supplemented with 50 μg/ml gentamicin, 10% foetal bovine serum (FBS, Corning cellgro®, Mediatech), 6 mM L-glutamine (Corning cellgro®, Mediatech) and 40 μM β-mercaptoethanol (Gibco®, Life Technologies). Cells were incubated at 16°C without CO_2, and the medium was replaced once a week.

2.2 | Generation of rISAV using a plasmid-based reverse genetics system

All viruses in this study were generated from plasmids as described by Toro-Ascuy et al., 2015. Briefly, the eight pSS-URG-based plasmids

for the synthesis of vRNA that contain the complete genome of ISAV 752_09 (pSS-URG; pSS-URG/1 to 8) and the four expression plasmids (pCDNA3.1/PB1, pCDNA3.1/PB2, pCDNA3.1PA and pCDNA3.1/NP) were synthetized by Genescript (USA). The GenBank numbers of the eight ISAV 752_09 segments are MN885647, MN885648, MN885649, MN885650, MN885651, MN885652, MN885653 and MN885654. The plasmids were transformed into *Escherichia coli* DH5α and purified using PureYield Midiprep System (Promega) according to the manufacturer's instructions. Purified plasmids were transfected in triplicate (clones 1 to 3) using Fugene 6 (Promega) at a 2:3 ratio (Fugene:DNA) into ASK cells seeded at a density of 2.5×10^4 cells/cm^2 in 12-well plates with L-15 medium. All plasmids were maintained to generate the HPRΔ and HPR0 viruses, with the exception of the pSS-URG/S6 vector, which was replaced by a homolog pSS-URG/S6 with the Open Reading Frame (ORF) of segment 6 (Table 1). We selected these HPR genotypes because they are the most representative in the deletion group (Figure S1). The 5' and 3' UTRs of pSS-URG/S6 belong to the virulent ISAV 752_09. Transfection media was replaced by fresh L-15 medium at 4 hr post-transfection. Cells were then incubated at 16°C, and the culture supernatant was harvested at 7 days post-transfection. The supernatants were designated passage 0. Additionally, for each generated virus, a "NP control" was included. This is performed transfecting eleven plasmids used in reverse genetic system for Isavirus, in this case not using the pCDNA3.1/NP. This control is useful for testing the plasmid contribution in detection of ISAV genome and replication time-course analysis by RT-PCR.

2.3 | Infection of ASK cells with rISAV

ASK cells were seeded as described above and incubated at 16°C until the monolayer reached 90% confluence. The cells then were washed twice with phosphate-buffered saline (PBS), and 500 μl of the passage 0 or post-infection supernatant of rISAV was added and diluted 1:10 in L-15 medium with 50 μg/ml gentamicin. Cells were incubated for 4 hr at 16°C and then washed twice with PBS, and 1 ml of fresh L-15 medium with 10% SFB was added. Cell culture supernatants were harvested 7 days post-infection (dpi) and stored at −20°C for further experiments.

TABLE 1 Highly polymorphic region (HPR) of the HE gene genotypes cloned in pSS-URG/S6. GenBank accession number and genotype of segment 6 ORF used in this study

Name	Genotype	ISAV Segment 6 (GenBank)
pSS-URG/S6^0	HPR0	EU118820
pSS-URG/S6^2	HPR2	HQ259676
pSS-URG/S6^3	HPR3	DQ785253
pSS-URG/S6^{10}	HPR10	AY973179
pSS-URG/S6^{14}	HPR14	FR796469
pSS-URG/S6^6	HPR7b	GU830900

2.4 | RT-PCR of viral gene and sequencing

Viral RNA segments were purified with the harvested supernatants treated with buffer lysis (20 μl/ml β-mercaptoethanol supplemented) using the E.Z.N.A.® Total RNA Kit (Omega Bio-tek) according to the manufacturer's instructions. First-strand cDNA from the HPR and segment 5 putative cleavage site regions was synthesized using Moloney murine leukaemia virus reverse transcriptase (Promega) with the specific primers S5_Fw (TACAACGGAAAGGATTAAGACTG), S5_Rv (TCTCCCTCTAGCAGCAGGTTC), HPR_3_Fw (5'-GCCCAGACATTGACTGGAGATG-3') and 18_HPR_Rv (5'-GATGGTGGAATTCTACCTCTAGACTTGTA-3') (Xiao et al., 2018). Subsequently, the transcribed cDNA was used as a template in PCR using GoTaq® Green Master Mix (Promega) with the same primers used in cDNA synthesis for either segment 5 or 6. PCR cycling conditions were 2 min at 95°C, followed by 30 cycles of 95°C for 30 s, 53°C for 30 s, 72°C for 25 s and a final extension step at 72°C for 5 min. PCR products were resolved by 2% agarose gel electrophoresis, visualized by MaestroSafe Nucleic Acid loading dye (MAESTROGEN) and purified using Wizard® SV Gel and PCR Clean-Up Kit (Promega). PCR products were cloned into the pGEM-T Easy Vector (Promega) according to the manufacturer's instructions. Purified PCR products and cloned vectors were sequenced by the Sanger method by Macrogen Inc. (Seoul, South Korea).

2.5 | Kinetics of rISAV infection

ASK cells were seeded in 48-well plates at a density of 2.5×10^4 cells/cm^2 and incubated at 16°C until the culture reached 80% confluence. The infection was performed as described above at a multiplicity of infection (MOI) of 0.01, with the 1st passage of rISAV. Supernatants were harvested at 0, 3, 5 and 7 days post-infection (dpi) for further RNA extraction. cDNA of segment 8 was synthesized as previously described with primers F5 (5'-GAAGAGTCAGGATGCCAAGACG-3') and R5 (5'-GAAGTCGATGATCTGCAGCGA-3') (F. S. B. Kibenge, Lyaku, Rainnie, Larry, & Hammell., 2000). The cDNA was used as a template for qRT-PCR, and qRT-PCR was performed with the following mix: 5 μl of Takyon Syber ROX 2X (Eurogentec), 2 μM of each primer used in retro-transcription and 2 μl of target cDNA, to a final volume of 20 μl with nuclease-free water. cDNA amplification conditions were 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 15 s, using an AriaMX Real-Time PCR System (Agilent, USA). Absolute quantitation of the viral copy number was determined using a 10-fold plasmid serial dilution. The experiments were performed twice and in duplicate wells.

2.6 | Computer analyses

DNA sequences were searched against nucleotide collections (nr/nt) using BLAST default parameters. High identity hits (≥99%) were

selected and used for multiple sequence alignment with the segment 6 templates used in pSS-URG/S6 synthesis with ClustalW2 1.2.2 version. RNA was modelled with the Mfold program (2.3 version) set at a folding temperature of 15°C. The default parameters were maintained for the other parameters. The joining sites and inserted sequences in the highly polymorphic regions of other viral segments were identified using IntaRNA at 15°C and Andronescu energy parameter. The default options were maintained for the other parameters.

2.7 | Statistical analysis

The statistical analysis involved the non-parametric Mann–Whitney *U* test for comparison of viral titres of different reassortants against rISAV^{HPR7b}. $\alpha = 0.05$, $^{*}p < .05$, $^{**}p < .005$.

3 | RESULTS

3.1 | Rescue of rISAV reassortants using reverse genetics

To study HPR sequence stability, we first rescued and cultured a set of reassortant recombinant ISAV that contained the HE gene from different HPR genotypes (Table 1, Figure S2) using the genetic backbone of the virulent Chilean isolate ISAV 752_09 (HPR7b). ISAV detection by endpoint reverse transcriptase PCR (RT-PCR) analysis revealed the presence of rISAV in most of the supernatants. Only the clones 2 rISAV^{HPR2}, rISAV^{HPR7b} and rISAV^{HPR10} were present in all the analysed passages (Table 2). All viruses were detected in passage 0 and tended to be RT-PCR positive due to the PCR product observed until passage 2, where rISAV^{HPR0} clones 2 and 3, rISAV^{HPR10} clones 1 and 3, rISAV^{HPR14} clone 2 and rISAV^{HPR7b} clone 1 were negative due to the absence of PCR product. In passage 3, most viruses were still positive for RT-PCR. Interestingly, all viruses that were negative in passage 2 became positive in passage 3, and only rISAV^{HPR2} clone 3, rISAV^{HPR3} clones 1 and 2, and rISAV^{HPR14} clones 1 and 3 were negative in passage 3. Surprisingly, rISAV^{HPR0} clone 3 was detected in passages 1 and 3, and rISAV^{HPR0} clone 2 was detected in all passages except 2, suggesting that viruses containing the HE gene derived from unculturable avirulent HPR0 can infect and replicate in ASK cells as will be showing below.

3.2 | Kinetics of infection of rISAV in vitro

We analysed the capacity of recombinant viruses to infect and replicate in permissive salmon ASK cells. All the rISAVs were infected at a MOI of 0.01 over 7 days, using rISAV^{HPR0} and rISAV^{HPR2} clone 2. Clone 1 was used for the other viruses. Viral titres were determined by real-time RT-PCR (qRT-PCR) of segment 8 at 0, 3, 5 and 7 dpi and compared to the viral titres with rISAV^{HPR7b}. All viruses were able to replicate in ASK cells (Figure 1), and at 3 dpi, the titres of most of the viruses were similar to that of rISAV^{HPR7b} (4.86×10^6 copies/ml on average), with the exception of rISAV^{HPR10}, which recorded the highest titre at 3 days, with 1.07×10^7 copies/ml on average. Differences in the replication curves were evident at 5 dpi, when all

TABLE 2 List of rISAV reassortants and their detection among the passages

Virus	Clone	Passage 0	Passage 1	Passage 2	Passage 3
rISAV^{HPR0}	3	+	–	–	+
rISAV^{HPR0}	2	+	+	–	+
rISAV^{HPR2}	2	+	+	+	+
rISAV^{HPR2}	3	+	+	+	–
rISAV^{HPR3}	1	+	+	+	–
rISAV^{HPR3}	2	+	+	+	–
rISAV^{HPR10}	1	+	+	–	+
rISAV^{HPR10}	2	+	+	+	+
rISAV^{HPR10}	3	+	+	–	+
rISAV^{HPR14}	1	+	+	+	–
rISAV^{HPR14}	2	+	+	–	+
rISAV^{HPR14}	3	+	+	+	–
rISAV^{HPR7b}	1	+	+	–	+
rISAV^{HPR7b}	2	+	+	+	+

Detection of segment 6 HPR from supernatant of ASK cells co-transfected (Passage 0) with the twelve plasmids of the reverse genetics system, and analysis of the initial three blind passages. Supernatants were analysed by end point RT-PCR 7 days post-transfection/inoculation. Transfections were performed in triplicate (clones 1 to 3), but here we present only viable clones. +, RT-PCR positive; –, RT-PCR negative.

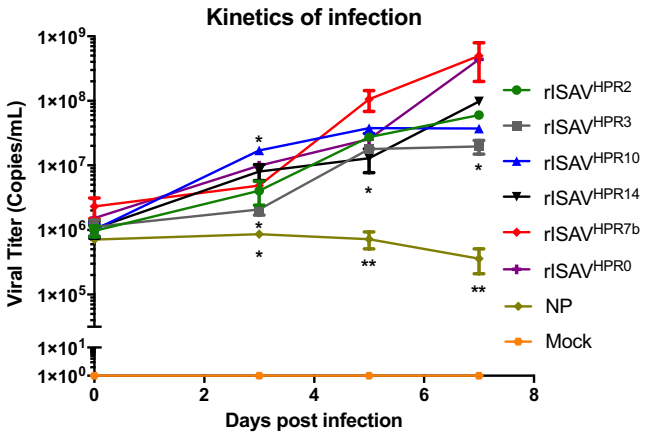

FIGURE 1 Kinetics of rISAV infection in ASK cells determined by qRT-PCR. Progress of infection over time analysed at 0, 3, 5 and 7 days post-infection in ASK cells at 0.01 MOI and viral titre (copies/ml) detection of segment 8 vRNA by qRT-PCR. NP, cells incubated with NP control supernatant; Mock, non-infected cells. Viral titre presented ± *SEM* of two independent experiments. $\alpha = 0.05$; $^{*}p < .05$; $^{**}p < .005$ [Colour figure can be viewed at wileyonlinelibrary.com]

viruses had significantly lower titres than that of rISAV^{HPR7b}, which was 1.06×10^8 copies/ml on average. The highest number of titres occurred at 7 dpi, while the virus rISAV^{HPR7b} was the most replicative, with 1.97×10^8 copies/ml on average. Most of viruses had significantly fewer titres ($p = .00512$). Surprisingly, rISAV^{HPR0} showed a titre of 3.86×10^8 copies/ml, without statically significant differences from that of rISAV^{HPR7b}. In addition, the NP control (transfection without NP plasmid expression) was evaluated in the kinetics of infection and showed no replicative activity due to the lack of amplification of segment 8 contrasting with the viruses rescued using the twelve plasmids. With these results, the plasmid contribution as template in the qRT-PCR is discarded. As was expected, the uninfected cells did not show segment 8 amplification.

3.3 | Dynamism on HPR sequence

The stability of the HPR sequence was analysed in detail using the sequences obtained from the HPR region PCR products from the first three passages. The results show that clone 3 in passage 0 of rISAV^{HPR0} had the expected genotype, but in passage 3 its genotype changed to a HPR7b genotype. This modification involved 69 nucleotide (nt) deletions, as can be observed in the alignment (Figure S2). In contrast, clone 2 of rISAV^{HPR0} maintained its genotype in passages 0, 1 and 3, and was genetically stable among these passages. In addition, clone 2 in passage 1 of rISAV^{HPR2} showed a HPR3 genotype, which required a 9 nt insertion. We detected an extremely saturated electropherogram signal in passage 2, indicating a high degree of polymorphism (Figure S3). We also detected an HPR0 genotype in passage 3. The expected genotype was found in passage 0 of rISAV^{HPR2} clone 3, but a saturated electropherogram was obtained in the first passage, while a HPR14 genotype was observed in passage 2 (the last virus detected). This change involved a 47-nt insertion and 20-nt deletion, based on a HPR2 starting. The genotypes in passage 0 both rISAV^{HPR3} were the transfected HPR3 genotype, while a polymorphic or saturated electropherogram was obtained in passage 1, and an HPR7b genotype was obtained in passage 2. Generating the HPR7b genotype requires a 39-nt deletion, and a 21-nt insertion based on a HPR3 genotype as a template. Clones 1 and 2 of rISAV^{HPR10} showed the expected genotype in the first 2 passages, but interestingly they evolved to an HPR2 genotype in passage 2, which means that a 14-nt deletion and a 20-nt insertion must have occurred. Clone 3 was not detected in this passage. But most interestingly, clone 3 was detected in passage 3 of rISAV^{HPR10} and showed a HPR0 genotype. If we assume that the modifications started with an HPR10 genotype (the last genotype detected), a 54-nt-long sequence must had been inserted. In addition, clone 2 in passage 2 of rISAV^{HPR10} showed a polymorphic electropherogram. The HPRs of all the clones in passages 0 and 1 of rISAV^{HPR14} had the same evolution. The HPR14 genotype in passage 1 changed to a HPR7b genotype, which involved a 23-nt deletion, and an 8-nt insertion. Clone 2 of rISAV^{HPR14} was the only one detected in all passages, but in passage 3 we detected an HPR0 genotype. The last genotype studied was HPR7b. Notably, clone 1 in passage 3 evolved to an HPR0 genotype. But in contrast, the genotype of clone 2 in the third passage was the expected HPR7b, so we assume this clonal virus was genetically stable among all the HPR passages. Most of the viruses sequenced in passage 3 had an HPR0 genotype, with the exception of clone 3 itself of rISAV^{HPR0}. On the other hand, we detected viral HPR0 clone 2 and HPR7b clone 2 genotypes, which were genetically stable among all the passages (Table 3).

3.4 | rISAV evolves into multiple genotypes in the same cell culture passage

As described above, the electropherograms of some PCR products were well-defined upstream and downstream of the HPR sequence, but with signal saturation in the HPR region, which suggests polymorphism. To determine whether more than one sequence is present in the polymorphic sequencing results, the PCR products were cloned into the pGEM-T Easy Vector for further sequencing. Surprisingly, all clone vectors had distinct inserts of different genotypes (Table 4).

An interesting example of sequence switching is observed in clone 2 of rISAV^{HPR2}, which evolved into the genotypes HPR4c and HPR2 (Table 4), going through genotype HPR3 (Table 3), which was extinguished to evolve into the HPR0 genotype (Figure 2). In detail, the evolution of an HPR3 genotype HPR modification into an HPR4c requires a 7-nt deletion and a 6-nt insertion (Figure 2). In this way, to obtain an HPR2 genotype, the described 9-nt insertion in passage 1 must be deleted. Another possibility is that HPR4c and HPR2 convert into each other. It is also possible that the HPR2 detected in passage 0 was displaced by HPR3 in passage 1, and with HPR3 displacement in passage 2, the virus can replicate normally in a way that can be detected. The insertions and deletions required for the evolution of the genotypes detected in the remaining viruses to an HPR0 genotype were described in the previous section.

The PCR products obtained in passage 3 were cloned to determine whether there was more than one sequence present. In this experiment, we only detected an HPR0 genotype of all bacterial clones sequenced (data not show).

Finally, in order to determinate the segment 5 stability in each virus that showed segment 6 polymorphism in passage 3, the putative cleavage site region was sequenced. The sequence analysis showed that the insertion IN4 was stable in all viruses as was expected for the ISAV 752_09 genotype transfected (data not show).

3.5 | Insertion sequences suggest intra-segmental recombination

The analysis of the sequences obtained in an alignment of the HPR region showed that the insertions previously described are the result of the addition of multiple short insertions whose sequences are found in the viral genome. We performed multiple

TABLE 3 Genotypes determined by PCR product sequencing in the different passages of the rISAV reassortants

Virus	Clone	Genotype			
		Passage 0	Passage 1	Passage 2	Passage 3
rISAV^{HPR0}	3	HPR0	–	–	HPR7b
rISAV^{HPR0}	2	HPR0	HPR0	–	HPR0
rISAV^{HPR2}	2	HPR2	HPR3	Polymorphism	HPR0
rISAV^{HPR2}	3	HPR2	Polymorphism	HPR14	–
rISAV^{HPR3}	1	HPR3	Polymorphism	HPR7b	–
rISAV^{HPR3}	2	HPR3	Polymorphism	HPR7b	–
rISAV^{HPR10}	1	HPR10	HPR10	HPR2	–
rISAV^{HPR10}	2	HPR10	Polymorphism	HPR2	–
rISAV^{HPR10}	3	HPR10	HPR10	–	HPR0
rISAV^{HPR14}	1	HPR14	HPR7b	–	–
rISAV^{HPR14}	2	HPR14	HPR7b	HPR7b	HPR0
rISAV^{HPR14}	3	HPR14	HPR7b	–	–
rISAV^{HPR7b}	1	HPR7b	HPR7b	–	HPR0
rISAV^{HPR7b}	2	HPR7b	–	–	HPR7b

Genotype identification result of rISAV segment 6-HPR by sequencing the viral HPR fragment detected in supernatants by RT-PCR for all analysed passages. – Not sequenced.

TABLE 4 Genotype determined by plasmid cloning sequencing of the polymorphic PCR products

Virus	Clone	Genotype	
		Passage 1	Passage 2
rISAV^{HPR2}	2	–	HPR2 HPR4c
rISAV^{HPR2}	3	HPR7b HPR2 HPR4c HPR3	–
rISAV^{HPR3}	1	HPR2 HPR3	–
rISAV^{HPR3}	2	HPR7b HPR3	–
rISAV^{HPR10}	2	HPR7b HPR4c HPR0 HPR14	–

PCR products that yielded polymorphic electropherograms were cloned, and 10 bacterial clones were sequenced. Genotype was assigned with a multiple sequence alignment with the pSS-URG/S6 templates used for transfection and BLASTn.

sequence alignments with most of the sequenced clones and noted that insertions follow a particular pattern of short insertions in the HPR (Figure 3). The first putative sequence of clone 3 of rISAV^{HPR2} inserted in passage 1 is present in segment 8 (Table 5), which for this sequence (TTCATTT) is referred to as insertion A and is present in most rISAVs (Figure 3). In addition, we detected a 9-nt-long sequence (AGCAACATC) in all HPR0, HPR10 and HPR14 genotypes. This insertion sequence, which we termed insertion B, is also present in segment 6. This insertion has different lengths in HPR of rISAV (Figure 3) including rISAV^{HPR2} clone 2 in passage 2 (HPR4c genotype), rISAV^{HPR10} clone 2 in passage 1 (HPR4c and HPR0 genotypes), rISAV^{HPR2} clone 2 in passage 3 (HPR0 genotype) and rISAV^{HPR3} clones 1 and 2 (HPR7b genotype). We detected a 7-nt-long inserted sequence, (TGTGTTG) called insertion C, which can be found in segment 8 neighbouring insertion A. This sequence was detected in the HPR of all clones with genotype HPR0 or HPR14. Interestingly, an additional repeat of insertion B was found upstream from insertion C, although it is shorter than other nucleotides described above, suggesting sequence duplication. This insertion was observed in HPR10, HPR14 and HPR0 genotypes. Upstream from short insertion B, a 7-nt-long sequence (GCAACCT) called insertion D was detected in the segment 1 of the HPR10, HPR14 and HPR0 genotypes. Finally, a 11-nt-long sequence (AACCAAGTGGA) called insertion E was detected in segment 5, but only in the HPR0 genotype. Upstream from insertion E, we observed 7-nt-long sequence (CAATACA) that is partly complementary to insertion C, differing only in one thymine, which suggests that recombination uses vRNA and cRNA as templates.

4 | DISCUSSION

Recombination rarely occurs in the *Orthomyxoviridae* family, probably because vRNPs do not disassemble during viral replication and

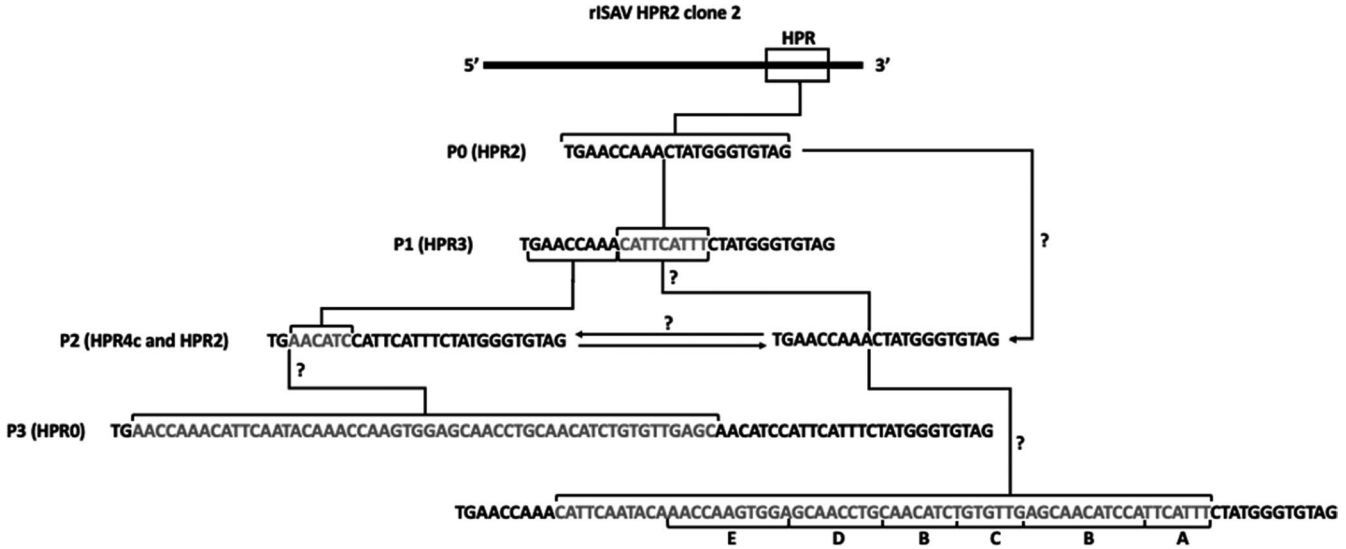

FIGURE 2 Dynamic of rISAV^{HPR2} clone 2 sequence modifications among passages. Insertions and deletions detected in rISAV^{HPR2} clone 2. Passage 1 genotype (HPR3) is obtained by a 9-nt insertion. Two genotypes (HPR2 and HPR4c) were detected in passage 2. The previous insertion of 9 nt had to be deleted to obtain an HPR2 genotype. An HPR4c genotype is also present, generated by a 6-nt insertion and a 7-nt deletion. A. The only genotype detected in passage 3 was HPR0, starting from an HPR4c genotype with a 54-nt insertion. On the other hand, a 60-nt insertion is required if recombination begins with an HPR2 genotype

are a physic barrier to recombination (Han & Worobey, 2011). Only a few recombinant viruses have been reported in influenza A H1N1 (Hao, 2011), including an insertion of 54 nt in hemagglutinin gene (HA), whose sequence is in host 28S rRNA (Khatchikian, Orlich, & Rott, 1989). It has been shown that the pathogenicity of these viruses increases through the acquisition of new cleavage sites that facilitate human to human transmission (Maurer-Stroh, Lee, Gunalan, & Eisenhaber, 2013). Another evidence is the recombination between nucleoprotein and the hemagglutinin gene described by Orich et al., and in this case, a 60-nt-long sequence from the nucleoprotein gene was inserted in hemagglutinin gene adjacent to the cleavage site, and the recombinant virus was described as highly pathogenic (Orlich, Gottwald, & Rott, 1994).

This evidence supports the hypothesis that RNA recombination is an evolutionary mechanism in which viruses create new genotypes with advantageous phenotypes (Simon-loriere & Holmes, 2011). In this sense, five insertions in the segment 5 ORF of ISAV have been reported (Cárdenas et al., 2019). The inserted sequences are found in others segments of the viral genome, which strongly suggests template switching recombination (Markussen et al., 2013). However, no junction sites have been found, nor have there been complementary analyses to shed light on the recombination mechanism. Additionally, a field study demonstrated an evolutionary event in which an ISAV HPR0 became a highly virulent HPR-deleted genotype (Christiansen et al., 2017), suggesting that ISAV HPR0 is a reservoir that can give rise to virulent genotypes. Nevertheless, two main mechanisms have been proposed for how the ISAV HPR sequence evolves. The first argues that ISAV deletes sequences from the HPR, making it virulent, while the second argues that ISAV becomes avirulent by inserting sequences in the HPR (Kibenge et al., 2012). The two hypotheses explain the high number of HPR genotypes, but do not address the molecular mechanism by which they are driven.

In this work, we found that most recombinant ISAV viruses evolve rapidly, changing HPR sequences in succeeding cell culture passages. This indicates that a single genotype can evolve into multiple genotypes as was previously described by Kibenge et al., 2012. Surprisingly, most of the viruses detected had an HPR0 genotype. As well, the sequences inserted in segment 6-HPR were found in different genomic segments of rISAV, suggesting intra-segmental recombination.

In this study, we used a reverse genetics system to obtain different genotypes of segment 6 of rISAV reassortant. We successfully detected all viruses in most of the passages in ASK cells (Table 2), which supports the formation of infective viral particles and suggests that all genotypes give rise to an infective virus for some clones. However, it is interestingly that not all clones could infect ASK cells, which suggests a stochastic recombination mechanism. Given that some segments may be incompatible in a determined gene constellation (in our case HPR7b) (Arai, 2019), we propose that the segments 6 of all genotypes used in this work are compatible with an HPR7b gene constellation, including when segment 6 is an HPR0 genotype. However, it is important to note that all the synthetic reassortant viruses have the 5' and 3' UTR from the virulent ISAV 752_09 strain (HPR7b genotype) this is relevant due to the UTR ends variation between ISAV strains that have been described (Fourrier, Heuster, Munro, & Snow, 2011). Changes in those UTR sequences might affect the virus replicative capacity as was described for influenzavirus A (Rodriguez, Marcos-Villar, Zamarreño, Yángüez, & Nieto, 2019; Sun et al., 2014) although in ISAV it has not been studied the effect of UTRs variations yet.

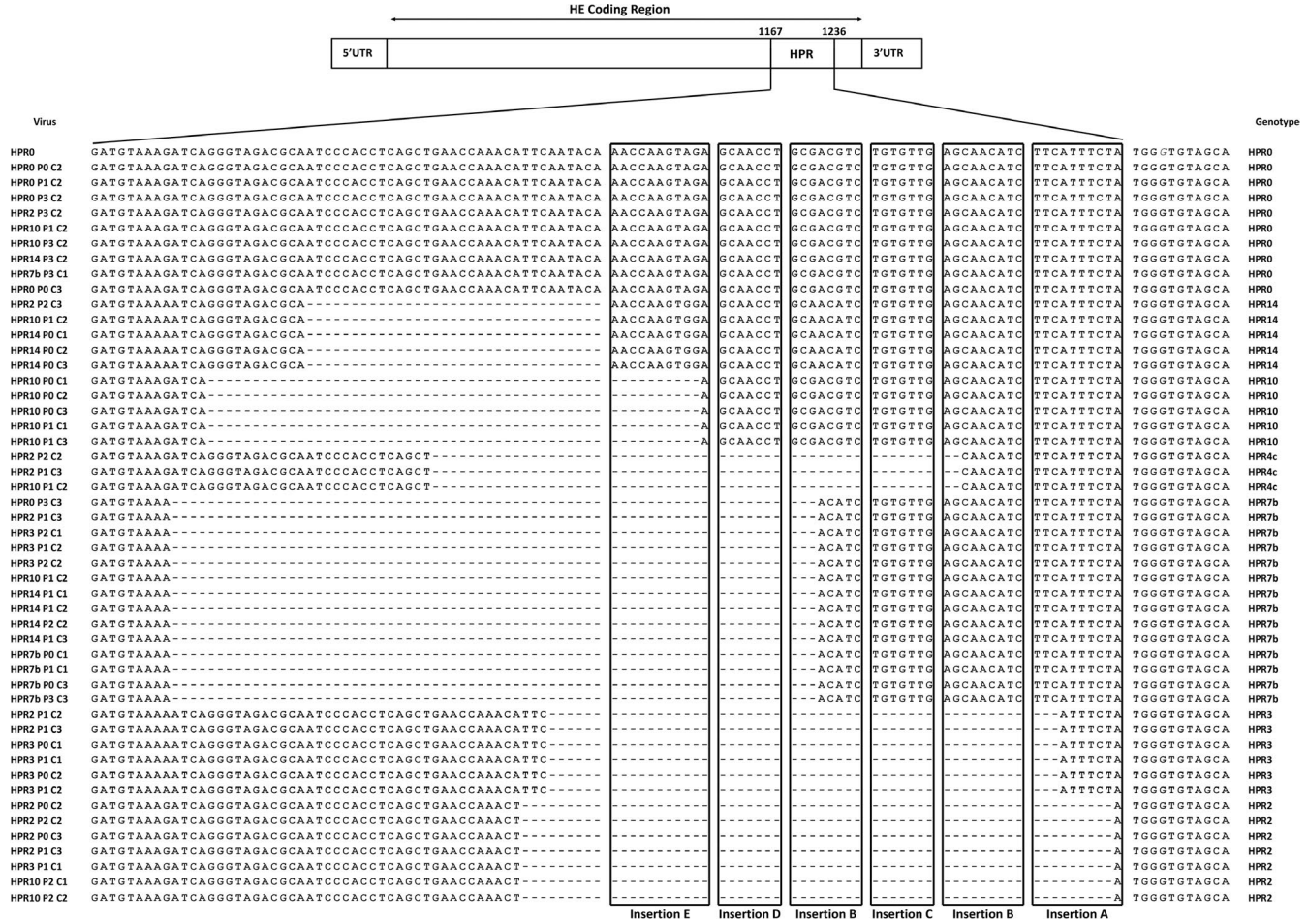

FIGURE 3 Multiple sequence alignment of different genotypes detected by sequencing and proposed insertion fragments. Multiple sequence alignment of different HPR sequences that show insertions detected in different HPR genotypes

TABLE 5 Proposed insertions detected in HPR

Insertion	Length (nucleotides)	Progenitor sequence (nucleotide position)	Sequence (5′-3′)
A	7	830–836 Segment 8	TTCATTT
B	9	1104–1,112 Segment 6	AGCAACATC
C	7	839–845 Segment 8	TGTGTTG
D	7	1,240–1,246 Segment 1	GCAACCT
E	11	1,080–1,090 Segment 5	AACCAAGTGGA

Name, length, sequence, and acceptor template of putative insertions found in the HPR. The position and sequence are described in function of vRNA.

This is the first work that shows a successful ISAV HPR0 cultivation in ASK cell line, but is important to note that this recombinant virus had the genetic backbone of an ISAV HPR7b, replacing the segment 6 ORF for an HPR0 genotype and keeping all gene constellation of an HPR7b genotype, and both 5′ and 3′UTR ends of ISAV 752_09, which taken together would explain the replicative capacity in ASK salmon cell line contrasting with any wild-type ISAV HPR0. With this strategy, using the UTR ends of virulent ISAV 752 09 and the reassortment, the ISAV HPR0 genotype became cultivable and prevails until passage 3, although it would be successfully cultivated adding passages as was previously described for rISAV (Toro-Ascuy, 2015), the explanation being the lack of immune response and of selection pressure on the strain, so that is no need to evolve into a virulent genotype (HPRΔ).

It is widely accepted that the HPR0 genotype is a reservoir for further change to a virulent genotype when conditions favour viral infection and spread (Christiansen et al., 2017). We have shown that when the HPR sequences are aligned they have a pattern of short insertions (Figure 3). These sequences are found in segments 1, 5, 6 and 8 (Table 5), which strongly suggest intra-segmental heterologous

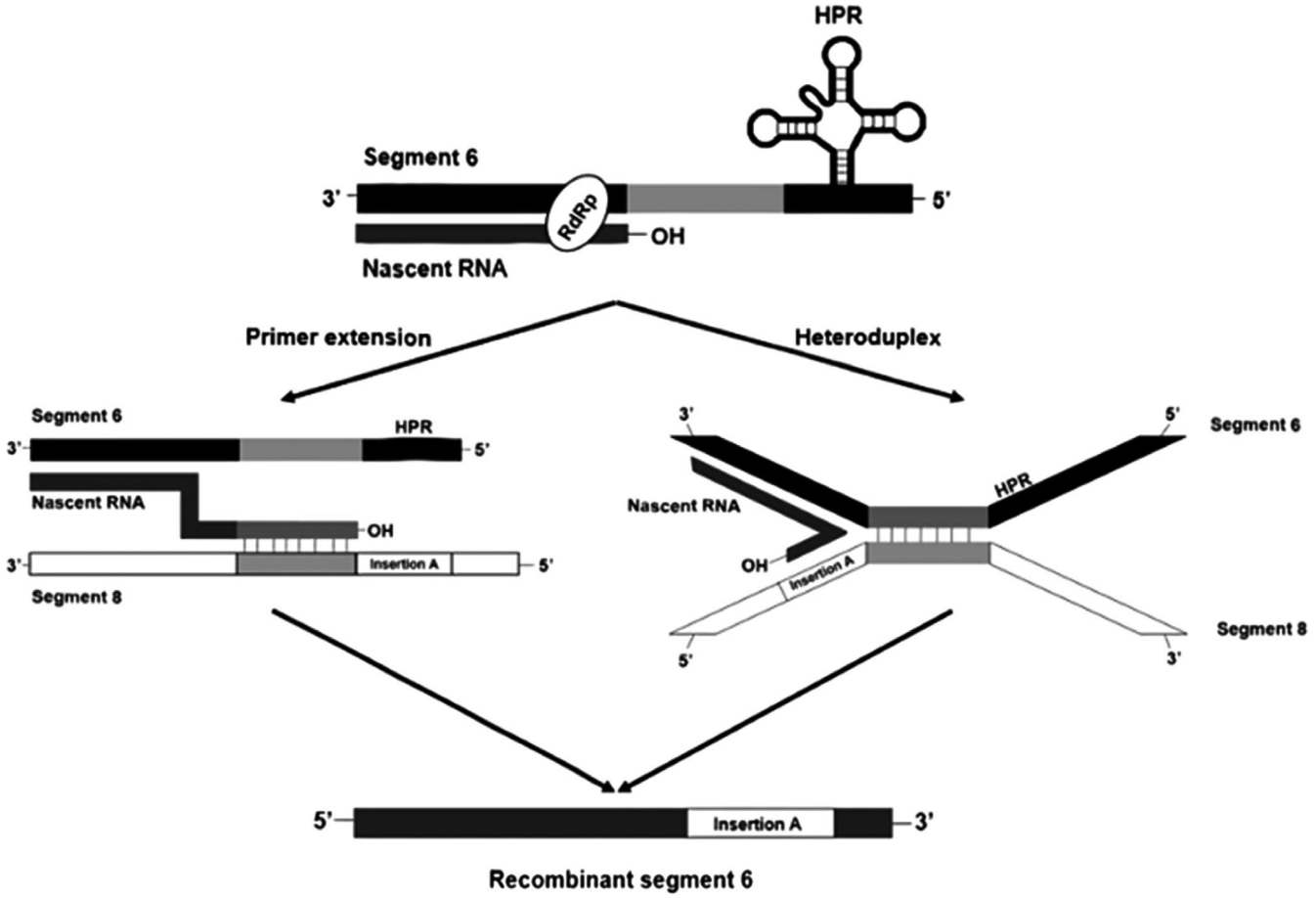

FIGURE 4 Molecular mechanisms involved in RNA recombination allowing insertion A. Segment 6 is represented in black, and segment 8 in grey (acceptor RNA), the oval represents RNA-dependent RNA polymerase (RdRp). It is likely that when RdRp is near the HPR sequence, its secondary structure stops and releases polymerase. Nascent RNA, which acts as a primer, must have a junction site (represented in grey), making it possible to anneal with a complementary sequence in segment 8 to resume synthesis. The second mechanism involves heteroduplex formation between segments 6 and 8, mediated by a base pair in junction site. This mechanism allows RdRp to pass through both segments. The two mechanisms generate a recombinant segment 6, with insertion A

recombination as previously described for ISAV segment 5 (Cárdenas et al., 2019). It has been proposed that insertions in segment 5 take place via template switching (Markussen et al., 2008) and it is possible that segment 6-HPR evolved via the same mechanism.

Interestingly, the finding of segment 8 sequences into the HPR region allows questioning the detection of Isavirus using the qRT-PCR methods suggested by World Organization for Animal Health and designed by Snow et al., 2006. The sequence analysis is shown that the primers do not anneal in the segment 8 sequence found into HPR, discarting the interference in the molecular diagnosis, but in future primer design for diagnostic purposes, these HPR region with highly recombination probability, should be considering.

The molecular mechanism that allows ISAV to recombine is unclear, and in general, this viral mechanism is poorly understood. A sequence in segment 6 was found downstream from insertion A that is complementary to segment 8 (TCTATG), which may act as a junction site for annealing segments 6 and 8 for recombination (Figures 3 and 4). This in turn strongly suggests class I template switching recombination. We propose, based on our RNA hybridization analysis (data not show), that recombination takes place by two main mechanisms: first, after RNA replication of RNA polymerase stops synthesis by the secondary HPR structure (recombination motif), and nascent RNA annealing with a new template (Figure 4 left panel) or second, the two segments anneal each other, forming an heteroduplex (Figure 4 right panel). These mechanisms have the same result, a recombinant segment 6 with insertion A, because both mechanisms allow polymerase to pass through segment 8 inserting sequence of insertion A in segment 6. More experiments are necessary to determine whether recombination takes place via primer extension or heteroduplex formation. Polymerase stops are the critical step in template switching, which are believed to be promoted by secondary structures and the incorporation of nucleotide mismatches (Chetverin, Chetverina, Demidenko, & Ugarov, 1997; Pilipenko, Gmyl, & Agol, 1995). Given that we did not find incorrect nucleotides in the HPR sequence, and given that the RNAs of all the proposed acceptor templates have a common stem-loop structure

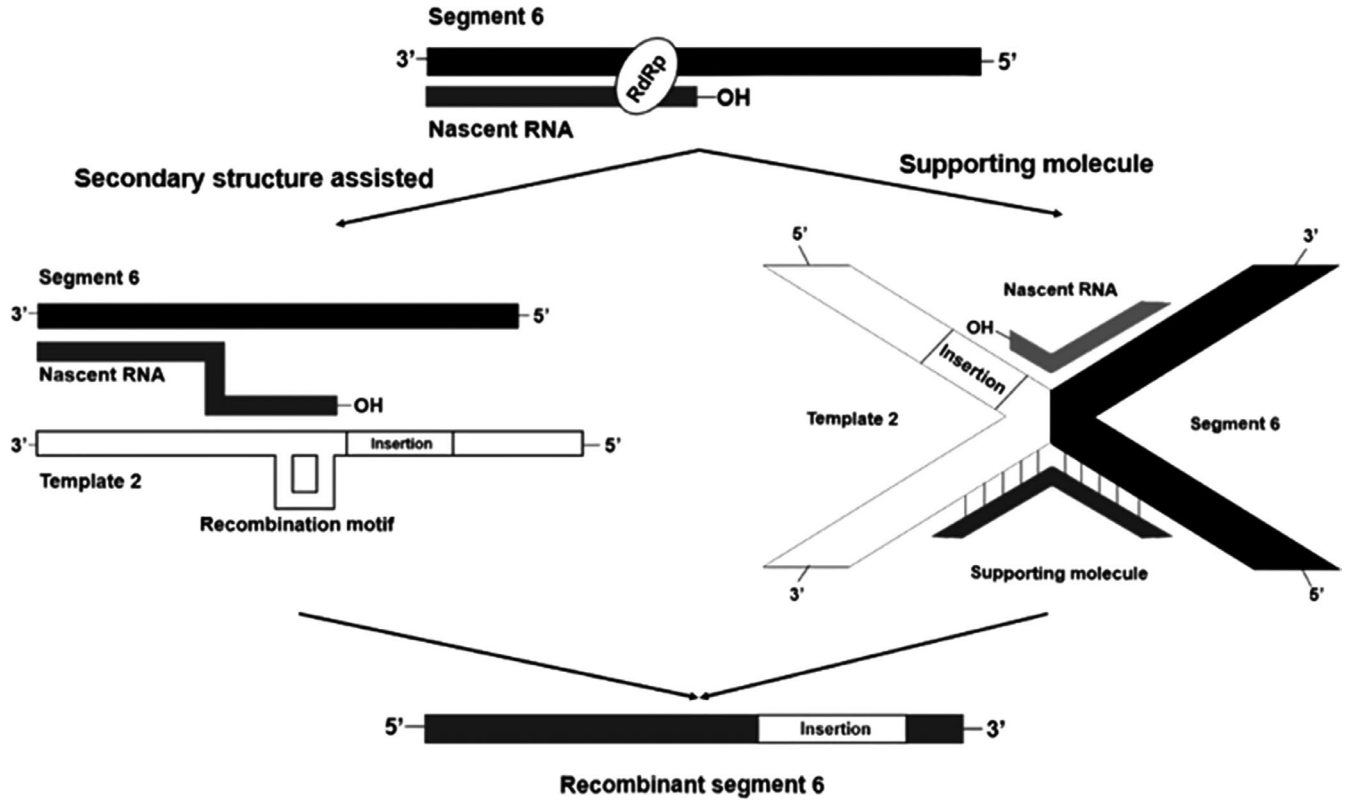

FIGURE 5 Molecular mechanism involved in RNA class II similarity non-essential recombination proposed for segment 6. Segment 6 is represented in black and acceptor RNA (template 2) in white. Before HPR replication, polymerase (represented as an oval) dissociated from the initial donor RNA (segment 6). A new template is selected because of its secondary structure (represented as a square), which acts as a *cis-acting* element. This allows resumption of replication. Segment 6 and the acceptor RNA can be bound together via a supporting RNA molecule (represented in dark grey) that must be partially complementary to both segment 6 and the new template. This mechanism allows polymerase to pass through both segments without dissociation. Both mechanisms permit the synthesis of a recombinant segment 6

observed in RNA modelling in silico (Figures S4 to S7), we propose that a secondary HPR structure is the main agent in recombination allowing polymerase stops and dissociation from the donor template.

We propose that that the secondary structure of the acceptor RNA has more affinity for the RNA-dependent RNA polymerase (RdRp) than the original template in all insertions that do not have junction sites. This would allow polymerase template switching (left panel in Figure 5) without requiring a base pair between nascent and acceptor RNA by *cis-acting* properties (Sztuba-soli, Fanning, Horn, & Bujarski, 2012) being the most probable mechanism due to the high complexity of the secondary structure of the vRNAs observed in the in silico analysis above mentioned. Models have been described in which a third supporting molecule is required for recombination (right panel in Figure 5) (Kuge, Kawamura, & Nomotot, 1989). The RNAs in these models recombine when held together by a third RNA molecule that is partly complementary to both parental RNAs. This molecule can be a host or short viral RNA. Small RNAs are produced during an infection by influenza A (Umbach, Yen, Poon, & Cullen, 2010), which suggests that they determine the change from transcription to replication (Scull & Rice, 2010). These small RNAs may be produced during ISAV infection and at the same time act as support molecules for recombination since they are complementary to parts of the viral genome. As well, secondary structures and joining sites are not required for this kind of recombination. Secondary structures in the human immunodeficiency virus (HIV) have been described as driving recombination between strands guided by a stem-loop motif, with recombination always occurring in a hairpin and never in the stem (Galetto & Negroni, 2005).

Finally, we propose that deletions are the product of nascent RNA dissociation and re-association in the same template guided by a secondary structure. We did not find a junction site, indicating that deletions take place via polymerase stops guided by a secondary HPR structure and re-association in the same template (Figure 6a). Another possibility is that deletions are produced because by the secondary HPR structure itself. If a section of the HPR is in a hairpin, polymerase can pass through the base of the tail deleting the sequence of the hairpin (Figure 6b). The last proposed mechanism for deletions is the supporting molecule mechanism (Figure 6c) (Kuge et al., 1989), in which two RNA molecules are necessary. Segment 6 adopts a hairpin-like structure produced by the second supporting RNA molecule via a pair base between two sections of segment 6. This allows polymerase to pass throw the loop-like structure deleting the sequences that annealed with the supporting molecule. However, based on the computational models of ISAV vRNAs, additional studies will be necessary in order to determinate

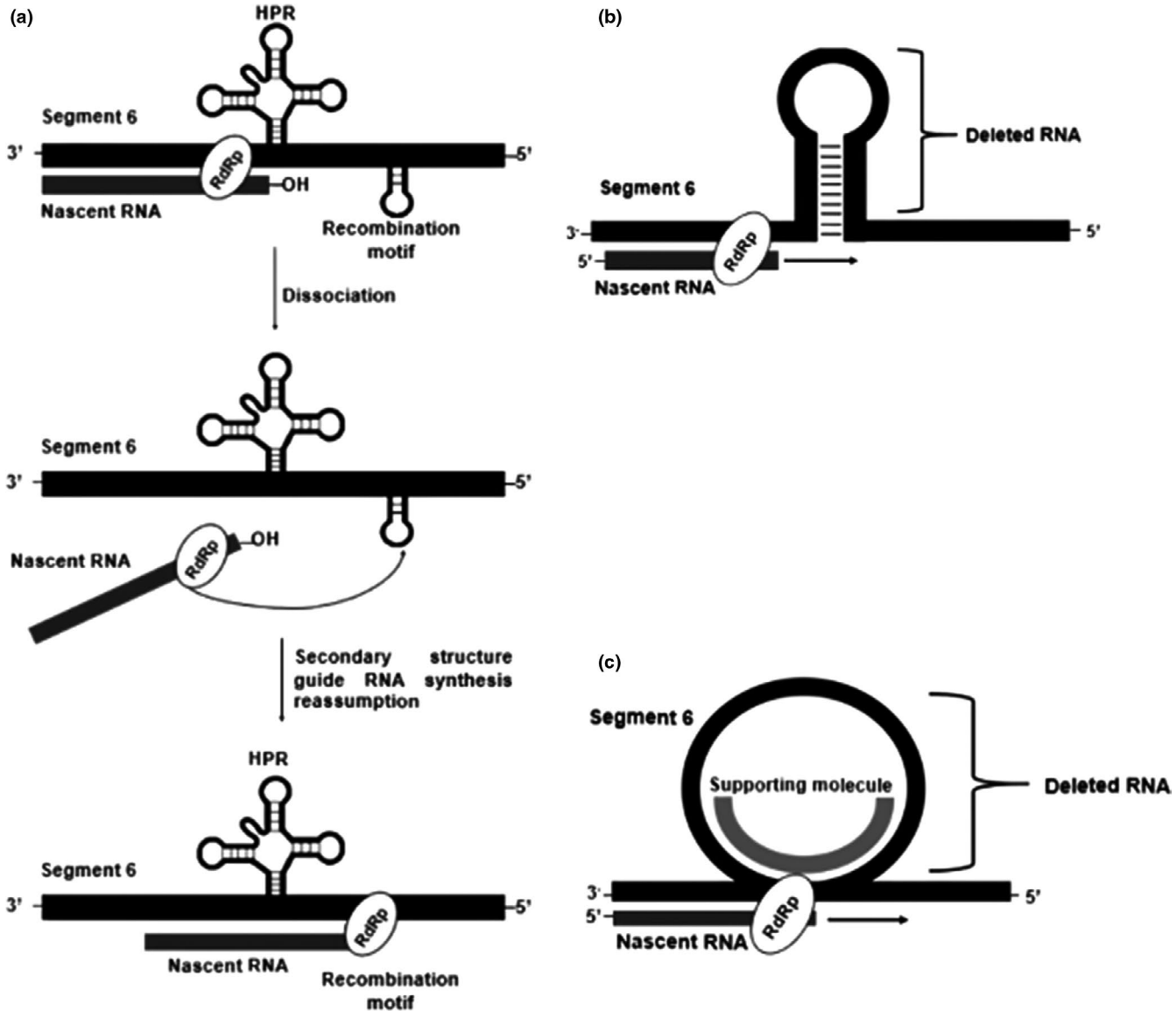

FIGURE 6 Proposed mechanism for deletion in the HPR sequence. A molecular mechanism to make deletions in segment 6-HPR to form different genotypes. (a). RdRp synthetize segment 6 downstream from HPR, but a secondary HPR structure allows polymerase stops and dissociation. RNA synthesis is reassumed because of a secondary structure with *cis-acting* properties upstream from the HPR. (b) Segment 6 allows the formation of a hairpin upstream or downstream (depending where the deletion is) from the HPR permits polymerase to pass through the base of the hairpin tail deleting all the sequences that made up the hairpin in the nascent RNA. (c) A second RNA that is partially complementary to segment 6 anneals with it and polymerase pass through the base of this loop-like structure, deleting the sequence in the loop

experimentally the relevance of the secondary structure for insertion/deletion mechanisms suggested for viral segments, being this work the first study approach using synthetic Isavirus.

We did not find unknown or intermediate HPRs, which could be the result of analysing the supernatant viruses. This implies that the analysed viruses pack their RNA and that the RNA sequences do not have defects. Many packaging sequences have been reported for influenza A viruses that allow for packaging only one of each segment (Goto, Muramoto, Noda, & Kawaoka, 2013; Hutchinson, Von Kirchbach, Gog, & Digard, 2010; Liang, Hong, & Parslow, 2005). These sequences are detected via RNA-RNA interactions (Gerber, Isel, Moules, & Marquet, 2014), and therefore, a defective RNA that does not incorporate the correct packaging sequences is not packaged in viral particles and cannot be detected in the analysis of the genome of viruses obtained in the supernatant. However, if these RNAs are formed in the cell, they can act as templates for recombination, allowing the synthesis of a complete segment 6 that can be packaged.

As discussed above, vRNPs can be a barrier to recombination, especially because of nucleoproteins that bind vRNA and cRNA to protect them from nucleases (Ruigrok & Baudin, 1995) and to prevent secondary structure formation (Dadonaite et al., 2019). The viral particles of wild-type ISAV have NPs, the genes of which are the first to be expressed and among the most expressed (Valenzuela-Miranda,

Eugenia, Manuel, & Gallardo-escárate, 2014). The reverse genetics system may not keep this early gene and initiates genome replication without a sufficient number of NPs in the cell, so that secondary structure formation results in recombination and aberrant virus genome replication, which in turn results in the viruses recombining until a functional HPR is obtained. This explains why there are no reports of the wild-type ISAV virus recombining as rapidly as our ISAV reassortants do in the cell culture.

We detected sequences in the different passages that we initially worked with, such as HPR7b, HPR10, HPR14, HPR3, HPR2 and HPR0. However, we also detected an HPR4c genotype that was not transfected, which proves that the viruses evolved in their HPR region and the results obtained were not an artefact resulting from cross-contamination. We also detected nucleotides upstream of the HPR in our sequenced PCR products that were unique to a particular genotype (data not shown) and are naturally found in wild-type genotypes. The nucleotides were conserved in all passages, even when the HPR evolved. Thus, we can confirm that a detected genotype was the product of recombination based on nucleotides, which act as fingerprints that can be detected in the different genotypes.

In conclusion, we propose that the HPR sequence evolves through insertions and deletions, and that the HPR0 is not the original ancestor of the current ISAV HPRΔ according with previously hypothesis exposed by Kibenge et al., 2012. We further propose that the virus uses a class I and or II template switching mechanism for rapid HPR modification, which allows evolution, given that we obtained the HPR0 genotype from the majority of tested genotypes and generated HPR7b from HPR0. Our results are evidence of our proposed HPR genesis mechanisms. The prevalence of ISAV HPR0 among farmed salmon in Chile may be the consequence of the evolution of previously virulent ISAV to an avirulent HPR0. However, our data also indicate that ISAV HPR0 can evolve to a virulent strain and therefore represent a latent risk to salmon farms.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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