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Short communication

Characterization and comparison of the full 3′ and 5′ untranslated genomic regions of diverse isolates of infectious salmon anaemia virus by using a rapid and universal method

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The 3′ and 5′ untranslated regions (UTRs) of the gene segments of orthomyxoviruses interact closely with the polymerase complex and are important for viral replication and transcription regulation. Despite this, the 3′ and 5′ RNA UTRs of the infectious salmon anaemia virus (ISAV) genome have only been partially characterized and little is known about the level of conservation between different virus subtypes. This report details for the first time, the adaptation of a rapid method for the simultaneous characterization of the 3′ and 5′ UTRs of each viral segment of ISAV. This was achieved through self circularization of segments using T4 RNA ligase, followed by PCR and sequencing. Dephosphorylation of 5′ ends using tobacco acid pyrophosphatase (TAP) proved to be a specific requirement for ligation of ISAV ends which was not essential for characterization of influenza virus in a similar manner. The development of universal primers facilitated the characterization of 4 genetically distinct ISAV isolates from Canada, Norway and Scotland. Comparison of the UTR regions revealed a similarity in organization and presence of conserved terminal sequences as reported for other orthomyxoviruses. Interestingly, the 3′ ends of ISAV segments including segments 1, 5 and 6, were shorter and 5′ UTRs generally longer than in their influenza counterparts.

Infectious salmon anaemia (ISA) is a multisystemic viral disease which can cause high mortality rates in farmed Atlantic salmon (*Salmo salar* L.) and is characterized by symptoms which include anaemia, ascites, haemorrhagic liver necrosis, renal intestinal haemorrhage and tubular necrosis (Godoy et al., 2008). Over the years, ISA has caused severe economic loss in all major Atlantic salmon producing nations which include Norway, North America, Scotland, the Faroe Islands and Chile (Bouchard et al., 1999; Godoy et al., 2008; Kibenge et al., 2001b; Mullins et al., 1998; Rodger et al., 1998; Scyth et al., 2003; Thorud and Djupvik, 1988).

Morphological, biochemical and genetic characteristics of the infectious salmon anaemia virus (ISAV) are consistent with those of the *Orthomyxoviridae* (Falk et al., 1997; Mjaaland et al., 1997). This family includes the influenza viruses A, B, C, Thogoto and Dhorivirus genera (Palese and Shaw, 2007). ISAV is an enveloped virus with a negative sense, single-stranded RNA genome consisting of 8 segments (Clouthier et al., 2002). Each segment contains an open reading frame (ORF), encoding for one or two specific viral proteins which is flanked by 5′ and 3′ untranslated regions (UTRs) (Clouthier et al., 2002; McBeath et al., 2006, Sandvik et al., 2000).

The protein coding ORFs of the ISAV genome have been determined fully, and the biological function of some segments, including segments 3, 5 and 6 have been demonstrated completely (Aspehaug et al., 2004, 2005; Rimstad et al., 2001). However, very little attention has been applied to the characterization of the non-coding end regions with only partial sequences of the 3′ and 5′ UTRs of ISAV segments 6 and 8 being reported (Sandvik et al., 2000). The complete characterization of all ISAV UTRs is fundamental to understanding their function, which based on knowledge of other orthomyxoviruses may be expected to involve overall regulation of the transcriptional and replication process of the viruses (Bergmann and Muster, 1996; Crescenzo-Chaigne et al., 2008).

Viral UTRs can be considered as two parts: highly conserved 3′ and 5′ terminal sequences and non-conserved sequences flanking each ORF (Crescenzo-Chaigne et al., 2008). The role of the conserved UTRs of influenza viruses has been studied extensively and these regions are heavily involved in transcription initiation (Fodor et al., 1994) and cap snatching (Hagen et al., 1994). Since ISAV transcription and replication strategies appear comparable to those of influenza A, it is likely that the 3′and 5′ ends act in a similar fashion (Sandvik et al., 2000; Toennessen et al., 2009).

In influenza viruses, the 3′ and 5′ conserved sequences display partial and inverted complementarity, which results in base pairing between the two ends and the formation of a partially double-

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stranded structure also known as a 3′ and 5′ duplex (Palese and Shaw, 2007). Previous work, has demonstrated that this double stranded structure harbors the promoter for the initiation of transcription and that this function is only activated upon base pairing (Catchpole et al., 2003; Crow et al., 2004; Fodor et al., 1994; Leahy et al., 1997). Disruption of the 3′ and 5′ duplex or mutation in the 3′ and 5′ end region has been shown to drastically reduce the binding affinity of vRNA to the polymerase complex (Fodor et al., 1994; Lee et al., 2003). Both the binding of the 3′ and 5′ ends appear to be essential for the complete activation of the PB1 endonuclease and cleavage of pre-mRNA (Catchpole et al., 2003; Hagen et al., 1994; Lee et al., 2003). Base pairing of the two conserved ends also results in the vRNA forming distinctive double-stranded panhandle structures (Hsu et al., 1987; Palese and Shaw, 2007) which are involved in subsequent transcription stages including elongation and polyadenylation (Palese and Shaw, 2007). Similar base pairing patterns of the conserved 3′ and 5′ ends were also recorded for ISAV segments 6 and 8 (Sandvik et al., 2000).

The non conserved 3′ and 5′ UTRs have not been subject to the same degree of investigation as the conserved termini. However, a growing body of evidence suggests that these regions may be implicated in expression, replication and packaging of the genomic RNA segments into newly formed virions (Crescenzo-Chaigne et al., 2008; Dos Santos Afonso et al., 2005; Fujii et al., 2005; Watanabe et al., 2003) and that they may also play a role in vRNA synthesis (Bergmann and Muster, 1996; Zheng et al., 1996).

In the past, methods involving the circularization of viral genomic segment by T4 RNA ligase have permitted a rapid and simple characterization of the UTRs of orthomyxoviruses like Thogoto and influenza virus (De Wit et al., 2007; Szymkowiak et al., 2003; Weber et al., 1997). This report details for the first time the adaptation and application of a T4 RNA ligation-based method to characterize the full 3′ and 5′ UTR sequences from all eight viral segments of ISAV and direct comparison between 4 isolates of diverse origin. This information is of fundamental importance to a range of fields concerning ISAV biology including understanding of viral function and the development of so called "reverse genetic" strategies aimed at the generation and study of recombinant viruses generated entirely from cloned DNA and viral epidemiology.

Four different ISAV isolates, including 390/98 and 982/08 (Scotland); Glesvaer/2/90 (Norway) and RPC NB 98-049 (Canada), were inoculated once onto TO cells (Wergeland H., Department of Fisheries and Marine Biology, University of Bergen). The cells were cultured according to methods described previously (Office International des Epizooties, 2009) and incubated at 15 °C for 14 days until full cytopathic effect developed. A total of 60 ml of viral supernatant was ultra centrifugated at 43,000 × *g*/4 °C for 2 h and the virus pellet collected and dissolved in 200 μl of Sigma water. RNA was extracted from the resuspended virus using the QIAamp MinElute Virus Spin kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The final elution was performed with 20 μl of Buffer AVE (Qiagen, Hilden, Germany) and concentrations determined by using a ND 1000 nanodrop (NanoDrop Technologies, Wilmington, USA). Reactions were performed in a microcentrifuge tube kept on ice. 16 μl of RNA was mixed with 2 μl of 10× reaction buffer and 2 μl of tobacco acid pyrophosphatase (TAP, 10 U/μl, Cambio, Epicentre Biotechnologies, Cambridge, UK). This corresponded to a total RNA concentration of 100 ng per reaction for 390/98 (Scot), 34 ng 982/08 (Scot), 62 ng for Glaesver 2/90 (Nor), and 90 ng for RPC/NB 98-04 (Can). Reactions were then vortex mixed and incubated at 37 °C for 1 h. 15 μl of TAP treated RNA was then ligated by T4 RNA ligase (New England Biolabs, Hitchin, UK) following the methods described previously (De Wit et al., 2007). A first denaturation step was performed at 70 °C for 5 min to minimize the formation of secondary structures and the samples placed directly on ice. cDNA of the ligated vRNA segment was synthesised by Random Hexamer priming using the Multiscribe Reverse Transcription Reagent kit (Applied Biosystem, Warrington, UK) as per manufacturer's instructions. Universal primers were designed following alignments of several ISAV strains of European and North American origins for each segment. Primers were selected in regions which displayed a high degree of conservation within the various strains, 200–400 bp up and down stream from the end of the ORF. Primers sequences are described in Table 1. The joined 3′ and 5′ ends were amplified from the ligated cDNA in a 50 μl reaction containing 5 μl of 10× NH_4 PCR buffer, 2 μl of $MgCl_2$ (50 mM), 1 μl of dNTPs (25 mM), 1 μl of each primers and 0.5 μl of Biotaq polymerase (5 U/μl) (Bioline, London, UK). The reactions were performed on a Techne Genius IV PCR machine (Techne, Cambridge, UK) using the following conditions: a first denaturing step at 95 °C for 2 min, 35 cycles each including 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. A minimum of two independent PCR were performed for each isolate and segment and sequenced independently to guard against the introduction of sequencing error. PCR products were analysed by electrophoresis through a 1% agarose (Invitrogen, Paisley, UK) gel with ethidium bromide (Sigma, Surrey, UK) staining and displaid sizes ranging between 400 and 800 bp. PCR products were purified from agarose gels using the MinElute Gel extraction Kit (Qiagen, Hilden, Germany) as per manufacturer's instruction and the concentration determined with a ND 1000 nanodrop as described above. Sequencing was performed with the same primers described above for the PCR and DTCS Quickstart mix (Beckman Coulter, High Wycombe, UK) on a Beckman Coulter CEQ™ 8800 Genetic Analysis System (Beckman Coulter, High Wycombe, UK) according to the manufacturers' protocol. Resulting sequences were analysed using Sequencer software version 4.5 (Gene Codes Corp, Ann Harbor, USA).

A rapid characterization method relying on segment self-circularization by T4 RNA ligase was adapted successfully for the amplification and sequencing of the 3′ and 5′ UTRS of ISAV. Ligation was only possible after previous TAP dephosphorylation treatment of the semi-purified vRNA segments. Once joined together, the 3′ and 5′ vRNA ends were amplified in a single RT/PCR with segment specific sets of primers spanning the 3′ and 5′ junction of the circularized RNA. Products between 400 and 800 bp were obtained for the 8 ISAV segments. The design of primers in conserved areas resulted in the method being universal and applicable to ISAV strains of diverse origin. Using this procedure, the complete 3′ and 5′ UTRs were sequenced successfully from all segments of 4 distinct ISAV isolates: 390/98 (Scot), 982/08 (Scot), Glaesver 2/90 (Nor) and RPC/NB 98-049 (Can).

Table 1
Universal primers used for the PCR amplification and sequencing of 3′ and 5′ UTRs of ISAV isolates from diverse origin.

Segment and UTR	Protein	Primer sequence
Segment 1 (3′)	PB2	5′-CCAGCCTTGGTCCTGTTGCTCC-3′
Segment 1 (5′)	PB2	5′-CGGCCAAACTGAAGAAGGGAATGAC-3′
Segment 2 (3′)	PB1	5′-AGATGTTTGTTCCCTATGTCCCACAT-3′
Segment 2 (5′)	PB1	5′-AGGCCAAGAGGATGTACGAAACAGT-3′
Segment 3 (3′)	NP	5′-CTCCAGCCAGAATTCATCATCTGTC-3′
Segment 3 (5′)	NP	5′-GCTTTTCTCTATTGACTTTGAAGGGGT-3′
Segment 4 (3′)	PA	5′-CCCTCCTTTGATTCCATGTTTGGC-3′
Segment 4 (5′)	PA	5′-ATGGAGGACATGGTCAAGGAAGT-3′
Segment 5 (3′)	F	5′-GGAATTGTTAAACCTAGACATGTTGGGTT-3′
Segment 5 (5′)	F	5′-AGTGACAGGGACGAGTTGGTGGATG-3′
Segment 6 (3′)	HE	5′-CTTGGAAACCTTCCACTCATCTGCTTCA-3′
Segment 6 (5′)	HE	5′-ATTGCACAAGGAGATGATCAGTAAACTT-3′
Segment 7 (3′)	ORF1	5′-GACAAACAGAAGTCATCTCCATTCCC-3′
Segment 7 (5′)	ORF2	5′-CAGGGTTGTATCCATGGTTGAAATGGAC-3′
Segment 8 (3′)	ORF1	5′-CGGTGGATCTTTCATCACACAGTAGAG-3′
Segment 8 (5′)	ORF2	5′-GAAGATGGTGCTCCTGGGGTACCTG-3′