In vitro and *in vivo* characterization of molecular determinants of virulence in reassortant betanodavirus

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SUMMARY

We have previously reported that betanodavirus reassortant strains (RGNNV/SJNNV) isolated from Senegalese sole exhibited a modified SJNNV capsid amino acid sequence, with two amino acid changes at positions 247 and 270. In the current study, we have investigated the possible role of both residues as putative virulence determinants. Three recombinant viruses harbouring site-specific mutations in the capsid protein sequence, rSs160.03$_{247}$ [S247A], rSs160.03$_{270}$ [S270N], and rSs160.03$_{247+270}$ [S247A/S270N], have been generated using a reverse genetics system. These recombinant viruses were studied in cell culture and *in vivo* in the natural fish host. The three mutant viruses were shown to be infectious and able to replicate in E-11 cells, reaching final titers similar to the wild-type virus, although with a somewhat slower kinetics of replication. When the effect of the amino acid substitutions on virus pathogenicity was evaluated in Senegalese sole, typical clinical signs of betanodavirus infection were observed in all groups. However, fish mortality induced by all three mutant viruses was clearly affected. Roughly 40% of the fish survived in these 3 groups in contrast to the wild-type virus which killed 100% of the fish. These data demonstrate that residues 247 and 270 play a major role in the betanodavirus virulence although when both mutated amino acids 247 and 270 are present, corresponding recombinant virus was not further attenuated.

INTRODUCTION

Betanodaviruses are the causative agents of Viral Encephalopathy and Retinopathy (VER) also known as Viral Nervous Necrosis (VNN), a pathological condition observed in a great number of fish species from different geographical areas (Munday *et al.*, 2002). The infection with betanodavirus is associated with histopathological lesions such as vacuolization in brain and retina. The clinical signs depend on fish species, biological stage, phase of the disease, and temperature. As a general remark, infected larvae have usually hyperacute disease leading to loss of appetite and death. Juveniles at high water temperature are prone to acute disease characterized by abnormal swimming behaviour. Betanodaviruses, members of the *Nodaviridae* family, are nonenveloped icosahedral viruses consisting of a bisegmented, single-stranded positive sense RNA genome. RNA1 (3.1 kb) encodes the viral RNA-dependent RNA polymerase, and RNA2 (1.4 kb) encodes the capsid protein (Comps *et al.*, 1994; Mori *et al.*, 1992). In addition, a subgenomic RNA3 is synthesised during RNA replication from the 3' terminus of RNA1. Betanodaviruses have been classified into four genotypes: striped jack nervous necrosis virus (SJNNV), tiger puffer nervous necrosis virus (TPNNV), red-spotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) based on a variable sequence of the RNA2, called T4 region (Nishizawa *et al.*, 1997). However, the isolation of reassortant strains between RGNNV and SJNNV genotypes from sole, sea bream and sea bass has been increasingly reported in the last years in the South of Europe (Névarez *et al.*, 2005; Olveira *et al.*, 2009; Panzarin *et al.*, 2012; Toffolo *et al.*, 2007). Although both combinations of genomic segments, SJNNV/RGNNV and RGNNV/SJNNV, have been

observed in viral isolates obtained from fish, the second type has been detected more frequently (Névarez *et al.*, 2005; Olveira *et al.*, 2009; Panzarin *et al.*, 2012).

Sequence analysis of three reassortant strains (RGNNV/SJNNV) isolated from Senegalese sole at both the genomic and protein levels revealed the existence of differences compared with type strains of both genotypes (Olveira *et al.*, 2009). Regarding the capsid protein, these changes are the residues 247 and 270, both on the C-terminal side of the protein, a region reported to be involved in host specificity (Ito *et al.*, 2008; Iwamoto *et al.*, 2004). One of these reassortants, SpIAusc160.03, was proven to be highly virulent for Senegalese sole, causing 100% mortality in juveniles challenged by immersion (S. Souto *et al*., unpublished data). For this reason, this strain was chosen in the current study.

The aim of this work was to investigate the possible role of residues at positions 247 and 270 as putative host and virulence determinants in betanodavirus reassortant strains. For this purpose recombinant viruses harbouring mutations in the coat protein sequence were generated by reverse genetics and compared in cell culture and in fish.

RESULTS

Betanodavirus-derived infectious cDNA constructs

Genomic RNA sequences were completed by performing a 5’ and 3’ RACE based on the sequences from Olveira *et al.* (2009). Complete sequences were published updating the previous ones (GenBank accession no. FJ803911 and FJ803923). On the RNA1 segment, 3 and 50 nucleotides (nt) were missing at 5’ and 3’ end, respectively. On the RNA2, 22 nt were missing at the 5’ end and 37 nt at the 3’ end. A comparison of the completed sequences for the virus strain Ss160.03 used in this study with the RGNNV and SJNNV genotypes was achieved. The RNA1 sequence was compared with the

sequence of the strain RGNNV-type SGWak97 (GenBank accession no. NC_008040) and the RNA2 with the sequence of the strain SJNNV-type SJ93Nag (GenBank accession no. AB056572) (Fig. S1). At the 5' ends no difference either in the RNA1 or in the RNA2 was evidenced. In contrast, at the 3'end of RNA1 differences were found at positions 3073 (C instead of T) and 3093 (T instead of C), and in the RNA2 two differences at nt 1408 (T instead of C) and 1411 (A instead of T), reflecting a polymorphism at those positions. At position 3093 of the RNA1, the genome of Ss160.03 contains a Thymine which differs from the cytosine in the SJ93Nag and SGWak97 strains. Full length RNA1 and RNA2 cDNA segments were cloned in the pBSδRiboT7t plasmid (Fig. 1 and Methods). Sequence of the final constructs pBS160R1 and pBS160R2 were identical to the Ss160.03 genome sequence previously published (Olveira *et al.*, 2009)

Recovery of rSs160.03 recombinant viruses

Recombinant rSs160.03-derived viruses were recovered from BSR T7/5 cells transfected together with pBS160R1 and either pBS160R2 or pBS160R2_247, or pBS160R2_270 or pBS160R2_247+270 plasmids. Supernatants from transfected cells were harvested at 7 days post transfection, used to infect fresh E-11 cells. At 48 hr post infection cells were fixed and analysed by immunofluorescence assays to confirm the successful recovery of the various recombinant viruses. Typical result of such assay is presented in Fig. 2 (a,b). The Fig. 2 (c) shows the RT-PCR products generated from the supernatants of E-11 infected with the wild-type and recombinant Ss160.03 viruses after 2 passages. As expected, a band at 427 bp was observed for both viruses confirming the successful recovery of the recombinant rSs160.03 virus. When RT is omitted from the reaction mixture no product was amplified. The sequence analysis of the RT-PCR products generated from supernatants of E-11 cells infected with the 3 Ss160.03

117 recombinant viruses after 2 passages confirmed the presence of the expected mutations
118 in the RNA2 genome at the positions 766 (rSs160.03$_{247}$, rSs160.03$_{247+270}$) and 836
119 (rSs160$_{270}$, rSs160.03$_{247+270}$) (Fig. 3). The mutagenesis led to the substitution of two
120 amino acid residues compared to the sequence of SJ93Nag (Acc No. AB056572). It
121 involved one nucleotide change per amino acid substitution: TCG to GCG leading to
122 substitution of serine to alanine at amino acid position 247 and AGT to AAT resulting
123 in a substitution of serine to asparagine at residue 270. Virus titers obtained from P2
124 were 1.8×10^8 $TCID_{50}$ ml^{-1} for rSs160.03$_{247}$ and 3.2×10^7 $TCID_{50}$ ml^{-1} for both
125 rSs160.03$_{270}$ and rSs160.03$_{247+270}$. The rSs160.03 reached maximal virus production (1.8
126 $\times 10^9$ $TCID_{50}$ ml^{-1}).

127 ## Growth curve kinetics of all the viruses

128 At 24 h post inoculation, all the viruses showed very similar titers in E-11 cells (Fig. 4).
129 After 48 h, it was observed a difference of 1 log titer between rSs160.03$_{247+270}$ and
130 rSs160.03. At 72 h p.i., the difference between these two viruses remained slightly
131 reduced. However, at the end of the experiment all the viruses showed titers reaching
132 10^7 $TCID_{50}$ ml^{-1}. No differences in the development of the cytopathic effect could be
133 observed among the viruses.

134 ## In vivo virulence of the wild-type versus recombinant Ss160.03 viruses

135 In order to compare the virulence of the recovered r160.03 to the wild-type Ss160.03,
136 Senegalese sole were infected by bath immersion. Fish mortality started at day 5 p.i for
137 fish group challenged with wSs160.03, while in the fish group infected with the
138 rSs160.03 the mortality was delayed to day 11. However, at day 30, mortality rates
139 reached 100% in both groups (Fig. 5). Abnormal behaviour and lack of appetite were
140 observed in both infected groups. Statistical analysis indicated that there was no

difference in virulence among the wild-type Ss160.03 and the recombinant virus ($p>0.05$).

In vivo virulence of each mutated recombinant Ss160.03 viruses

Mortality in all fish groups was first recorded between 2 and 4 days post infection. At the end of the experiment, 35 days p.i., the cumulative percent of mortality reached 100% in the positive control group (wSs160.03). Whereas roughly 40% of the fish challenged with the 3 mutants, rSs160.03$_{247}$, rSs160.03$_{270}$, and rSs160$_{247+270}$ survived to the infection (Fig. 6). VER characteristic clinical signs (loss of appetite and abnormal swimming behaviour) were observed in all challenged groups. These signs were observed throughout the experiment in the group challenged with the wSs160.03, whereas in the other groups these clinical signs were only observed in the first 15-20 days p.i corresponding to the acute phase of the disease. No clinical signs or mortality were recorded in the negative control groups.

Virus was detected by RT-PCR and recovered from all pools of fish except those from negative control groups. The nucleotide sequences of the PCR products confirmed the presence of the respective mutations in the RNA2 segment of each recombinant virus. The statistical analysis of the mortality curves revealed that the pair comparison between each mutant group and the wild-type Ss160.03 group showed a significant difference in virulence ($p<0.002$). However, there were no significant differences ($p>0.05$) in the virulence among the mutant groups (rSs160.03$_{247}$, rSs160.03$_{270}$ and rSs160$_{247+270}$).

Virus load in the brain of infected fish

To analyse the ability to infect the brain tissues by the three mutant viruses and the wild type strain wSs160.03, an experimental infection in Senegalese sole was performed.

After immersion challenge all four viruses (wSs160, rSs160.03$_{247}$, rSs160.03$_{270}$, and rSs160.03$_{247+270}$) were detected as soon as day 1 post infection in the brain (Fig. 7). The four viruses replicated in a similar way during the first five days. However, from the 6th day the genome copy number of wSs160 was clearly higher than that of the mutants. At day 6 p.i., it was observed a difference in the viral load of about 1 log with rSs160.03$_{247}$ and rSs160.03$_{270}$ and at day 10 p.i. the difference was especially high with rSs160.03$_{270}$ (more than two logs).

DISCUSSION

The viral factors that contribute to the pathogenicity and virulence of betanodavirus have not been yet clearly characterized. It has been reported that host specificity is controlled by RNA2 and/or its encoded coat protein (CP) (Ito *et al.*, 2008; Iwamoto *et al.*, 2004) and the protruding positions in the C-terminal region (residues 238 to 340 in SJNNV) have been considered good candidates for host specificity determinants (Iwamoto *et al.*, 2004). In addition, previous studies from our laboratory indicate that amino acids at positions 247 and 270 of the capsid of reassortant strains (RGNNV/SJNNV) might be putative determinants that could broaden the spectrum of the fish hosts (Olveira *et al.*, 2009).

In this study, we analysed the role of two residues (247 and 270) as putative host and virulence determinants of betadonavirus reassortant strains. For that purpose recombinant betanodaviruses were produced in transfected BSRT7/5 cells by a DNA-based reverse genetics system as previously described (Takizawa *et al.*, 2008). This approach has been widely used to identify the determinants of virulence in RNA virus such as influenza virus (Horimoto & Kawaoka, 1994; Lycett *et al.*, 2009), Lujo virus

188 (Bergeron *et al.*, 2012), West Nile virus (Diamond, 2009), alphanodavirus (Ball &
189 Johnson, 1999) and fish RNA virus (Biacchesi, 2011).
190 The rSs160.03 virus was successfully recovered by reverse genetics. The recovered
191 virus presented similar replication in E-11 cell culture compared to the wild-type after
192 two passages (not shown). The virulence of the rSs160.03 virus was tested *in vivo* using
193 the natural host species, Senegalese sole. Pathogenicity and virulence were
194 demonstrated to be similar to the wild-type virus in the experimental infection on fish,
195 although a delay was observed in the first days post infection. The three mutants
196 rSs160.03$_{247}$ (Ser→Ala), rSs160.03$_{270}$ (Ser→Asn), and rSs160.03$_{247+270}$ were readily
197 recovered and amplified in E-11 cells.
198 When the effect of the amino acid substitutions on fish pathogenicity was evaluated,
199 typical clinical signs of betanodavirus infection were observed in all groups,
200 questioning that residues 247 and 270 may act as host determinants. However, in the
201 groups infected with the mutant viruses clinical signs were only observed from day 3p.i.
202 to days 15-20. p.i., whereas in the fish infected with the wild-type virus these signs
203 lasted throughout the experiment (35 days). In addition, in the fish challenged with the
204 mutant viruses, when clinical signs disappeared, mortalities ended shortly after and
205 roughly 40% of the fish survived. However, in fish challenged with the wild-type strain,
206 mortalities reached 100%. The lower cumulative percent of mortality induced by mutant
207 viruses could be due to a slower spread in the fish host as supported by the data of viral
208 replication in the brain of infected Senegalese sole. Indeed, throughout the 10 days of
209 the experiment, a gradual and continuous increase of the wild-type virus RNA1 copy
210 number was observed, while the RNA1 copy number for the mutant viruses reached a
211 plateau at day 5. On the basis of the mortality curve data it would be expected that all
212 the individuals challenged with the wild virus should be infected. In the case of the fish

213 challenged with the mutants, with a observed mortality of 60%, it could be expected
214 that 4 out of 10 individuals would have no or few virus, but we cannot rule out the
215 possibility of horizontal transmission (fish to fish or through the water), which could
216 lead to infection of the whole group. This hypothesis is supported by the fact that the
217 virus was detected in the surviving fish, which also indicates that fish do not clear the
218 virus. A different level of infection could account for the differences in the genome
219 copy number between the wild type strain and the mutant group, but would not explain
220 the differences observed among the mutants. It has been previously shown that the
221 region from residues 217 to the C-terminus is displayed at the outer surface of the
222 capsid and therefore may be involved in the interaction with the cell surface (Tang *et*
223 *al.*, 2002). It can be speculated that these amino acid changes (247 and 270) at the viral
224 surface might modify the affinity of the virus for cellular receptors, thus affecting the
225 kinetics of virus spread. As an example it has been reported that a single residue at
226 position 226 of the hemagglutinin receptor binding domain of influenza virus could
227 change the binding recognition of sialic receptors (Zhang *et al.*, 2013). Moreover, the
228 efficiency of virus spread has been reported to be a critical determinant for
229 neurovirulence for different animal viruses like polytropic murine retrovirus (Robertson
230 *et al.*, 1997), rabies virus (Dietzschold *et al.*, 1985) and sindbis virus (Lee *et al.*, 2002).
231 It is interesting to note that when both mutated amino acids 247 and 270 are present,
232 corresponding recombinant virus was not further attenuated. Both residues are probably
233 located in the surface-protruding domain of the capsid reported to form 60 protrusions
234 (Tang *et al.*, 2002), but their relative position and orientation is unknown. It can be
235 speculated that conformational changes related to mutation could account for the results
236 obtained when both amino acids were mutated. However, further studies will be

237 necessary to determine their position in the capsid and also their involvement in the
238 interaction with cell receptors.
239 The results obtained from the *in vitro* replication experiments indicated there was no
240 effect of the amino acid change on the growth curve of the viruses when compared with
241 the rSs160.03 and the wild strain. Because SSN-1 and E-11 cells are susceptible to the 4
242 betanodavirus genotypes, it would not be expected any difference in the replication on
243 E-11 cells of rSs160.03$_{247}$, rSs160.03$_{270}$, rSs160.03$_{247+270}$, since the change of one or
244 two amino acids makes Ss160.03 more similar to SJNNV genotype The differences
245 with the *in vivo* experiments might be due to the receptors on the cell surface. It has
246 been reported that the sialic acid is involved in the binding of betanodavirus to SNN-1
247 cells (Liu *et al*., 2005) and therefore E-11, which are a clone of SSN-1 (Iwamoto *et al*.,
248 2000). Although until present there is no data regarding the neuronal receptors that
249 mediate entry of betanodavirus in fish brain, it is quite probable that neuronal receptors
250 are different to those present in SSN-1, because this cell line was derived from a whole
251 snakehead fry tissue (Frerichs *et al*., 1991). For example it has been reported that most
252 of the fixed rabies virus laboratory strains have acquired the ability to use ubiquitous
253 receptors that are present at the surface of non-neuronal cell types, which are different
254 from the neuronal receptors that use to propagate in the nervous system (Seganti *et al*.,
255 1990)
256 In conclusion, we have demonstrated that two amino acid residues in the C-terminus of
257 the capsid protein play a major role in the betanodavirus pathogenicity. Virulent natural
258 reassortant strains have a Ser at both 247 and 270 residues position, when these amino
259 acids were substituted by an Ala and Asn at 247 and 270 positions, respectively,
260 attenuated recombinant viruses were generated. Nevertheless, other motifs must be
261 involved in virulence because these changes were not sufficient enough to yield a totally

attenuated virus. Olveira *et al.* (2009) described a third change in the capsid protein sequence of the reassortant strains located in the N-terminal region (position 20), that must be considered. This region has been associated with virus assembly and RNA packaging (Tang *et al.*, 2002). Finally, in the current study, we have also observed differences in the 3' non coding region (NCR) of both genomic segments of Ss160.03 with respect to the genotypes SJNNV and RGNNV. It is known that NCR may play an important role in virulence, presumably through its effects on genome translation, replication or transcription. The importance of a single change in the 3'NCR has been reported in Influenza A strains and related to regulation of protein expression (Wang & Lee, 2009).

METHODS

Viruses and cells

The betanodavirus strain used in this work was the SpSs-IAusc160.03 (Olveira *et al.*, 2009), called Ss160.03 in the text, a reassortant strain isolated from diseased Senegalese sole (*Solea senegalensis*) with a genome consisting on a RGNNV-type RNA1 segment and SJNNV-type RNA2 segment (RGNNV/SJNNV).

The virus was propagated in E-11 cells grown in L-15 Leibovitz (Lonza) medium supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 mg ml^{-1}) and 2% foetal bovine serum (FBS, Lonza) and maintained at 25ºC.

The BSRT7/5 cells kindly provided by Dr KK Conzelmann (Buchholz *et al.*, 1999) were grown in a 5% CO_2 humidified atmosphere at 37 °C and maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS, 2 mM L-glutamine (Lonza), penicillin (100 units ml^{-1}) and streptomycin (100 mg ml^{-1}). Cells

were incubated in culture medium containing Geneticin (G418, 1 mg ml^{-1}, final concentration) every two subcultures.

Determination of the RNA1 and RNA2 complete sequences

The bulk of the Ss160.03 sequence was taken from the GenBank accession no. FJ803911 and FJ803923 (Olveira *et al.*, 2009). In order to obtain the complete sequence, the 5' and 3' termini of Ss160.03 of both genomic segments were determined by rapid amplification of cDNA ends (RACE) using the FirstChoice RLM-RACE kit (Ambion) according to the manufacturer's instructions. For the 3'RACE, a poly(A) tail was added to the viral RNA using Poly(A) Tailing Kit (Ambion). First strand cDNA was synthetized using the supplied 3' RACE Adapter. For both ends, cDNA was synthetized using the M-MLV Reverse Transcriptase. The cDNA was then subjected to PCR with the AccuPrime™ Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific) using the specific primers described in Table 1.

The 5'-Ss160.03 and 3'-Ss160.03 PCR products from RACE (RNA1 and RNA2) were purified from agarose gel and cloned using the pGEM®-T Easy Vector System (Promega) and transformed into *E. coli* and sequenced with M13F and M13R primers. Five individual clones were used to determine the 5' and 3' sequences of both segments.

Construction of full-length cDNA clones

cDNA clones of Ss160.03 segments 1 and 2 were independently prepared. Viral RNA from purified virus was used as the template to generate the full-length cDNA. RNA1 and RNA2 were synthetized using the SuperScript® III Reverse Transcriptase (Invitrogen) using random primers. The cDNA was then amplified using the Platinum Pfx DNA Polymerase (Invitrogen) with the specific primers for the 5' and 3' termini of each RNA, 5T7R1/3*Nae*R1 and 5T7R2/3*Nae*R2 (table 1). As shown in table 1, the 5T7

primers included the T7 promoter sequence followed by 21 or 22-base sequence corresponding to the 5' terminus of RNA1 and RNA2, respectively. Two additional G residues, which were considered to improve T7-driven RNA transcription (Takizawa *et al.*, 2008), were added to the 5'of each segment as well. The 3*Nae* primers introduced a *NaeI* restriction enzyme site artificially at 3' to facilitate further cloning steps. Each viral segment was inserted in the pJET1.2/blunt Cloning Vector (Thermo Scientific).

The full-length T7p-Ss160.03R1-*NaeI* and T7p-Ss160.03R2-*NaeI* cDNA genome inserts were recovered from pJET1.2/blunt vectors by digestion with *SacII* and *NaeI* restriction enzymes and inserted into the pBSδRiboT7t, containing the hepatitis delta ribozyme sequence and the T7 terminator sequence (Biacchesi *et al.*, 2000), digested with *SacII* and *SmaI* restriction enzymes leading to the expression vectors named pBST7160δRiboT7t_R1 and pBST7160δRiboT7t_R2 (to simplify pBS160R1 and pBS160R2). After blunt-ended digestion with *NaeI*, the 3' ends of the Ss160.03 sequence were fused to the ribozyme sequence (Fig.1) followed by the T7 terminator sequence and the 5' ends fused to the T7 promoter. Prior to transfection the mutation originated with the 3*Nae* primers at the 3'end of each RNA was reverted using site-directed mutagenesis using the primers 3RevR1 and 3RevR2 (Table 1).

Construction of pBS160R2_247, pBS160R2_270 and pBS160R2_247+270

To construct the mutants pBS160R2_247, pBS160R2_270 and the pBS160R2_247+270 nucleotide substitutions were introduced in the triplets that codified the positions 247, 270, and both positions respectively (amino acid capsid numbering). The plasmids were generated using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions using 100 ng of the pBS160R2 as DNA

template and each specific primer that contained the desired nucleotide change (Table 1). The success of the mutagenesis was confirmed by sequencing using the CEQTM Dye Terminator Cycle Sequencing (DTCS) Quick Start Kit (Beckman Coulter) in a CEQTM 8000 Genetic Analysis System (Beckman Coulter). Sequence was confirmed once by sequencing upstream with primer F2 and downstream with primer R3 (Table 1). The sequences were analysed using DNASTARLasergene® v.7.1 SeqMan II and MegAlign (DNASTAR).

Transfection of BSR T7/5 cells and recombinant Ss160.03 recovery

For the recovery of recombinant viruses, BSR T7/5 cells were used as previously described (Buchholz *et al.*, 1999). The cells were plated in a 6-well plate and transfected with 1 µg pBS160R1, 1 µg of either pBS160R2, pBS160R2_247, pBS160R2_270 or pBS160R2_247+270 and the reagents of Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. After incubation for 12h at 37°C, the transfection mix was removed and the cells were shifted to 28°C with DMEM containing 2% FBS for 7 days. After that, cells were suspended in the supernatant by scratching the wells and then subjected to three cycles of freezing-thawing. Supernatant P0 was clarified by low-speed centrifugation at 1500 × *g* in a microcentrifuge and used to inoculate E-11 cells monolayers in a 24-well plate at 25°C. After 7 days, the supernatant from this culture (supernatant P1) was collected and used to infect fresh cells. Supernatant P2 was titered and inoculated into T-25 flasks in order to produce a virus stock of each recombinant betanodavirus (rSs160.03, $rSs160_{247}$, $rSs160_{270}$ and the $rSs160_{247+270}$)

Reverse transcription and polymerase chain reaction (RT-PCR) on genomic RNA from recombinant viruses

RNA genome of recombinant virus was directly extracted from the P2 supernatant by using the E.Z.N.A. Total RNA Kit I (Omega Bio-Tek), treated with DNase I and analysed by RT-PCR using Superscript III (Invitrogen) for the cDNA synthesis and the Gotaq Polymerase (Promega) for the PCR. The pairs of primers used, F2/R3 for the RNA2 and F7/R7 for the RNA1, are indicated in Table 1. PCR products were subjected to nucleotide sequencing.

Indirect immunofluorescence analysis on cells

E-11 cells grown in 24-well plates were infected with the rSs160.03. At 48h post-infection, cells were fixed with a mixture of ethanol and acetone (1:1) at –20°C for 20 min and washed with PBS. Primary mouse monoclonal antibody against the coat protein of Ss160.03 (Abmart) was incubated in PBS-Tween 0.05% for 1 h at room temperature (RT) and washed 3 times with PBS-Tween 0.05%. Cells were then incubated with Fluorescein isothiocyanate (FITC)-conjugated anti mouse immunoglobulins (F0257, Sigma) in PBS-Tween 0.05% for 1 h at RT in the dark. Cells were stained with DAPI (Sigma-Aldrich) according to the manufacturer's instructions and were then visualized with a UV-light microscope (Nikon).

Growth curve of the various viruses

E-11 cells in 24-well plates were infected with the following viruses: wSs160.03, rSs160.03, $rSs160.03_{247}$, $rSs160.03_{270}$ and $Ss160_{247+270}$ at a multiplicity of infection of 0.1. After 1 h adsorption, the inoculum was removed and fresh medium (supplemented with 2% FBS) was added. Aliquots of viral supernatant were taken at different times post-infection: 24h, 48h, 72h, 5 days and 7 days. The supernatants were titrated in

duplicate on E-11 cells by the endpoint dilution method on 96-wells plates. The 50% tissue culture infective dose ($TCID_{50}$) was estimated by the method of Reed & Müench (1938).

Fish challenge

Two different experimental infections in sole were performed in order to assess the virulence of each recombinant virus produced by reverse genetics. The number of fish included in each experiment was calculated following the test of Logrank (Ahnn & Anderson, 1995). A total of 430 Senegalese sole juveniles (mean weight 2 g) were obtained from a commercial fish farm and maintained in the fish facilities of the Universidad de Santiago de Compostela. The fish were fed dry commercial pellets daily. All animals were handled in strict accordance with good animal practice as defined by the European Union guidelines for the handling of laboratory animals (directive 2010/63/UE). The protocol was approved by the Galician Committee of experimental animals welfare and the Xunta de Galicia (Permit Id. 15004AE/12/INV MED02/ANIMAL[05]/IBM4). All efforts were made to minimize animal suffering. The fish were placed in opaque tanks containing seawater and acclimated at 22°C for 10 days. During the experiment oxygen, nitrogen compounds, pH, and salinity were monitored continuously. Temperature, lighting and noise were also strictly controlled in order to minimize stress. Before each experimental infection, 10 fish were sacrificed with an anaesthetic overdose (MS-222, Sigma-Aldrich). Fish were examined for the presence of nodavirus by RT-PCR (Cutrín *et al.*, 2007), and all were found to be negative for the virus. In a first assay, an experimental infection was carried out with the wild-type Ss160.03 (wSs160.03) strain and the recombinant virus recovered by reverse genetics (rSs160). Two groups of 25 fish were infected with: a) the wSs160.03, b) the rSs160.03 and a third group was mock infected with L-15 medium (10 fish). In a second

403 experiment, duplicate groups of 40 Senegalese sole were infected with the recombinants
404 rSs160.03$_{247}$, rSs160.03$_{270}$ and rSs160.03$_{247+270}$ and with the wild strain (wSs160.03).
405 Duplicate negative control groups (15 fish/group) were also set up. Fish were bath-
406 challenged for 3h at a final viral concentration of 10^5 $TCID_{50}$ ml^{-1} and maintained for 35
407 days at 22°C. Abnormal behaviour and mortality were recorded twice a day throughout
408 the experimental period and dead fish were removed from the tanks. Surviving fish at
409 the end of each experiment were euthanized using a MS-222 overdose.

410 ## Virological analysis

411 Brains from 5 dead fish were aseptically collected and pooled. The organs were
412 homogenized and diluted 1:10 (w/v) in Earle's balanced salt solution (Hyclone
413 Laboratories Inc.) supplemented with penicillin (1000 UI ml^{-1}), streptomycin (1000 µg
414 ml^{-1}), gentamycin (500 µg ml^{-1}) and fungizone (20 µg ml^{-1}). The homogenates were
415 clarified by centrifugation at 2000 × *g* for 20 min at 4ºC. An aliquot of 0.1 ml of each
416 sample was used for RNA extraction and subjected to RT-PCR and sequencing as
417 previously described (Olveira *et al.*, 2009). The supernatants obtained from brain
418 homogenates were incubated overnight at 4 °C and inoculated (diluted at 1:10 and
419 1:100) onto 48-well plates of semi-confluent monolayers (roughly 80% confluence) of
420 E-11 cells. After 10 d, samples were subcultured by inoculating 0.1 ml of the scraped
421 cell suspension onto new cultures. Plates were observed daily for cytopathic effect
422 (CPE) detection. Non-infected cells were used as controls. Identity of viral isolates was
423 confirmed by RT-PCR.

424 ## Characterization of replication in vivo

425 Four groups of sole (mean weight 2 g, 100 fish/group) were infected as described above
426 (Fish challenge) with either: wSs160.03, rSs160.03$_{247}$ rSs160.03$_{270}$, and rSs160$_{247+270}$ by

bath immersion (10^5 $TCID_{50}$ ml^{-1}) at 22°C. A negative control group of 10 fish were mock-infected (L-15). Ten fish from each group were randomly sampled daily from day 1 to 10 post-infection and euthanized with a MS-222 overdose. RNA extraction and synthesis of cDNA was performed as described above from duplicate pools of five fish. The RNA1 copy number was quantified by SYBR Green Real Time PCR using CFX96^{TM} Real-Time PCR Detection System (Biorad) with the primers SnodR1 F/R (Olveira *et al.*, 2013) and according to the manufacturers' instructions. To prepare the standard curve, 20-fold dilutions of a plasmid containing the RNA1-full length of SGWak97 strain were prepared up to 1 viral copy in DEPC water. Viral load data were calculated as RNA1 copies per gram of brain tissue and reported as the mean of the two sampled pool of fish at a given time point.

Statistical analysis

Results from experimental infection in fish with the recombinant viruses were analysed using GraphPad Prism 5.0. A *p value* of <0.05 was considered to be statistically significant. Survival analysis was undertaken with life tables and Kaplan–Meier analysis. Survival curves were compared by performing the log-rank Mantel-Cox test. Post analysis was performed using a significance set at $p<0.02$ incorporating a Bonferroni correction for multiple testing.

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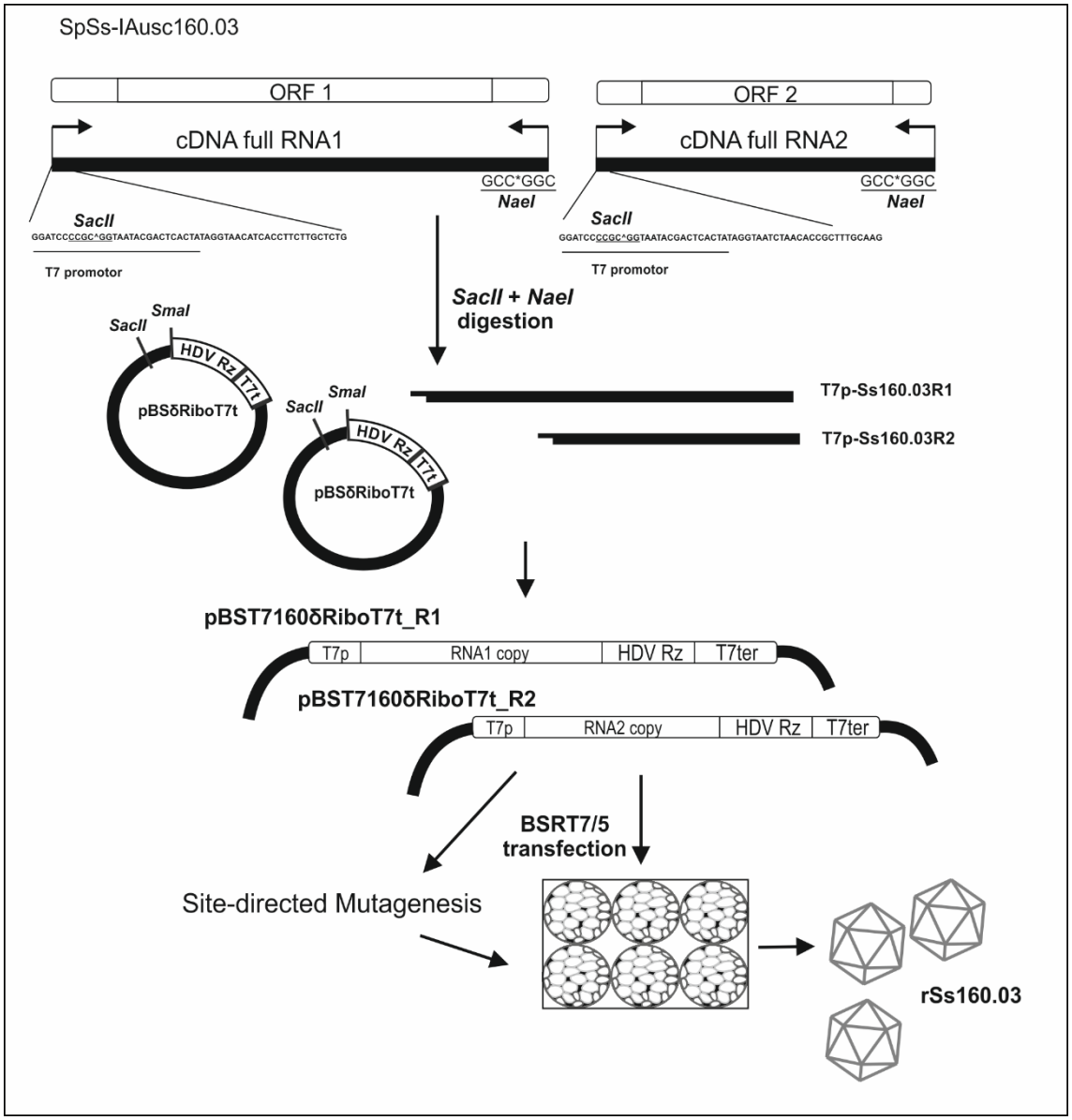
549 **FIGURES**



550

551 Figure 1**.** Plasmid constructs and reverse genetics system. The full-length cDNA of the
552 Ss160.03 RNA1 and RNA2 was amplified by PCR adding the T7 promoter at the 5'
553 ends and the hepatitis delta virus (HDV) ribozyme sequence fused to the 3' ends. Both
554 segments were assembled into a modified pBSδRiboT7t vector using the *SacII*, *NaeI*
555 and *SmaI* restriction. Recombinant viruses were recovered from 6-well plates of BSR-
556 T7/5 cells transfected with 1 µg of both pBS160R1 and pBS160R2 (pBS160R2,

pBS160R2_247, pBS160R2_270 and pBS160R2_247+270) (see “Materials and Methods”).

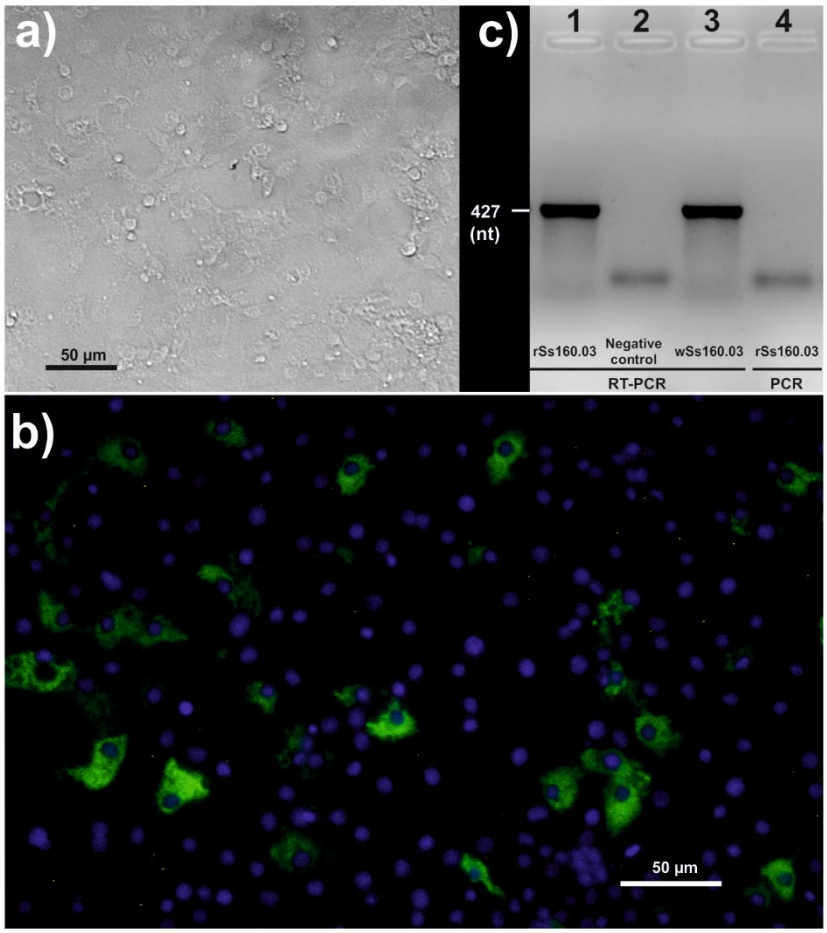
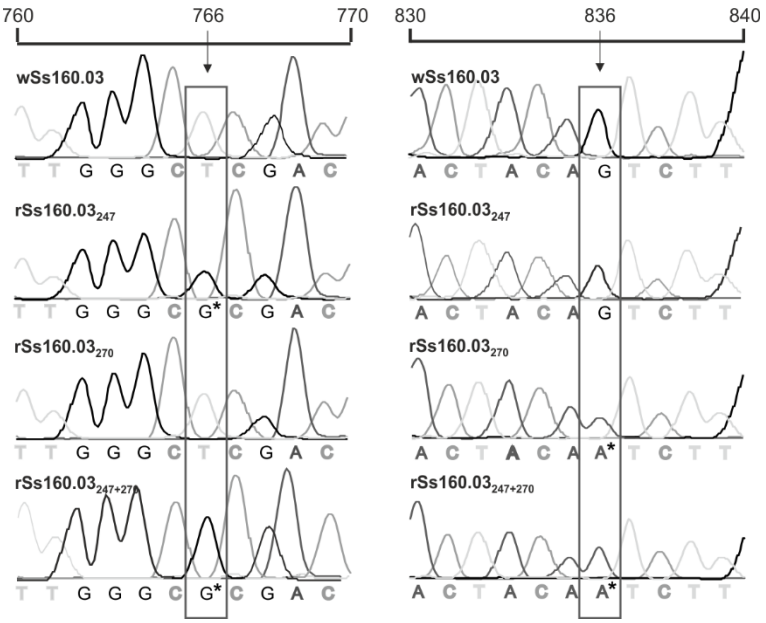
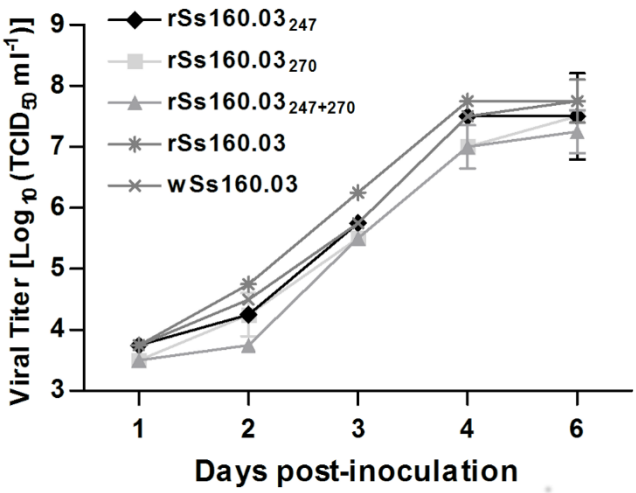


Figure 2. Confirmation of the recovery of the rSs160.03 virus. UV microscopy observation following immunofluorescence assay using a monoclonal antibody directed against the coat protein in E-11 cells: a) rSs160.03-infected cells 48 h post-infection at the first passage b) Immunofluorescent labeling of the infected cells. Green: recombinant virus on infected cells. Blue: nuclear DAPI staining. c) Visualisation on agarose gel of RT-PCR and PCR products amplified from the RNA extracted from the supernatant of infected E-11 cells (Passage 2). Lane 1: RT-PCR from DNase-treated RNA. Lane 2: negative control of RT-PCR (DEPC-treated distilled water). Lane 3: positive control wild-type Ss160.03. Lane 4: PCR (Reverse transcription omitted).



570

571 Figure 3. Site-specific mutations in RNA2 ORF sequence of recombinant Ss160.03
572 viruses. Sequence electropherograms are shown for RT-PCR products obtained from
573 control viruses Ss160.03 and from recombinant virus rSs160.03$_{247}$, rSs160.03$_{270}$, and
574 rSs160$_{247+270}$. The nucleotide positions correspond to the complete SpSs-IAusc160.03
575 RNA2 sequence. The box contains the mutated positions. The asterisk indicates the
576 muted nucleotide.



577

578 Figure 4: Replication kinetics of the recovered viruses. E-11 cells were infected with the
579 rSs160.03$_{247}$ (rhombus), rSs160.03$_{270}$ (square), rSs160.03$_{247+270}$ (triangle), rSs160.03
580 (cross) and wSs160.03 (asterisk) viruses at an MOI of 0.1. Aliquots of the culture

supernatants were taken at the indicated time points and titered in duplicate. Data are plotted as mean and SD.

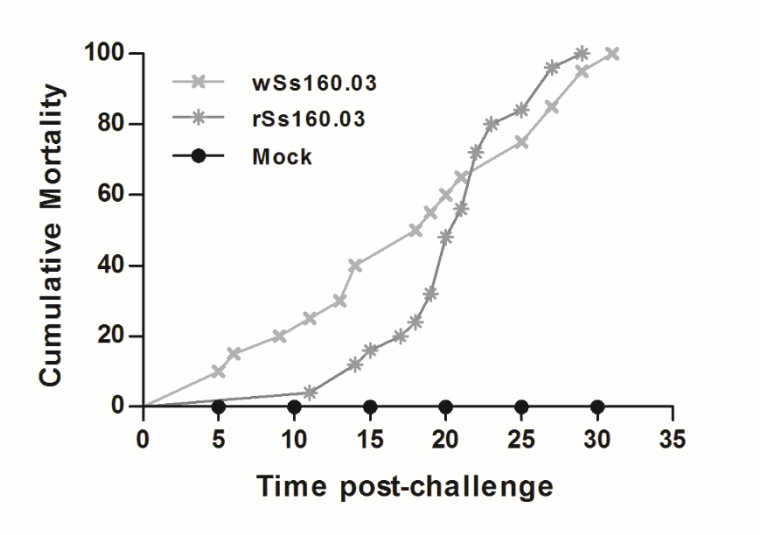


Figure 5. Percent cumulative mortality caused by wild-type Ss160.03 and the rSs160.03. Juveniles were infected by bath (10^5 $TCID_{50}$ ml^{-1}) with the wild-type Ss160.03 (cross) and the rSs160.03 (triangle) recovered by reverse genetics. Fish were kept at 22 °C.

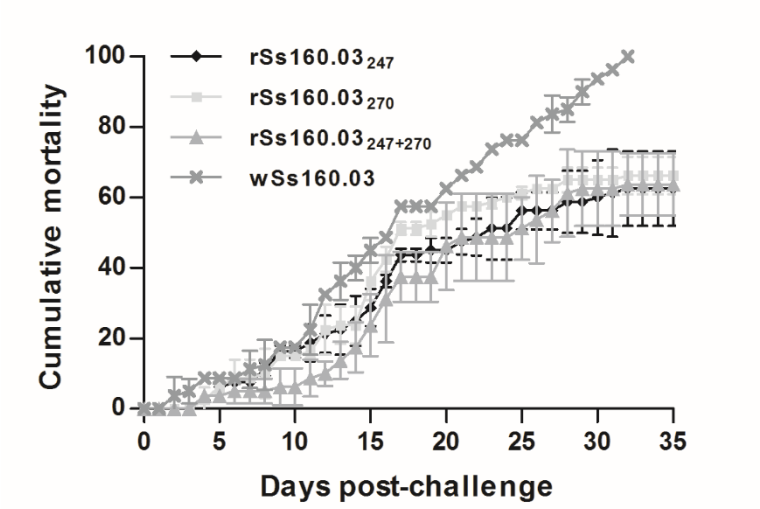
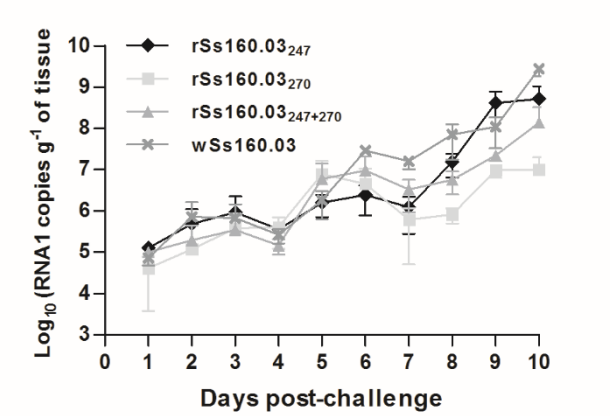


Figure 6. Percent cumulative mortality caused by wild-type Ss160.03 and the three mutant strains. Senegalese sole were bath-challenged with 10^5 $TCID_{50}$ ml^{-1} of wild-type Ss160.03 (cross, positive control) and the different rSs160.03 strains recovered by reverse genetics $rSs160.03_{247}$ (rhombus), $rSs160.03_{270}$ (square) and $rSs160.03_{247+270}$ (triangle). Data presented are the mean of two replicates. Fish were kept at 22 °C.



593

594 Figure 7: Time course of the viral replication in brain. RNA1 replication in brain tissues
595 of the sole infected with rSs160.03$_{247}$ (*rhombus*), rSs160.03$_{270}$ (*square*), rSs160.03$_{247+270}$
596 (*triangle*) and wSs160.03 (*cross*). Numbers on the *y* axis represent the $\log_{10}$ of the
597 number of RNA1 copies detected by qRT-PCR per gram of brain tissues and the
598 numbers on the x axis indicate the days post-challenge. Fish were infected by bath at a
599 final concentration of 10^5 $TCID_{50}$ ml^{-1} and maintained at 22 °C. Samples were analysed
600 in pools of five fish.

601

TABLES

Table 1. Oligonucleotides primers used in in this study. Restriction enzyme sites are in bold; mutated nucleotides are underlined

Primer	Sequence	Segment
RACE specific primers		
5'FchR1	GGTGTTATGTGCTCGCGGCTCTTT	RNA1
3'FchR1	GCAAAGAGCCGCGAGCACATAACACC	RNA1
5'FchR2	GGCGACGACTGCACCACGAG	RNA2
3'FchR2	TTGAGACACCTGAGGACACCACCGCT	RNA2
Full-length cDNA copies of genomic RNA1 and RNA2		
5T7R1	GGATCC**CCGCGG**TAATACGACTCACTATAGGTAACATCACCTTCTTGCTCTG	RNA1
5T7R2	GGATCC**CCGCGG**TAATACGACTCACTATAGGTAATCTAACACCGCTTTGCAAG	RNA2
3*Nae*R1	**G**CCGGCCGAAGCGTAAGACAGCATAAAGC	RNA1
3*Nae*R2	**G**CCGGCCGAGTATTGTAGCGATCAGCGGCC	RNA2
Site direct Mutagenesis		
MUTaa247	CCATTCTCTTGGGC**G**CGACCCAACTCGACCTCGCTCCTGC	RNA2
MUTaa270	GCCCATTGACTACA**A**TCTTGGAGTGGGCGACGTCGACCGG	RNA2
3RevR1	CATTTGGTGCCGATTGC**G**GGCACTGGCCGTCCC	RNA1
3RevR2	GCGATTGACGTCACCTGGCCG**C**CTGGTACTCCTGTGTG	RNA2
Specific primers pair for viral identification and sequencing		
F7/R7	ATATCACGATGAGTTCACTA/ CGAGTCAACACGGGTGAAGA	RNA1
F2/R3	CGTGTCAGTCATGTGTCGCT/ CGAGTCAACACGGGTGAAGA	RNA2
M13F	GTTTTCCCAGTCACGAC	pGem-T
M13R	CAGGAAACAGCTATGAC	pGem-T

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