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

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We previously reported that betanodavirus reassortant strains [redspotted grouper nervous necrosis virus/striped jack nervous necrosis virus (SJNNV)] isolated from Senegalese sole (*Solea senegalensis*) exhibited a modified SJNNV capsid amino acid sequence, with changes at aa 247 and 270. In the current study, we investigated the possible role of both residues as putative virulence determinants. Three recombinant viruses harbouring site-specific mutations in the capsid protein sequence, rSs160.03247 (S247A), rSs160.03270 (S270N) and rSs160.03247+270 (S247A/S270N), were 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 titres similar to the WT 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 three groups in contrast with the WT virus which killed 100% of the fish. These data demonstrated that aa 247 and 270 play a major role in betanodavirus virulence although when both mutated aa 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 – a pathological condition observed in a great number of fish species from different geographical areas (Munday *et al.*, 2002). Betanodavirus infection is associated with histopathological lesions such as vacuolization in the brain and retina. The clinical signs depend on fish species, biological stage, phase of the disease and temperature. As a general remark, infected larvae usually have hyperacute disease leading to loss of appetite and death. Juveniles at high water temperature are prone to acute disease characterized by abnormal swimming behaviour. Members of the genus *Betanodavirus*, family *Nodaviridae*, are non-enveloped icosahedral viruses consisting of a bisegmented, positive-sense ssRNA 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 synthesized 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), redspotted grouper nervous necrosis virus (RGNNV) and barfin flounder nervous necrosis virus (BFNNV) based on a variable sequence of the RNA2, called the T4 region (Nishizawa *et al.*, 1997). However, isolation of reassortant strains between RGNNV and SJNNV genotypes from sole, sea bream and sea bass has been increasingly reported in recent years in the south of Europe (Névez *et al.*, 2005; Olveira *et al.*, 2009; Panzarini *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 (Névez *et al.*, 2005; Olveira *et al.*, 2009; Panzarini *et al.*, 2012).

Sequence analysis of three reassortant strains (RGNNV/SJNNV) isolated from Senegalese sole (*Solea senegalensis*)
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 at aa 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, SpSs-IAusc160.03 (hereafter called Ss160.03), was proven to be highly virulent for Senegalese sole, causing 100% mortality in juveniles challenged by immersion (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 aa 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 5' and 3' RACE based on the sequences from Olveira et al. (2009). Complete sequences were published, updating the previous sequences (GenBank accession numbers FJ803911 and FJ803923). On the RNA1 segment, 3 and 50 nt were missing at the 5' and 3' end, respectively. On the RNA2 segment, 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 number NC_008040) and the RNA2 sequence was compared with the sequence of the strain SJNNV-type SJ93Nag (GenBank accession number AB056572) (Fig. S1, available in the online Supplementary Material). No difference in either RNA1 or RNA2 was evidenced at the 5' ends. In contrast, differences were found at the 3' end at nt 3073 (C instead of T) and 3093 (T instead of C) in RNA1, and two differences were found at the 3' end at nt 1408 (T instead of C) and 1411 (A instead of T), reflecting a polymorphism at those positions. At nt 3093 of RNA1, the genome of Ss160.03 contains a T, which differs from the C in the SJ93Nag and SGWak97 strains. Full-length RNA1 and RNA2 cDNA segments were cloned in the pBSRibo77 plasmid (Fig. 1 and Methods). Sequences of the final constructs pBS160R1 and pBS160R2 (see Methods) were identical to the Ss160.03 genome sequence published previously (Olveira et al., 2009).

Recovery of recombinant Ss160.03 viruses

Recombinant Ss160.03-derived viruses were recovered from BSRT7/5 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 and used to infect fresh E-11 cells. At 48 h post-infection (p.i.), cells were fixed and analysed by immunofluorescence assays to confirm the successful recovery of the various recombinant viruses. A typical result of such an assay is presented in Fig. 2 (a, b). Fig. 2(c) shows the reverse transcription (RT)-PCR products generated from the supernatants of E-11 infected with the WT and recombinant Ss160.03 viruses after two passages. As expected, a band at 427 bp was observed for both viruses, confirming the successful recovery of the recombinant Ss160.03 virus. No product was amplified when reverse transcriptase was omitted from the reaction mixture. The sequence analysis of the RT-PCR products generated from supernatants of E-11 cells infected with the three Ss160.03 recombinant viruses after two passages confirmed the presence of the expected mutations in the RNA2 genome at nt 766 (rSs160.03_247, rSs160.03_247+270) and 836 (rSs160.270, rSs160.03_247+270) (Fig. 3). The mutagenesis led to the substitution of two amino acid residues compared with the sequence of SJ93Nag (GenBank accession number AB056572). It involved one nucleotide change per amino acid substitution: TCG to GCG leading to a substitution of serine to asparagine at aa 227. Virus titres obtained from passage 2 were 1.8 × 10^8 TCID50 ml^-1 for rSs160.03_247, and 3.2 × 10^7 TCID50 ml^-1 for both rSs160.03_270 and rSs160.03_247+270. rSs160.03 showed maximum virus production (1.8 × 10^9 TCID50 ml^-1).

Growth curve kinetics of all viruses

At 24 h p.i., all viruses showed very similar titres in E-11 cells (Fig. 4). After 48 h, a difference of 1 log titre was observed between rSs160.03_247 and rSs160.03_270. At 72 h p.i., the difference between these two viruses remained slightly reduced. However, at the end of the experiment all the viruses showed titres reaching 10^7 TCID50 ml^-1. No differences in the development of the cytopathic effect could be observed amongst the viruses.

In vivo virulence of the WT versus recombinant Ss160.03 viruses

In order to compare the virulence of the recovered recombinant Ss160.03 viruses with the WT (wSs160.03), Senegalese sole were infected by bath immersion. Fish mortality started at day 5 p.i. for the group challenged with wSs160.03, whilst mortality was delayed to day 11 in the group infected with rSs160.03. However, at day 30, mortality rates reached 100% in both groups (Fig. 5). Abnormal behaviour and lack of appetite were observed in both groups. Statistical analysis indicated that there was no difference in virulence amongst the WT Ss160.03 and the recombinant virus (P>0.05).
In vivo virulence of each mutated recombinant Ss160.03 virus

Mortality in all fish groups was first recorded between 2 and 4 days p.i. At the end of the experiment, 35 days p.i., the cumulative percentage mortality reached 100% in the positive control group (wSs160.03), whereas ~40% of the fish challenged with the three mutants, rSs160.03247, rSs160.03270 and rSs160.03247+270, survived the infection (Fig. 6). Characteristic clinical signs of VER (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 wSs160.03,
whereas in the other groups these clinical signs were only observed during 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. Statistical analysis of the mortality curves revealed that the pairwise comparison between each mutant group and the WT Ss160.03 group showed a significant difference in virulence ($P<0.002$). However, there were no significant differences ($P>0.05$) in virulence amongst 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 of the three mutant viruses and the WT strain $wSs160.03$ to infect brain tissue, 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 p.i. in the brain (Fig. 7). The four viruses replicated in a similar way during the first 5 days. However, from day 6 the genome copy number of $wSs160$ was clearly higher than that of the mutants. At day 6 p.i., a difference in the viral

**Fig. 2.** Confirmation of the recovery of the $rSs160.03$ virus. UV microscopy observation following immunofluorescence assay using a mAb directed against the coat protein in E-11 cells. (a) $rSs160.03$-infected cells 48 h p.i. at the first passage. (b) Immunofluorescent labelling of the infected cells. Green, recombinant virus on infected cells; blue, nuclear DAPI staining. (c) Visualization 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, RT-PCR negative control (DEPC-treated distilled water); lane 3, positive control WT Ss160.03 ($wSs160.03$); lane 4, PCR (reverse transcriptase omitted). Scale bars=$50 \mu m$. 

![Image](https://example.com/image.png)
load of ~1 log with rSs160.03 247 and rSs160.03 270 was observed, and at day 10 p.i. the difference was especially high for rSs160.03 270 (>2 log).

**DISCUSSION**

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

In this study, we analysed the role of two residues (aa 247 and 270) as putative host and virulence determinants of betanodavirus reassortant strains. For that purpose, recombinant betanodaviruses were produced in transfected BSR77/5 cells by a DNA-based reverse genetics system as described previously (Takizawa et al., 2008). This approach has been widely used to identify the determinants of

**Fig. 3.** Site-specific mutations in the RNA2 ORF sequence of recombinant Ss160.03 viruses. Sequence electropherograms are shown for RT-PCR products obtained from control virus WT Ss160.03 (wSs160.03), and recombinant viruses rSs160.03 247, rSs160.03 270 and rSs160.03 247+270. The nucleotide positions correspond to the complete Ss160.03 RNA2 sequence. The box contains the mutated positions. Asterisks indicate mutated nucleotides.

**Fig. 4.** Replication kinetics of the recovered viruses. E-11 cells were infected with recombinant viruses rSs160.03 247, rSs160.03 270, rSs160.03 247+270 and rSs160.03, and WT Ss160.03 (wSs160.03) at m.o.i. 0.1. Aliquots of the culture supernatants were taken at the indicated time points and titrated in duplicate. Data are plotted as mean ± SD.
virulence in RNA viruses, such as influenza virus (Horimoto & Kawaoka, 1994; Lycett et al., 2009), Lujo virus (Bergeron et al., 2012), West Nile virus (Diamond, 2009), alphanodavirus (Ball & Johnson, 1999) and fish RNA virus (Biacchesi, 2011).

The rSs160.03 virus was successfully recovered by reverse genetics. The recovered virus presented similar replication in E-11 cell culture compared with the WT after two passages (not shown). The virulence of rSs160.03 was tested in vivo using the natural host species, Senegalese sole. Pathogenicity and virulence were demonstrated to be similar to the WT virus in experimental infection, although a delay was observed in the first days p.i. The three mutants, rSs160.03_{247} (S247A), rSs160.03_{270} (S270N) and rSs160.03_{247+270} (S247A/S270N), were readily recovered and amplified in E-11 cells.

When the effect of the amino acid substitutions on fish pathogenicity was evaluated, typical clinical signs of betanodavirus infection were observed in all groups, questioning that aa 247 and 270 may act as host determinants. However, clinical signs were only observed from day 3 to 15–20 p.i. in the groups infected with the mutant viruses, whereas in the fish infected with the WT virus these signs lasted throughout the experiment (35 days). In addition, in the fish challenged with the mutant viruses, when clinical signs disappeared, mortalities ended shortly after and ~40% of the fish survived. However, clinical signs were only observed from day 3 to 15–20 p.i. in the groups infected with the mutant viruses, whereas in the fish infected with the WT virus these signs lasted throughout the experiment (35 days). In addition, in the fish challenged with the mutant viruses, when clinical signs disappeared, mortalities ended shortly after and ~40% of the fish survived. However, in fish challenged with the WT strain, mortalities reached 100%. The lower cumulative percentage mortality induced by mutant viruses could be due to a slower spread in the fish host as supported by the viral replication data from the brain of infected Senegalese sole. Indeed, throughout the 10 days of the experiment, a gradual and continuous increase of the WT virus RNA1 copy number was observed, whilst the RNA1 copy number for the mutant viruses reached a plateau at day 5. On the basis of the mortality curve data it would be expected that all the individuals challenged with the WT virus should be infected. In the case of the fish challenged with the mutants, with a observed mortality of 60%, it could be expected that four out of 10 individuals would have no or few virus, but we cannot rule out the possibility of horizontal transmission (fish to fish or through the water), which could lead to infection of the whole group. This hypothesis is supported by the fact that the virus was detected in the surviving fish, which also indicates that fish do not clear the virus. A different level of infection could
account for the differences in the genome copy number between the WT strain and the mutant group, but would not explain the differences observed amongst the mutants. It has been previously shown that the region from aa 217 to the C terminus is displayed at the outer surface of the capsid and therefore may be involved in the interaction with the cell surface (Tang et al., 2002). It can be speculated that these changes (aa 247 and 270) at the viral surface might modify the affinity of the virus for cellular receptors, thus affecting the kinetics of virus spread. As an example, it has been reported that a single residue at aa 226 of the haemagglutinin receptor binding domain of influenza virus could change the binding recognition of sialic receptors (Zhang et al., 2013). Moreover, the efficiency of virus spread has been reported to be a critical determinant for neurovirulence for different animal viruses, such as polytropic murine retrovirus (Robertson et al., 1997), rabies virus (Dietzschold et al., 1985) and sindbis virus (Lee et al., 2002). It is interesting to note that when both mutated aa 247 and 270 were present, corresponding recombinant virus was not further attenuated. Both residues are probably located in the surface-promoting domain of the capsid reported to form 60 protrusions (Tang et al., 2002), but their relative position and orientation is unknown. It can be speculated that conformational changes related to mutation could account for the results obtained when both amino acids were mutated. However, further studies will be necessary to determine their position in the capsid and also their involvement in the interaction with cell receptors.

The results obtained from the in vitro replication experiments indicated there was no effect of the amino acid change on the growth curve of the viruses when compared with rSs160.03 and WT. As SSN-1 and E-11 cells are susceptible to the four betanodavirus genotypes, we would not expect any difference in the replication on E-11 cells of rSs160.03247, rSs160.03270 and rSs160.03247+270 as the change of one or two amino acids makes Ss160.03 more similar to the SJNNV genotype. The differences in the in vivo experiments might be due to the receptors on the cell surface. It has been reported that sialic acid is involved in the binding of betanodavirus to SNN-1 cells (Liu et al., 2005) and therefore E-11, which are a clone of SSN-1 (Iwamoto et al., 2000). Although there is no data regarding the neuronal receptors that mediate entry of betanodavirus in fish brain, it is quite probable that the neuronal receptors are different from those present in SSN-1, because this cell line was derived from a whole snakehead fry tissue (Frerichs et al., 1991). For example, it has been reported that most of the fixed rabies virus laboratory strains have acquired the ability to use ubiquitous receptors that are present at the surface of non-neuronal cell types, which are different from the neuronal receptors that they use to propagate in the nervous system (Seganti et al., 1990).

In conclusion, we have demonstrated that two residues in the C terminus of the capsid protein play a major role in betanodavirus pathogenicity. Virulent natural reassortant strains have a serine at both aa 247 and 270. Attenuated recombinant viruses were generated when these amino acids were substituted by alanine and asparagine at aa 247 and 270, respectively. Nevertheless, other motifs must be involved in virulence because these changes were not sufficient to yield a totally attenuated virus. Oliveira et al. (2009) described a third change in the capsid protein sequence of the reassortant strains located in the N-terminal region (aa 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 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 Ss160.03 (SpSs-IAusc160.03; Oliveira et al., 2009) – a reassortant strain isolated from diseased Senegalese sole with a genome consisting of 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 U ml⁻¹), streptomycin (100 mg ml⁻¹) and 2% FBS (Lonza), and maintained at 25 °C.

The BSRT7/5 cells kindly provided by Dr K. K. Conzelmann (Ludwig-Maximilians-Universität Munich) (Buchholz et al., 1999) were grown in a 5% CO₂ 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 U ml⁻¹) and streptomycin (100 mg ml⁻¹). Cells were incubated in culture medium containing Gentamicin (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 numbers FJ803911 and FJ803923 (Oliveira 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 RACE using a 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 a Poly(A) Tailing kit (Ambion). First-strand cDNA was synthesized using the supplied 3′ RACE Adaptor. For both ends, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. The cDNA was then subjected to PCR with 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, cloned using a pGEM-T Easy Vector system (Promega), transformed into Escherichia 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 prepared independently. Viral RNA
from purified virus was used as the template to generate the full-length cDNA. RNA1 and RNA2 were synthesized using SuperScript III Reverse Transcriptase (Invitrogen) using random primers. The cDNA was then amplified using Platinum Pfx DNA Polymerase III Reverse Transcriptase (Invitrogen) using random primers. The length cDNA. RNA1 and RNA2 were synthesized using SuperScript II restriction enzyme sites are in bold; mutated nucleotides are underlined.

**Table 1. Oligonucleotide primers used in this study**

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<th>Primer</th>
<th>Sequence</th>
<th>Segment</th>
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<td><strong>RACE-specific primers</strong></td>
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<tr>
<td>5’TchR1</td>
<td>GTTGTATGCTCGCGGC</td>
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<tr>
<td>3’TchR1</td>
<td>GCAAAGCCCGGCAACATAAACC</td>
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<td><strong>Full-length cDNA copies of genomic RNA1 and RNA2</strong></td>
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<td><strong>Site directed mutagenesis</strong></td>
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<td><strong>Specific primers pairs for viral identification and sequencing</strong></td>
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<tr>
<td>M13R</td>
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**Construction of pBS160R2_247, pBS160R2_270 and pBS160R2_247+270.** To construct the mutants pBS160R2_247, pBS160R2_270 and pBS160R2_247+270, nucleotide substitutions were introduced in the triplets that codified position 247, position 270 and both positions, respectively (amino acid capsid numbering). The plasmids were generated using a QuikChange Multi Site-Directed Mutagenesis kit (Agilent) according to the manufacturer’s instructions using 100 ng 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 a CEQ Dye Terminator Cycle Sequencing Quick Start kit (Beckman Coulter) in a CEQ 8000 Genetic Analysis system (Beckman Coulter). Sequences were confirmed once by sequencing upstream with primer F2 and downstream with primer R3 (Table 1). The sequences were analysed using Lasergene version 7.1, SeqMan II and MegAlign (DNASTAR).

**RT-PCR of genomic RNA from recombinant viruses.** The RNA genome of recombinant virus was directly extracted from supernatant P2 using an 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 cDNA synthesis and the Gotaq Polymerase (Fromega) for PCR. The pairs of primers used, F2/R3 for RNA2 and F7/R7 for RNA1, are listed 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 rSs160.03. At 48 h p.i., cells were fixed with a mixture of ethanol/acetone (1:1) at –20 °C for 20 min and washed with PBS. Primary mouse mAb against the coat protein of Ss160.03 (Abmart) was incubated in PBS/Tween 0.05 % for 1 h at room temperature and washed three times with PBS/Tween 0.05%. Cells were then incubated with FITC-conjugated anti-mouse immunoglobulins (F0257; Sigma) in PBS/Tween 0.05% for 1 h at room temperature in the dark. Cells were stained with DAPI (Sigma-Aldrich) according to the manufacturer’s instructions and visualized with a UV-light microscope (Nikon).

Growth curve of the various viruses. E-11 cells in 24-well plates were infected with the viruses wSs160.03, rSs160.03, rSs160.03 247, rSs160.03 270 and rSs160 247 at m.o.i. 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 p.i.: 24 h, 48 h, 72 h, 5 days and 7 days. The supernatants were titrated in duplicate on E-11 cells by the end-point dilution method on 96-well plates. TCID 50 was estimated by the method of Reed & Muench (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 log-rank test (Ahn & Anderson, 1995). In total, 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. Oxygen, nitrogen compounds, pH and salinity were monitored continuously during the experiment. 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 (Cutrin 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 WT Ss160.03 (wSs160.03) strain and the recombinant virus recovered by reverse genetics. Two groups of 25 fish were infected with wSs160.03 and rSs160.03, and a third group was mock infected with L-15 medium (10 fish). The RNA1 copy number was quantified by SYBR Green real-time PCR using a CFX96 Real-Time PCR Detection system (Bio-Rad) with the primers SnodR1 F/R (Oliveira et al., 2013) and according to the manufacturers’ instructions. To prepare the standard curve, 20-fold dilutions of a plasmid containing the full-length RNA1of strain Ss160.03 (Abmart) was incubated in PBS/Tween 0.05% for 20 min at 4°C. An aliquot of 0.1 ml of the scraped cell suspension was inoculated onto new cultures. Plates were incubated at 22 °C for 10 days. The homogenates were centrifuged at 2000 g for 20 min at 4 °C. An aliquot of 0.1 ml of each sample was used for RNA extraction, and subjected to RT-PCR and sequencing as described previously (Oliveira et al., 2009). The supernatants obtained from brain homogenates were incubated overnight at 4 °C and inoculated (diluted at 1:10 and 1:100) onto 48-well plates of semi-confluent monolayers (~80% confluence) of E-11 cells. After 10 days, samples were subcultured by inoculating 0.1 ml of the scraped cell suspension onto new cultures. Plates were observed daily for cytopathic effect detection. Non-infected cells were used as controls. Identity of viral isolates was confirmed by RT-PCR.

Characterization of replication in vivo. Four groups of sole (mean weight 2 g, 100 fish per group) were infected as described above with either wSs160.03, rSs160.03 247, rSs160.03 270 or rSs160 247 by bath immersion (10⁴ TCID 50 ml⁻¹) at 22 °C. A negative control group of 10 fish was mock-infected (L-15). Ten fish from each group were randomly sampled daily from day 1 to 10 p.i. 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 a CFX96 Real-Time PCR Detection system (Bio-Rad) with the primers SnodR1 F/R (Oliveira et al., 2013) and according to the manufacturers’ instructions. To prepare the standard curve, 20-fold dilutions of a plasmid containing the full-length RNA1of strain Ss160.03 (Abmart) were prepared up to one viral copy in DEPC water. Viral load data were calculated as RNA1 copies per gram of brain tissue.

Statistical analysis. Results from experimental infection in fish with the recombinant viruses were analysed using GraphPad Prism 5.0. P<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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REFERENCES


S. Souto and others


