Aquabirnavirus polyplody: a new strategy to modulate virulence?

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One of the main research issues regarding infectious pancreatic necrosis virus (IPNV) is its virulence mechanisms. The basis for understanding the molecular virulence determinants of this virus was established over the last decade when it was demonstrated that certain amino acid domains in the VP2 and VP2–NS inter-region determined the level of virulence of IPNV. However, certain variability was still inexplicable and therefore other factors may also be involved. To this end, it was demonstrated recently that infectious bursal disease virus (IBDV), a virus in a different genus of the same family as IPNV, can package more than two dsRNA segments, and that polyplody may be associated with virulence. In the present report, we analysed the viral fractions obtained after gradient centrifugation to demonstrate that IPNV virions can also package more than two segments, thus indicating that polyplody is a common birnavirus trait. The differential replication ex vivo and virulence in vivo additionally suggested that such a characteristic is involved in the modulation of virus infectivity. However, although the ex vivo results clearly demonstrated that the replication capacity was enhanced as the viral ploidy increased, the in vivo results could not strongly support a direct relationship between ploidy and virulence to the host, thus suggesting that other virulence determinants are also involved.

INTRODUCTION

Aquatic birnaviruses, including infectious pancreatic necrosis virus (IPNV), have been detected and isolated from both asymptomatic and acutely diseased specimens belonging to an extensive range of aquatic animals worldwide. IPNV belongs to the genus Aquabirnavirus and Infectious pancreatic necrosis virus is the prototype species of the family Birnaviridae. IPNV particles are non-enveloped icosahedrons (T=13 1veno symmetry) of ~70 nm in diameter enclosing a bisegmented dsRNA genome (Lightner & Post, 1969; Cohen et al., 1973; Dobos, 1977). Segment A, which comprises nt 2962–3097 depending on the virus strain, contains two partially overlapping ORFs. The smaller one, located at the 5’ end, encodes VP5, a non-structural polypeptide (Duncan et al., 1987; Magyar & Dobos, 1994; Dobos, 1995a). VP5 is not essential for virus replication and, although it seems to be associated with virulence, its specific function remains to be characterized (Santi et al., 2005). The larger ORF encodes a polyprotein that, through co-translational proteolytic processing, yields pVP2 (the precursor of the VP2 capsid polypeptide), VP3 and VP4 polypeptides. VP2, the capsid protein, is related to virulence (Heppell et al., 1995; Blake et al., 2001; Santi et al., 2004). VP3 is a modular, self-oligomerizing protein that interacts with dsRNA and VP1, thus suggesting its involvement in RNA replication and genome packaging. VP3 also acts as a scaffolding protein, contributing to the correct assembly of the pVP2 capsomers (Pedersen et al., 2007). VP4 is the autocatalytic protease responsible for polyprotein cleavage. Segment B (~2900 bp) contains one ORF encoding VP1, the viral RNA-dependent RNA polymerase (Duncan et al., 1991; Dobos, 1995b).

Aquabirnaviruses were initially grouped into 10 serotypes (Hill & Way, 1995), with a certain relationship to the geographical origin. The genotyping of these viruses by Blake et al. (2001) also demonstrated some correlation between serotyping and the geographical origin of the strains. However, attempts to correlate the strain type with the level of virulence were fruitless; only with two strains, West Buxton (WB; serotype A1, genotype 1) and Abild (Ab; serotype A3, genotype 3), has a marked relatedness to high and low virulence, respectively, been presumed, at least for salmonid fish (Vesterga˚rd Jorgensen & Grauballe, 1971). However, in challenge studies performed by our group with different variants of WB and Ab strains, a certain variability in virulence levels was observed that could not be explained by either the type or the origin of the strain (Lago et al., 2010). Santi et al. (2004), studying the variable virulence of the Sp strain...
(serotype A2, genotype 5) demonstrated that these changes could be due to sequence variations in specific regions of the VP2 capsid protein. Other authors (Lago et al., 2010, 2013) suggested that segment reassortment could also contribute to the modulation of the virulence in these types of viruses.

Recently, data gathered on infectious bursal disease virus (IBDV), the only known member of the genus Avibirnavirus, have suggested the existence of an as-yet-unexplored biological feature that might significantly affect birnavirus virulence. Work by Luque et al. (2009) demonstrated that most IBDV infectious particles harbour more than a single genomic equivalent, and suggested that polyploidy could influence virus virulence. In the present study, we have shown that IPNV virions are also polyploid, thus indicating that polyploidy is a common birnavirus trait. Additionally, we assessed the impact of IPNV polyploidy on virus replication *ex vivo* and on virulence *in vivo*.

**RESULTS**

Although the results were similar regardless of the strain assayed, the data provided here are those from the 5B1 E strain, given that more replicates were performed in all assays.

**Composition of CsCl gradient viral subpopulations**

As shown in Fig. 1, CsCl gradient purification of the IPNV tested strains yielded two to six virus bands. The bands occurred at densities of $1.280 \pm 0.021$, $1.300 \pm 0.044$, $1.306 \pm 0.004$, $1.310 \pm 0.05$, $1.314 \pm 0.006$ and $1.323 \pm 0.007 \text{ g ml}^{-1}$ (from top to bottom). Following the notation used by Luque et al. (2009), fractions were named F1 (gradient top) to F6 (bottom). The relative abundance of the different virus fractions was roughly consistent in all experiments. F1 and F2 were consistently under-represented, while F6 always encompassed more than 50 % of the total purified virus. Occasionally, in some batches, the abundance of F1 was higher than all other bands except for F6 (Fig. 1c). It is worth noting that the number of bands visualized was highly dependent on the quantity of virus produced for purification.

Electron microscopy analysis of the different gradient fractions revealed the presence of three main particle species, namely $T=1$ subviral particles (SVPs) (25 nm in diameter), $T=7$ particles (~55 nm in diameter) and $T=13$ (with sizes ranging from 65 to 90 nm), similar to those described previously for IBDV.

The composition of the first two fractions was conspicuously different from the rest (Fig. 2). F1 and F2 comprised mixed populations formed by a high proportion of $T=1$ along with a low proportion of $T=7$ particles, as well as some empty, broken and apparently intact $T=13$ particles. Fractions F3–F6 exclusively contained complete $T=13$ particles with diameters ranging from 65 to 90 nm (as shown in Fig. 3, and described below). The high structural heterogeneity of F1 and F2, when compared with the F3–F6 subpopulations, prevented their use in subsequent stoichiometry analyses.

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**Fig. 1.** Viral fractions (of strain 5B1 E) visualized after ultracentrifugation through a CsCl gradient. A maximum of six fractions was observed and these were named F1–F6 from the lowest to the highest density. The densities of the bands were (mean $\pm$ s.d.) $1.280 \pm 0.021$, $1.300 \pm 0.044$, $1.306 \pm 0.004$, $1.310 \pm 0.05$, $1.314 \pm 0.006$ and $1.323 \pm 0.007 \text{ g ml}^{-1}$, respectively. Panels (a)–(e) show the different number of bands (fractions) visualized among all the purifications performed, which was regardless of the strain but dependent on the quantity of virus purified.
**Protein analysis**

SDS-PAGE revealed that fractions F3–F6 had a similar protein composition (Fig. 4) of VP1 (94 kDa), VP2 (54 kDa), VP3 (31 kDa) and VP4 (24 kDa), although VP4 was only detected by Coomasie blue staining and not by Western blotting. VP1 was not detected in F1 and only faintly visualized after Western blotting in F2. VP3 was only faintly visualized in F1 after Western blotting, and VP4 was not detectable in F1 and F2. It was remarkable that the quantity of VP2 in F1 was at least as high as in F6, considering the extremely low proportion of virions in the first fraction. We must point out that, although the results shown here were for strain 5B1E, the same pattern was observed when the Ab and WB strains were assayed.

**Genomic analysis**

Electrophoresis of the dsRNA from the six virus fractions was performed using fraction samples containing identical protein concentrations. Due to both the low abundance of fractions F1 and F2 and the observed structural heterogeneity detected in F1 and F2, F3 was used as the reference to equalize fractions F3–F6, in terms of protein concentration.

As shown in Fig. 5, the silver-stained gels revealed a proportionally increasing amount of viral RNA from F3 to F6. We assumed that, similarly to what has been shown for IBDV (Luque et al., 2009), F3 viral particles contained two genome segments. We used MultiGauge v.3.0 and ImageJ software to normalize the intensity of both dsRNA bands in the four fractions. The results gathered from the analysis of data corresponding to 15 independent purification batches indicated that the relative amounts of viral RNA in fractions F4, F5 and F6 were 1.58±0.13-, 2.18±0.13- and 3.04±0.33-fold (mean±SD) higher than that of F3, respectively. Analysis of these data indicated that F4 virions contained three dsRNA segments, while F5 and F6 harboured four (two genome equivalents) and six (three genome equivalents) segments, respectively. Interestingly, the relative intensities of the upper band with respect to the lower one in fractions F3–F6 were 1.1, 2.3, 1.2 and 1.2, respectively, which indicated that the majority of virions present in fraction F4 contained two copies of segment A and one of segment B. All these observations were similar, regardless of the IPNV strain assayed.

**Particle size**

Evaluation of the particle size found in each fraction, performed on over 30 virus purification batches, revealed that, although fractions F3–F6 were mostly made up of T=13 particles, there were significant size variations, both inter- and intra-fraction (Fig. 3). In F3, a few small particles of ~55 nm (most likely corresponding to T=7 particles) were visualized, and some of ~80 nm. Most of the particles in this fraction (~85%) were in the 65–75 nm range, although the most frequently visualized size had a mean value of 70.66±1.68 nm. Particles measuring ~55 nm were also observed, although in a low proportion (0.9%), in fraction F4, in which a few 80 nm particles were also present. The diameter of most virions in this fraction (~85%) were in the 65–75 nm range, although the most frequently visualized size had a mean value of 70.66±1.68 nm. Particles measuring ~55 nm were also observed, although in a low proportion (0.9%), in fraction F4, in which a few 80 nm particles were also present. The diameter of most virions in this fraction was ~75 nm (75.67±0.90), as in fractions F5 (75.99±1.43) and F6 (75.86±0.93). Particles of 55 nm were not detected in F5 and F6, where the smallest particles had diameters of ~65 nm. The largest visualized particles in F6 were ~80 nm, a particle size also present in F5. The largest particles (~90 nm) were occasionally detected only in fraction F5. It is worth highlighting that, in contrast to smaller particles, the contour of these large particles has a globose, less icosahedral appearance than their smaller counterparts.
counterparts (Fig. 3e). Similar particle size distributions were found in all tested IPNV strains.

**Differential replication ex vivo**

For this assay, fractions F3–F6 were used directly (no cell-culture propagation was applied before the ex vivo assay). The virus from all four fractions under study (F3–F6) induced the characteristic IPNV cytopathic effect in BF-2 monolayers, with those with a higher number of genome equivalents yielding higher titres: particles from F3 yielded $6.3 \times 10^7$ TCID$_{50}$ ml$^{-1}$ (7.8 ± 0.51, when represented as log of TCID$_{50}$ ml$^{-1}$ ± SD from five replicas); F4 particles provided titres of $8.9 \times 10^8$ TCID$_{50}$ ml$^{-1}$ (8.95 ± 0.37); F5 particles produced $3.2 \times 10^9$ TCID$_{50}$ ml$^{-1}$ (9.5 ± 0.18) and F6 particles produced $1 \times 10^{10}$ TCID$_{50}$ ml$^{-1}$ (10.0 ± 0.88).

**Differential virulence in vivo**

Challenges in fish [sole (*Solea senegalensis*) and salmon (*Salmo salar*)] were performed with fractions from each of the three strains listed above. Only with strain 5B1E was the assay repeated twice with both fish species. Although the results were basically similar in all cases, we present here only those obtained with 5B1E. The challenges with sole were performed for a maximum of 30 days (Fig. 6a). Mortality rates were low; thus, F5 induced a maximum cumulative mortality (CM) of 3 %, while F3 was 7 % and F6 was only 20 %. No deaths were recorded in the group challenged with F4. The fish did not show significant symptoms; however, it should be noted that the fish inoculated with fraction F6 developed anorexia. The virus was reisolated and detected by direct reverse transcription (RT)-PCR plus nested-PCR, as well as by
quantitative real-time RT-PCR (RT-qPCR), from all sampled individuals, both dead and surviving. The cycle threshold ($C_t$) values observed in RT-qPCR ranged from 19 to 31 in dead fish and from 36 to 39 in surviving individuals.

The salmon challenge experiment was performed in two stages. In an initial stage, no fish died (and no symptoms were recorded) for 20 days post-infection (p.i.) with any of the virus fractions. After a second dose with the corresponding fraction, mortalities were recorded over an extra 20-day period (Fig. 6b). Again, F4 yielded an extremely low CM (4 %) and F5 was just 17 %. Fish challenged with fraction F3 showed an intermediate mortality rate of 57 % at 14 days p.i., and fraction F6 yielded the highest rate (100 %) at day 15 p.i. The virus was reisolated and detected by RT-PCR plus nested-PCR, and by RT-qPCR from both dead and surviving fish. The $C_t$ values observed in RT-qPCR ranged from 18 to 23 in dead fish, and from 34 to 38 in surviving individuals. Some of the fish inoculated with F6 and (at a lower proportion) with F3 showed the classic symptoms of dark pigmentation, exophthalmia and abnormal swimming (data not shown).

**DISCUSSION**

At present, there are relatively few studies on the virulence factors of IPNV, and they are mainly focused on the connection of virulence to specific regions and residues of the genomic sequence (Sano et al., 1992; Bruslind & Reno, 2000; Santi et al., 2004; Shivappa et al., 2004; Song et al., 2005). Another well-known determinant of virulence is reassortment (Schrauwen et al., 2014), which has been suggested to be responsible for the existence of virulent variants among other viruses (Cárdenas et al., 2014). Although some studies conducted by our group with IPNV have suggested a possible implication of reassortment in the modulation of virulence (Lago et al., 2010), the poor reproducibility of results obtained in some challenge
trials led us to suspect that there had to be some additional strain-related virulence determinants.

It has been reported that the avibirnavirus IBDV is polyploid (Luque et al., 2009). These authors also showed that such characteristics played a role in modulation of virus infectivity through the packaging of different numbers of genome copies. Polyploidy has also been found in several non-icosahedral virus families (e.g. families Paramyxoviridae, Orthomyxoviridae and Filoviridae), which use it to modulate their virulence (Enami et al., 1991; Rager et al., 2002; Beniac et al., 2012). Therefore, our first objective was to determine whether aquabirnaviruses could also be polyploid. In the present study, the visualization of higher numbers of bands in CsCl gradients was clearly related to the original quantity of virus: the patterns of bands 5 and 6 were only obtained when high-titre virus stocks were used for purification. Among the six fractions, only F1 and F2 showed a protein composition clearly consistent with that reported for SVPs (Coulibaly et al., 2010), which were most represented in both fractions. The expected protein pattern of IPNV virions was obtained in the remaining four fractions, corresponding to complete T=13 icosahedral particles. This was why only these four populations were considered for the stoichiometry analysis of the genomic composition. The results – equalizing the quantity of virus among the fractions – clearly demonstrated that populations with higher density incorporated larger amounts of viral genome. Using F3 as the reference population, and hypothesizing that its particles would incorporate a single set of segments A and B (one genomic equivalent), the IPNV virions would be able to incorporate up to three genome equivalents (three copies of both

**Fig. 6.** Curves of cumulative mortality in challenges performed with 5B1e fractions on sole (*Solea senegalensis*) (a) and salmon (*Salmo salar*) (b). The curve for salmon represents the days post-infection (p.i.) after the secondary inoculation of the fraction.
segments in fraction F6). F5 virions would have two sets of segments (two genomic equivalents) and, interestingly, the genome of virions in fraction F4 would be comprised of two copies of segment A and a single copy of segment B (1.5 genomic equivalents). In a previous report by Luque et al. (2009), the authors demonstrated that IBDV virions could package up to four copies of dsRNA corresponding to two genome equivalents, and that all the fractions showed equimolar amounts of both segments. It could be argued that, like Luque et al. (2009) with IBDV, we should have considered the F2 fraction as the reference, which would have yielded a similar result of maximum numbers of genome equivalents for both viruses. However, unlike IBDV, the F2 IPNV fraction was not composed of T=13 virions but mostly by SVPs. Nevertheless, even considering the hypothetical case that the very few T=13 particles present in the F2 fractions were composed of two genome segments and that virions in fractions F3–F6 packaged increasing numbers of genome equivalents, to our understanding the important fact is simply that IPNV, like IBDV, is also polyploid.

Polyploidy can represent a problem when it comes to packaging the genome during the morphogenesis as, a priori, it needs the enlargement of the ‘container’, which is a well-known phenomenon in eukaryotic cells (Comai, 2005). This fact does not represent a problem for helical viruses such as those mentioned above, as they are more permissive with regard to genome equivalent variations. Furthermore, in the case of filovirus, the particle can modify its length to incorporate up to 22 genome copies (Beniac et al., 2012). However, this fact is not always related to an increase in virulence, as it has been demonstrated that high virulence is sometimes associated with shorter virus filaments (Kiley et al., 1982).

The increasing of the shell volume has tight constraints in icosahedral viruses, whose capsids contain a well-defined cargo space. In this regard, it was reported that IBDV is able to encapsidate four dsRNA copies without being associated with an increase in its size (Luque et al., 2009). This may be possible because these viruses have a much larger capsid than is necessary to enclose a single genome copy (Castón et al., 1997).

In the present study, IPNV virions were able to enclose up to at least three copies of the genome. However, in contrast to IBDV, IPNV virions do increase their size when they incorporate more than one genome equivalent. It has been reported that the aquabirnavirus capsid varies from 55 to 74 nm, with 60 nm being the most frequent size (Lightner & Post, 1969; Moss & Gravell, 1969; Kelly & Loh, 1972; Chang et al., 1978). However, our results showed that 60–65 nm particles were present – in variable but low percentages – in most fractions and that the most frequent sizes were ~70 nm (in F3) and 75 nm (in the remaining three populations). An increasing number of 80 nm particles were observed from the F3–F6 fractions and some particles measuring ~90 nm were only visualized in the F5 population. Considering the mean size of the most frequent particles, the volume of the virions would be approximately 2.720, 2.783, 2.787 and 2.785 nm$^3$ from F3 to F6, respectively. Therefore, a direct relationship between the number of packaged genome equivalents and the volume of the capsid was not observed. We cannot explain our observations. However, they are a fact demonstrated by a large number of measurements from many experiments.

The ex vivo replication study showed that all fractions were functional and able to replicate in BF-2 monolayers. In addition, it was observed that higher genome equivalents were associated with higher yields of viral titres in the infected monolayers. This clearly indicates that polyploidy is related to the infectivity of IPNV, a phenomenon already reported for IBDV (Luque et al., 2009). Regarding the in vivo challenges, we employed two fish species that we have been testing with IPNV-like particles over a long time, and therefore we could expect predictable results. In this regard, in the case of experiments using sole fry, the mortalities were lower than expected from previous experiments. In the case of the challenges performed with salmon, the natural host species of the virus, we used young juvenile instead of fry in order to reduce the expected virulence of the virus, which was demonstrated by the fact that neither morbidity nor mortality was observed 20 days after the first inoculation. Only after a second inoculation of the same fraction were morbidity and mortality recorded, and the virus was detected and reisolated from the fish tissues. Based on the results from the ex vivo experiment, we also expected to observe a clear correlation between the infectivity of each fraction and the number of genome equivalents of the virions in vivo. Unfortunately, only fraction F6 (with three genome equivalents) showed the highest virulence in both species, clearly higher than that of the remaining fractions; F4 and F5 showed very low infectivity in both species, and F3 yielded intermediate levels of CM in both cases.

Further studies must be conducted, combining the three virulence determinants (specific amino acid residues, reassortment and polyploidy) and the host factor, for a better understanding of the pathogenesis of these viruses.

In conclusion, we can say that our results clearly demonstrated that polyploidy also exists among aquabirnavirus, as has been demonstrated previously for avibirnavirus, and that it may represent a mechanism to modulate its infectivity.

**METHODS**

**Cell lines, viral strains and virus titration.** Monolayers of BF-2 cells (Bluegill caudal trunk; ATCC CCL-91) were grown at 20 °C in Eagle’s minimum essential medium supplemented with 10 % FBS, 100 U penicillin ml$^{-1}$ and 100 µg streptomycin ml$^{-1}$. Virus infections were performed at an m.o.i. of 0.1 in 25 confluent BF-2 cell monolayers grown in 150 cm$^2$ flasks. The IPNV strains used in our study were the WB and Ab reference strains, as well as SB1E, which is a
Virus purification. For each purification, at least 20 semi-confluent BF-2 monolayers grown in 150 cm² flasks were employed to propagate each of the three strains. When cytopathic effect was complete, the cell medium was collected and centrifuged at 3000 g for 10 min. The supernatant was incubated for 24 h at 4 °C with 5 % polyethylene glycol 8000 and 0.5 M NaCl. The virus was then pelleted at 8000 g for 30 min and resuspended in PBS buffer [25 mM piperazine-N,N-bis (2-ethanesulfonic acid) (pH 6.2), 150 mM NaCl and 20 mM CaCl₂] supplemented with protease inhibitors (Roche). After pelleting through a 25 % sucrose cushion at 35 000 r.p.m. in an SW55Ti Beckman Coulter rotor for 1 h at 4 °C, the virus was resuspended in 500 µl PBS buffer and loaded onto a CsCl gradient (1.33 g ml⁻¹) and centrifuged for 20 h at 31 000 r.p.m. at 4 °C in a SW32Ti rotor (Beckman Coulter). Virus bands were visualized by means of a light beam projected along the longitudinal axis from the bottom of the tubes. When the expected six virus fractions (named F₁–F₆, from the upper to the lower band) were visualized, they were collected by side puncture and dialyzed against PBS buffer over 24 h at 4 °C. When a lower number of bands was visualized, the assay was rejected.

Analysis of the protein and genomic composition of fractions F₁–F₆. Prior to electrophoresis, the amount of protein in fractions F₃–F₆ was determined using a BCA protein assay kit (Roche). Aliquots of each fraction were adjusted to the same protein concentration, and then subjected to overnight digestion with proteinase K (2 mg ml⁻¹) in the presence of 0.5 % SDS at 37 °C. Afterwards, the samples were denatured at 98 °C for 5 min and subjected to 7.5 % SDS-PAGE (Laemmli, 1970) at 150 V for 4 h. RNA bands were detected by staining with either Red Safe (ABM) or silver stained using a Silver Stain Plus kit (Bio-Rad). The bands were quantified using MultiGauge v3.0 (Fujifilm) and ImageJ software (NIH). Genomic proportions were normalized using the population harbouring a lower concentration of genomic segments (F₃) as a reference.

For the characterization of protein composition, one aliquot of each fraction was denatured by incubation at 98 °C for 3 min and subjected to 12 % SDS-PAGE. Gels were then either stained with colloidal Coomassie blue or subjected to Western blotting using an anti-IPNV Sp type polyclonal antibody and visualized by chemiluminescence (GE Healthcare).

Electron microscopy. Aliquots of 2 µl of each sample were loaded onto carbon-coated grids. After drying, the grids were stained with 2 % aqueous uranyl acetate for 2 min, drained and washed. Micrographs were recorded with a 200 kV FEOL electron microscope operating at 120 kV at a nominal magnification.

Experimental challenge. For the in vivo assays, two fish species were selected: Atlantic salmon (Salmo salar), a species widely recognized as susceptible to the virus, and Senegalese sole (Solea senegalensis), a species of great interest in our area and for which we have demonstrated that certain IPNV strains can produce morbidity and mortality (Lago et al., 2010). Two hundred Atlantic salmon (mean length 7 cm) and 150 Senegalese sole (mean weight 2 g) fry were obtained from commercial fish farms and acclimatized for at least 7 days. Fish were maintained at a maximum density of 100 fish in 100 l aquaria with aeration and were fed ad libitum with a commercial diet. All animals were handled in strict accordance with good animal practices, 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 Animal Welfare and the Xunta de Galicia (Permit ID 15004/13/002). All efforts were made to minimize animal suffering. Temperature, lighting and noise were strictly controlled in order to minimize stress. Prior to the experimental infections, 10 fish of each species were sacrificed with an overdose of the anaesthetic MS-222 (Sigma-Aldrich) and tested by RT-PCR and nested-PCR to rule out the presence of IPNV, viral nervous necrosis virus, viral hemorrhagic septicaemia virus and infectious haematopoietic necrosis virus.

For this assay, the F₃–F₆ fractions were used directly (no cell-culture propagation was applied before the in vivo assay). Fish were injected intramuscularly with 0.1 ml inoculum containing 10⁵ TCID₅₀ of the corresponding fraction or with L-15 medium (control group) and distributed into four groups (40 and 30 individuals per group, in the salmon and sole trials, respectively) named after the inoculated virus fraction (F₃–F₆). Fish were then maintained at 15 °C and mortalities were recorded daily for 40 days in the case of salmon, and for 30 days for infected sole. Dead fish were stored at ~80 °C and subsequently used for virus analysis.

In the salmon experiments, due to the absence of mortality, a second virus injection was applied at 20 days p.i., and fish were sampled (five fish per fraction) at days 30, 33 and 35 p.i. (10, 13 and 15 days after the second infection) with the aim of monitoring virus replication. Surviving and sampled fish were killed by an MS-222 overdose.

Virus detection. From dead and surviving fish, the heart, kidney, spleen and head were sampled aseptically and pooled in sets of five fish. The samples were disrupted and the homogenates diluted 1 : 10 (w/v) in Earle’s balanced salt solution supplemented with 1000 IU penicillin, 1000 µg ml⁻¹ streptomycin, 500 µg gentamicin ml⁻¹ and 10 µg amphotericin (Gibco) ml⁻¹. After centrifugation at 7500 g for 15 min at 4 °C, the supernatants were incubated overnight at 4 °C and then subjected to virus detection by isolation in cell culture, by RT-PCR plus nested-PCR, and by RT-qPCR.

For RT-PCR detection of IPNV, total RNA extraction was performed using a RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. cDNA synthesis was carried out with the SuperScript III First-Strand Synthesis System (Invitrogen). PCR and nested-PCR were carried out using the commercial kit Go Taq Flexi DNA Polymerase (Promega), using primers 5'-AGAGATCAGTACTGTCAGTACGTCAGTACTGAC-3' (forward) and 5'-TTGTCACCACAGGAAATGTAGTGAC-3' (reverse) (Heppell et al., 1992) for PCR, and 5'-AAAGGCAGTGCGGGGCTGAGAGAGG-3' (forward; designed by our laboratory at position 1403 in the genome sequence of the Jasper strain, GenBank accession no. M18049) and 5'-TTGTCACCACAGGAAATGTAGTGAC-3' (reverse) for semi-nested-PCR. Amplified DNA products were run in 2 % agarose gels and visualized with Red Safe (INTRON Biotechnology).

For RT-qPCR detection of IPNV, the total RNA extracted previously as described above was subjected to cDNA synthesis as above. For qPCR amplification, segment B was targeted using primers 5'-CHGCGCCGGCTACACATCATAAGG-3' (forward) and 5'-CCGC-AGCTBAGRTACCAGAATCGG-3' (reverse) at positions 838 and 976 in the Jasper strain (GenBank accession no. M58756), employing a GoTaq PCR Master Mix kit (Promega) with SYBR Green I, as described by the manufacturer. The amplification was performed in a C1000 Thermal Cycler CFX96 RealTime System (Bio-Rad).

For virus isolation in cell culture, homogenate supernatants were diluted 1 : 1 and 1 : 10 in L-15 medium, and inoculated in duplicate on semi-confluent BF-2 monolayers grown in 48-well plates. Plates were maintained at 15 °C and visualized daily to detect cytopathic effect. After 10 days, a new passage was applied and after two positive passages, the presence of IPNV was confirmed by RT-PCR.
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