Comparison of Mortality and Viral Load in Rainbow Trout (*Oncorhynchus mykiss)* infected with Infectious Pancreatic Necrosis Virus (IPNV) Genogroups 1 and 5

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ABSTRACT

Infectious Pancreatic Necrosis Virus (IPNV) is the etiological agent of a highly contagious disease that affects farmed salmonids. IPNV isolates have been phylogenetically classified into eight genogroups, of which two are present in Chile, genogroups 1 and 5. Here we compare the mortality rate caused by isolates from both genogroups in rainbow trout (*Oncorhynchus mykiss*) fry to determine if there is an association between host susceptibility and phylogenetic characterization of IPNV. Fish were challenged by immersion with one of four isolates (two of each genogroup) and mortality curves were assessed after 30 days. Viral load was measured in all mortalities and in live fish sampled at 1, 7 and 20 days post-infection. Although mortality was low throughout the challenge, differences were found between fish infected with different isolates.

Both isolates from genogroup 1, caused greater cumulative mortalities than either of the isolates from Genogroup 5. When combined, the overall mortality rate of fish challenged with genogroup 1 isolates was significantly higher than those infected with genogroup 5. However, viral load was lower on trout infected with genogroup 1 isolates. These results suggest that rainbow trout are more susceptible to IPNV isolates from genogroup 1 than genogroup 5.

KEYWORDS

IPNV, Rainbow trout, Mortality, Viral load, Phylogenetic Classification, Host susceptibility.

INTRODUCTION

Infectious Pancreatic Necrosis (IPN) is a highly contagious viral disease that causes great economic losses to trout and salmon aquaculture worldwide. The etiological agent of the disease, IPN virus (IPNV), a non-enveloped, double-stranded RNA (dsRNA) virus which affects the main salmonid species cultured worldwide, i.e. Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). The disease is transmitted horizontally as well as vertically, and after an outbreak surviving fish can become asymptomatic carriers of the virus, representing a risk to their offspring and other susceptible fish (Bootland et al., 1991). Salmonids are more susceptible to IPNV during their first feeding fry stage in fresh water, where prevalence of the disease is high, and as post-smolts shortly after transfer to the sea (Jarp et al., 1995; Evensen & Santi, 2008). During

an IPN outbreak, mortality rates can vary greatly from insignificant to almost 100%, and these differences have been ascribed to several environmental, viral and host-related factors. Host genetic variability associated to IPNV mortality has been confirmed in Atlantic salmon with the identification of a major-effect quantitative trait loci (QTL), associated with mortality levels in fresh water and seawater (Houston *et al.*, 2008; Moen et al., 2009), and the subsequent identification of epithelial cadherin associated with resistance to IPNV (Moen et al. 2015). In rainbow trout, resistance against IPNV has also shown significant genetic variation (Flores-Mara et al., 2017; Yoshida et al., 2019). Furthermore, a recent study detected a marker which moderately explains the genetic variance for this trait (Rodríguez et al., 2019).

Amino acids 217 and 221 of the IPNV capsid protein, VP2, are associated with differences in virulence and replication rates between isolates (Santi et al., 2004; Song et al. 2005). Thus, IPNV isolates with amino acids Thr_{217} and Ala_{221} are considered highly virulent and Pro_{217} and Thr_{221} as almost avirulent (Song et al., 2005). However, these markers have only been studied in IPNV type Spjarup (Sp, genogroup 5), and do not always correlate with field mortality rates seen in IPN outbreaks (Ruane et al., 2015; Tapia et al., 2015).

IPNV belongs to the genus *Aquabirnavirus*, from the *Birnaviridae* family. Its genome consists of two dsRNA segments: segment A, which encodes a viral capsid protein (VP2), an internal protein (VP3), a viral protease (VP4), and a non-structural protein (VP5); and segment B, which encodes the RNA polymerase (VP1) (Dobos, 1995). *Aquabirnaviruses* were initially classified based on antiserum neutralization assays directed against three IPNV reference serotypes: VR299, Sp and Ab (Macdonald & Gower, 1981). Subsequently, a

standard serological classification scheme was proposed, and the strains were divided into serogroups A and B. The former serogroup contains the majority of IPNV isolates associated with the disease and are grouped into 9 serotypes, A1-A9 (Hill & Way, 1995). More recently, *Aquabirnaviruses* were classified according to their nucleotides and deduced amino acid sequences. Phylogenetic analysis of the coding region of the VP2 protein from 28 aquatic birnavirus isolates revealed that the nine reference strains of serogroup A were grouped into six genogroups, some of which were composed of several genotypes (Blake et al., 2001). These genogroups are highly correlated with the serological classification and geographic origin of the strains, for example, genogroup 5 corresponds to serogroup A2 and includes several isolates from Europe such as Sp, Fr10 and N1, while genogroup 1 correlates to serotype A1 and includes strains from the United States, such as West Buxton (WB), VR-299 and Buhl (Blake et al., 2001). Following this approach, a seventh genogroup comprised only by Japanese aquabirnaviruses was proposed by Nishizawa et al. (2005). An Aquabirnavirus isolated from *Oncorhynchus mykiss* in Australia was classified in an eighth genogroup (McCowan et al., 2015; Mohr et al., 2015).

Chile is currently the largest producer of rainbow trout worldwide (FAO 2019); and it was in this species that IPNV was isolated for the first time in the country (McAllister & Reyes 1984). The isolate corresponded to serotype VR-299 or 1A (Espinoza et al., 1985, McAllister & Reyes, 1984), and it was later classified in genogroup 1 (Eissler et al., 2011). During the late nineties, IPN disease was confirmed in Atlantic salmon farms and since then the virus has spread through the country's different farming areas. Only two IPNV genogroups have been reported in the country, genogroup 1 (North American origin) and genogroup 5

(European origin), with the latter being more dominant and widely dispersed (Mutoloki & Evensen, 2011, Eissler *et al.*, 2011, Calleja *et al.*, 2012; Tapia *et al.*, 2015; Manríquez *et al.* 2017).

More recently, Torres *et al.* (2016) analysed data from Chilean isolates and suggested a host-specific relationship between the reported genogroups and the salmonid species cultivated in the country, since isolates belonging to genogroup 5 were mainly isolated from *S. salar*, while IPNV genogroup 1 was mostly isolated from *O. mykiss* or *O. Kisutch*. However, to date there are no experimental studies aimed to evaluate a relationship between these two genogroups and the differential susceptibility between host species. Thus, the objective of this study was to compare the mortality rates and viral load in rainbow trout infected with isolates from genogroups 1 and 5 to confirm the likely association between this species and the phylogenetic classification of IPNV.

MATERIALS AND METHODS

Virus isolates and fish:

Four IPNV isolates obtained from field outbreaks in Chile were used for the experimental challenges in this study. The isolates belong to the strain collection of the Laboratory of Virology of the University of Valparaíso and were molecularly characterized previously by sequencing both segments of the virus genome according to Jorquera *et al.* (2016). The four isolates represent the two IPNV genogroups present in the country: two isolates from genogroup 1 (type

125 strain WB), herein referred to as WB1 and WB2, respectively; and two from
126 genogroup 5 (type strain Sp), herein referred to as SP1 and SP2, respectively. All
127 isolates were propagated and titrated in the Chinook Salmon Embryo (CHSE-
128 214) cell line. For virus propagation, cells were grown in Leibovitz (L-15) culture
129 medium, supplemented with 10% fetal bovine serum (FBS, HyClone) and 50
130 µg·mL-1 gentamicin. Then the cells were infected with first passages of the
131 isolates at a multiplicity of infection (MOI) of 0.001 FF/cell and incubated at 15
132 °C until a massive cytopathic effect (CPE) was observed (around 3 to 5 days post-
133 infection). The cells were then subjected to two cycles of freezing and thawing
134 and centrifuged at 3000 × g for 15 min at 4°C. Supernatants were collected,
135 aliquoted and immediately stored at -80°C until titration or challenge
136 inoculation.

137 Titration was done twice, between two to three days after the isolates
138 were propagated and prior to the inoculation of the fish. For standard end-point
139 titration, aliquots of the isolates were thawed and serially diluted in L-15
140 medium and inoculated into confluent monolayers of CHSE-214 cells grown on
141 96-well plates. The cells were incubated at 15 °C for a week and then observed
142 under an inverted light microscope. The infected wells with clear ECP were
143 counted and the $TCID_{50}$ was calculated according to the Reed & Muench (1938)
144 method.

145 Rainbow trout fry (~ 0.9 g) were provided by the Rio Blanco hatchery
146 (Los Andes, Chile) and transferred to the aquaculture facilities of the
147 "Laboratorio Cerrillos", Veterquimica S.A. (Santiago, Chile). Prior to the challenge
148 test, the fish were sanitarily checked for the presence of IPNV, *Renibacterium*
149 *Salmoninarum, Piscirickettsia salmonis, Flavobacterium psychrophilum* and *F.*

150 *columnare* by qPCR. All of these diagnostic analyses were carried out at
151 Veterquimica diagnostic laboratory. Fish were held at 10-15 °C and fed daily on
152 commercial feed until the day preceding virus exposure.
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154 *Experimental challenges and Sampling:*
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156 In order to compare the mortality caused by the IPNV isolates in rainbow
157 trout, 1,440 fish were challenged during January 2018. Prior to challenge, fish
158 were acclimatized for 22 days and then allocated into eight 0.02 m^3 aerated
159 challenge tanks, with ~180 individuals per tank. The experimental design
160 included two tank replicates per treatment. Once distributed in their respective
161 tanks, fish were challenged by immersion using the four IPNV isolates. For this,
162 duplicate groups of fish were exposed to each isolate at 1 x 10^5 TCID50/mL^{-1}.
163 Thereafter, fish were maintained at 10 °C and mortality was recorded daily until
164 the trial ended 30 days post-infection. Dead fish were weighed and the entire
165 viscera, including kidney, was sampled to confirm the presence of IPNV and to
166 estimate viral load, following the procedures recommended by the World
167 Organisation for Animal Health (OIE 2006). Mortality samples were maintained
168 in L-15 medium at 4 °C until homogenization. Additionally, six fish were taken
169 randomly from each tank and sacrificed by benzocaine overdose (BZ-20;
170 Veterquimica, Chile) at days 1, 7 and 20 post-infection. The fish were weighed
171 and tissue samples were taken and stored in RNAlater® solution (Ambion, USA)
172 at −80 °C until use. All procedures for challenges and sampling were approved by
173 the Comite de Bioetica Animal, Facultad de Ciencias Veterinarias y Pecuarias,
174 Universidad de Chile (Certificate N° 17086-VET-UCH).

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176 *IPNV testing and viral load*

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178 Samples from mortality and survivor fish were tested to confirm the
179 presence of IPNV and to estimate viral load. A RT-qPCR assay for the VP1 region
180 of the virus (Eissler *et al.* 2011) was used for confirmation of the presence of the
181 virus in both mortality and survivor fish samples, while an indirect fluorescent
182 antibody test (IFAT) targeting the VP2 protein of the virus (Espinoza & Kuznar
183 2002) was used only on samples from mortalities. For the latter, homogenization
184 was done in 1 mL of L-15 medium using bead beating in the Minlys (Bertin)
185 homogenizer. The homogenates were pelleted at 2000 × g (15 min at 4°C) in a
186 MIKRO 22R centrifuge (Hettich Zentrifugen), and the supernatants were
187 collected and stored at -80°C until use. For the IFAT assay the methodology
188 described by Espinoza & Kuznar (2002) was followed with slight modifications.
189 Briefly, 100 µL of each supernatant was serially diluted in L-15 medium and
190 inoculated into duplicate confluent monolayers of CHSE-214 cells grown on 24-
191 well plastic plates with 12 mm circular glass coverslips. After 24 h, the cell
192 monolayers were fixed with cold methanol for 10 min, rinsed with PBS 1× buffer,
193 and incubated with a polyclonal antibody against VP2 IPNV during 1 h at room
194 temperature. Cells were rinsed again, and a secondary anti-rabbit antibody
195 (Sigma) conjugated with fluorescein isothiocyanate (FITC) was used to label the
196 infected cells (1/100 in PBS). After rinsing the cells for a third time, the circular
197 glass cover-slips containing the cell monolayers were mounted on glass slides
198 using an anti-fade mounting solution (DakoCytomation) and visualized using an
199 epifluorescence microscope (Olympus BX60). For the RT-qPCR assay, total RNA

was extracted from 200 µL of each supernatant from the mortality samples with the E.Z.N.A.$_{TM}$ Total RNA Kit I (Omega Bio-tek) according to the manufacturer's instructions. The extracted RNA was eluted with molecular biology grade water and stored at -80°C until use. In the case of samples taken from alive fish at different timepoints (1, 7 and 20 days), tissues (maintained in RNAlater) were homogenized in 1 mL of TRIzol reagent (Invitrogen) by bead beating, and total RNA was extracted following the standard TRIzol RNA isolation procedure.

Concentration and purity of the extracted total RNA was determined by measuring the absorbance ratio at 260 nm over 280 nm using a spectrophotometer (MaestroNano, Maestrogen). To ensure that contamination was strictly controlled during the RNA extraction process, a negative control using molecular biology grade water was included. The extracted RNA was reversely transcribed and amplified by a one-step RT-qPCR using a 48-well plate real-time PCR system Step-One (Applied Biosystems). The sets of primers and probes used for the VP1 and for the Elongation Factor -1 alpha (ELF1α) RT-qPCR assays are shown in Table 1. The AgPath-IDTM One-Step RT-PCR Kit (Applied Biosystems) was used for the amplification of the VP1 protein in segment B. Reaction was carried out in a 15 µL reaction volume containing 7.5 µL of RT-PCR Buffer (2X), 1.35 µL of each forward and reverse primers (0.9 µM), 0.3 µL of the VP1 Taqman probe (0.2 µM), 0.6 µL of RT-PCR Enzyme Mix (25X) and 2 µL of total RNA as template. The thermal profile used was 48°C for 10 min for reverse transcription, pre-denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 59°C for 45 s. In case of ELF1α amplification was carried out in a 15 µL reaction volume containing 7.5 µL of 2X Brilliant III Ultra-Fast SYBR® Green QRT-PCR Master Mix (Stratagene),

225 0.75 µL of each forward and reverse primers (0.5 µM), 0.8 µL of RT/RNAse block,
226 0.2 µL of ROX (0.3 µM) as passive reference and 2 µL of total RNA as template.
227 The thermal profile used was 50°C for 5 min for reverse transcription,
228 pre-denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C
229 for 5 s and annealing/extension at 60°C for 10 s. Finally, a melting curve analysis
230 from 70°C to 95°C was performed. The detection limit and the efficiency of the
231 assays were evaluated using 10-fold dilutions of total RNA from the virus and
232 from non-infected rainbow trout. The amplification efficiencies were 103% for
233 the IPNV RT-qPCR targeting segment B (Taqman) and 100% for the ELF1 RT-
234 qPCR (SYBR green), and the cut-off Ct values were 30.8 and 32.4, respectively.
235
236 *Statistical analysis*
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238 Kaplan-Meier mortality curves were calculated using the GraphPad Prism
239 7 software (GraphPad Software Inc., Ja Lolla, CA, USA). For this analysis,
240 mortalities were considered the result of IPNV infection only in cases when IPNV
241 presence was confirmed by IFAT and/or RT-qPCR, while live fish sampled at
242 different time points were not taken into account. Cumulative mortality rates
243 over time between fish infected with different isolates and genogroups were
244 compared using the Log-rank test ($p < 0.05$). In order to compare the results and
245 evaluate the agreement between the two diagnostic methods used, the Kappa
246 statistic was calculated. The scale used to interpret the Kappa statistic was as
247 follows: below 0.01 less than chance agreement, 0.01–0.20 slight agreement,
248 0.21–0.40 fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 substantial
249 agreement and 0.81–0.99 indicate almost perfect agreement (Viera & Garrett

2005). For the estimation of viral load in dead and survivor fish samples by RT-qPCR, relative expression of IPNV VP1 mRNA was calculated using the Pfaffl method (Pfaffl 2001) which accounts for differences in PCR efficiency. ELF1α was used as a housekeeping gene for normalization, and the sample with the lowest VP1 gene expression (*i.e.* highest Ct value) was set as calibrator. Finally, differences in viral load were analyzed using an analysis of covariance (ANCOVA), in which the dependent variable (viral load) was explained by a lineal model including tank nested to genogroup as factor and time of death and body weight of fish as covariate.

RESULTS

Mortality

Mortality due to IPNV started at day 4 post-challenge in fish infected with either WB1 or WB2. In fish challenged with isolates from genogroup 5, mortality began on day 11 (SP2) and day 13 (SP1) post infection. Cumulative mortality at day 30 post infection was low in all groups challenged, but especially in fish infected with isolates from genogroup 5, with final cumulative mortalities of 0.67% and 1% for SP1 and SP2, respectively. Fish infected with genogroup 1 isolates showed a higher cumulative mortality, with 2.33% for WB2 and 7.33% for WB1. Overall, trout infected with isolates belonging to genogroup 5 showed a total cumulative mortality of 0.84%. The total cumulative mortality reached 4.73% for genogroup 1. Kaplan–Meier (Log-Rank) analysis showed that the mortality curves of fish infected with the four isolates were significantly different

(p <0.0001), and that isolate WB1 had a significantly higher mortality rate over time than either of the two isolates from genogroup 5 (Figure 1). Furthermore, when comparing the two genogroups, the mortality rate of trout infected with genogroup 1 isolates was significantly higher than of those challenged with isolates from genogroup 5 (p <0.0001).

IPNV testing and viral load

Mortality samples

A total of 49 mortality samples were tested to confirm the presence of IPNV by RT-qPCR and IFAT. The virus was detected in 34 of the analyzed samples (69.4%). IPNV positive samples by IFAT showed cells with a distinctive fluorescent staining, indicating that these samples contained infective viral particles. Samples ranged from a few fluorescent cells to hundreds per coverslip in high viral titer samples (Figure 2). There was substantial agreement between the two diagnostics methods (Kappa = 0.62, p < 0.0001), however, RT-qPCR was more sensitive, detecting IPNV in 33 of the samples tested, where only 26 positive samples were detected by IFAT. From the 34 positive samples, 29 were infected with isolates from genogroup 1, and 5 with isolates from genogroup 5. On average, virus load measured by RT-qPCR was 2 folds higher in fish infected with isolates from genogroup 5 than from genogroup 1. When the viral loads were compared by means of a ANCOVA using tank nested to genogroup as factor a statically significant difference was found (p = 0.005).

Survivor fish samples

Survivor fish sampled at day 1, 7 and 20 post infection, were analyzed to test for the presence of IPNV and to assess viral load by RT-qPCR. A total of 144 samples were tested and IPNV was found in 48 (33.3%), 23 belonging to fish infected with genogroup 1 isolates and 24 from genogroup 5. At day one post infection, IPNV positive samples were only detected in fish infected with isolate WB2, but showed very little VP1 mRNA expression, with a Ct value near cutoff value. Thus, one of these samples was set as calibrator for relative quantification of IPNV. At day 7 and 20 post infection all fish groups challenged with the different isolates showed at least one sample with the presence of the virus. Fish infected with isolate SP2 had the highest prevalence of IPNV, with 17 positive samples, while SP1 infected fish had the lowest, with seven samples positive to the virus (data not shown). In fish infected with isolates WB1 and WB2 from genogroup 1 the virus was detected in 12 and 11 of the analyzed samples, respectively. There was an increase in viral load throughout the challenge in fish infected with both genogroups, reaching a peak at day 20 post infection (Figure 3). As with mortality samples, viral load in survivor fish was, on average, higher in fish infected with isolates from genogroup 5 than with genogroup 1, and when the means of both groups were compared by an ANCOVA test, there was a statistically significant difference ($p = 0.018$).

DISCUSSION

Previous reports have demonstrated that there is host genetic variation in resistance to IPN and that virulence may vary between IPNV isolates, causing great variation in mortality rates during outbreaks of the disease (Storset et al., 2007; Skjesol et al., 2011; Mutoloki et al., 2016). In this study, the mortality and viral load of rainbow trout fry individuals infected with Chilean IPNV isolates from genogroup 1 and 5 (more commonly known as strains WB and Sp, respectively) were compared by means of a 30-day experimental challenge. Our findings suggest that IPNV isolates from genogroup 1 were more virulent, i.e caused a higher mortality rate, than isolates from genogroup 5 in rainbow trout. This lends support to previous findings that suggested a host-specific relationship between IPNV isolates from genogroup 1 and salmonids from the genus *Oncorhynchus* farmed in Chile, based on the data from more than one hundred Chilean isolates from both genogroups (Tapia et al., 2015; Torres et al., 2016). Furthermore, since the publication of those studies these authors have continued to sequence more IPNV isolates, finding the same association between genogroups and salmonid species (Eissler et al. 2017). Conversely, Manriquez et al. (2017) recently suggested that there is no fixed relationship between salmonid species and genogroups of IPNV in Chile, since they found genogroup 1 isolates not only in *O. mykiss* and *O. kisutch* but also in *S. salar*. However, these authors molecularly characterized only 36 Chilean isolates, from which 10 where classified in genogroup 1, and only 4 came from *S. salar*. Hence, it is important to bear in mind that although there is a well known relationship between the genogroups of IPNV and salmonids present in Chile, this is not strict host specificity, and both salmon and trout can be infected with either genogroup of the virus.

352 This type of host-specific relationship has also been reported for other
353 salmonid viruses, such as Infectious Hematopoietic Necrosis Virus (IHNV) in
354 North America, where most of the isolates of the genogroups named U and M,
355 come predominantly from sockeye salmon (*O. nerka*) and rainbow trout,
356 respectively (Garver et al., 2003). Studies based on experimental challenges with
357 both species and genogroups have shown that the relationship was associated
358 with a specific virulence of IHN for each host, which depends mainly on the
359 ability of the virus to enter the host fish and replicate (Garver et al., 2006;
360 Peñaranda et al., 2009, 2011; Purcell et al., 2009). As with IPNV, IHNV isolates
361 from both the U and M genogroups can infect rainbow trout; however, only the M
362 genogroup viruses are highly virulent in this species. Using RT-qPCR they
363 showed that viral load was significantly higher in trout infected with the more
364 virulent M virus. Furthermore, a microarray analysis indicated that infection
365 resulted in a greater overall host transcriptome change, suggesting that the M
366 virus was more efficient at mediating host cell shutoff in order to enhance viral
367 replication (Purcell et al., 2011).

368 Despite low mortality rates, viral load with IPNV genogroup 5 isolates
369 was high and, these fish were indeed infected with IPNV and viral replication
370 occurred until the last day of sampling, 20 days post infection. More
371 interestingly, there is a tendency for genogroup 5 to have higher virus load on
372 average than fish infected with isolates from genogroup 1, in both mortality and
373 survivor fish samples. This would suggest that WB strain type isolates require
374 the same or even less viral load than Sp train to cause greater mortality in
375 rainbow trout. This is in contrast to what was seen in IHNV, and previous studies
376 that show a positive relation between mortality caused by IPNV and virus load in

377 Atlantic salmon, i.e. higher mortalities were associated with higher viral loads in
378 susceptible fish or in fish infected with more virulent isolates (Skjesol et al.,
379 2011; Reyes-Lopez et al., 2015; Robledo et al., 2016). It is important to point out
380 that both isolates from genogroup 5 had contained high virulence marker, Thr_{217}
381 and $Ala_{221,}$ in their VP2 sequence; whereas isolates from genogroup 1 had the
382 avirulent motif, Pro_{217} and Thr_{221}. Nonetheless, virulence was higher for
383 genogroup 1 isolates in trout, suggesting that these virulence motifs are specific
384 for the Sp strain, and that other genetic variants could have an effect on the
385 virulence of the WB strain. Furthermore, in a previous challenge in Atlantic
386 salmon, isolates SP1 and SP2 were able to cause significant mortality (data not
387 shown), indicating that these viruses were virulent only in this species.

388 As was expected, mortality varied between fish infected with different
389 isolates. However, overall mortality levels for the challenge were low, and only
390 moderate differences in mortality were noted. It is well known that induction of
391 overt IPN disease and mortality in experimental challenges is difficult to achieve,
392 and is determined by several input variables like virus dose, fish age,
393 temperature, infection route and host genetic susceptibility. For the challenge
394 performed in this study we tried to meet the criteria for most of the variables
395 recommended to obtain IPN-induced mortality in rainbow trout, by infecting
396 first feeding fry via waterborne with a relatively high virus dose and maintaining
397 them at 10 °C. However, it is plausible that a number of limitations could have
398 influenced the results obtained and overall low mortalities reached in the
399 challenge. One of the possible limitations was the virus dose.

400 Some authors recommend a dose as high as $1x10^7$ $TCID_{50}$/mL per fish to
401 attain higher mortalities in a cohabitation challenge with IPNV (Munang'andu et

al., 2016). However, we were unable to obtain higher viral titers without increasing the number of passages of the isolates in cell culture. Nevertheless, several researchers have used the same dose used in this study in immersion challenges and have obtained considerably higher mortalities in rainbow trout and Atlantic salmon (Okamoto et al., 1987; Skjesol et al., 2011; Robledo et al., 2016).

Another technical limitation of this study is that neither phenotypic nor genotypic data for IPNV resistance of the rainbow trout individuals challenged was available. Recently it has been reported that mortality due to IPN in rainbow trout can range between 0% and 100% for the most resistant and susceptible families, respectively (Yoshida et al., 2019). Munang'andu et al. (2016) pointed out the importance of using highly susceptible fish to establish a challenge model for IPNV in Atlantic salmon that achieves mortalities above 75% in infected fish. Thus, it would be recommended that in future trials rainbow trout from known susceptible families are used in order to reach higher mortality levels that allow greater differentiation between groups.

Together these results show that IPNV isolates from genogroup 1 and genogroup 5 could infect rainbow trout but only the former caused important mortality in this species. This would suggest that IPNV isolates from the WB type strain are more virulent in rainbow trout than those from the Sp strain, and there is an association between the phylogenetic classification of IPNV and host susceptibility.

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456 REFERENCES

457

458 Blake, S., Ma, J. Y., Caporale, D. A., Jairath, S., & Nicholson, B. L. (2001).
459 Phylogenetic relationships of aquatic birnaviruses based on deduced amino
460 acid sequences of genome segment A cDNA. *Dis Aquat Organ*, 45(2), 89-102.
461 doi:10.3354/dao045089

462 Bootland, L. M., Dobos, P., & Stevenson, R. M. (1991). The IPNV carrier state and
463 demonstration of vertical transmission in experimentally infected brook
464 trout. *Diseases of aquatic organisms*, 10, 13-21. doi:10.3354/dao010013

465 Calleja, F., Godoy, M.G., Cárcamo, J.G., Bandín, I., Yáñez, A.J., Dopazo, C.P., Kibenge,
466 F.S. & Avendaño-Herrera, R. (2012). Use of reverse transcription-real time
467 polymerase chain reaction (real time RT-PCR) assays with Universal Probe
468 Library (UPL) probes for the detection and genotyping of infectious
469 pancreatic necrosis virus strains isolated in Chile. *Journal of Virological*
470 *Methods*, 183(1), 80-85. doi:10.1016/j.jviromet.2012.03.022

471 Dobos, P. (1995). The molecular biology of infectious pancreatic necrosis virus
472 (IPNV). *Annual Review of Fish Diseases, 5*(0), 25-54. doi:10.1016/0959-
473 8030(95)00003-8

474 Eissler, Y., Pavlov, M. S., Conejeros, P., Espinoza, J. C., & Kuznar, J. (2011).
475 Detection and quantification of Chilean strains of infectious pancreatic
476 necrosis virus by real-time RT-PCR assays using segment B as a target. *Lat.*
477 *Am. J. Aquat. Res., 39*(3), 9. doi:10.3856/vol39-issue3-fulltext-14

478 Eissler, Y., Rosenfeld, C., Espinoza, J. C., Kuznar, J., Tapia, D., & Torres, P. (2017).
479 Determinación de factores epidemiológicos de riesgo en la presentación
480 clínica de la enfermedad Necrosis Pancreática Infecciosa. Informe Final
481 Proyecto Fondo de Investigación Pesquera y de Acuicultura FIPA 2014-
482 60. Retrieved from http://www.subpesca.cl/fipa/613/articles-
483 89418_informe_final.pdf.

484 Espinoza, E., Farías, G., Soler, M., & Kuznar, J. (1985). Identity between Infectious
485 Pancreatic Necrosis Virus VR-299 and a Chilean Isolate. *Intervirology, 24*(1),
486 58-60. doi:10.1159/000149619

487 Espinoza, J. C., & Kuznar, J. (2002). Rapid simultaneous detection and
488 quantitation of infectious pancreatic necrosis virus (IPNV). *Journal of*
489 *Virological Methods, 105*(1), 81-85. doi:Pii S0166-0934(02)00083-6

490 Evensen, Ø., & Santi, N. (2008). Infectious Pancreatic Necrosis Virus. In B. W. J. M.
491 H. V. V. Regenmortel (Ed.), *Encyclopedia of Virology (Third Edition)* (pp. 83-
492 89). Oxford: Academic Press. doi:https://doi.org/10.1016/B978-
493 012374410-4.00772-X

494 FAO. (2019, 26 April). Fisheries Global Information System (FIGIS) Integrated
495 web-based dissemination system. Retrieved from
496 http://www.fao.org/fishery/

497 Flores-Mara, R., Rodríguez, F. H., Bangera, R., Lhorente, J. P., Neira, R., Newman,
498 S., & Yáñez, J. M. (2017). Resistance against infectious pancreatic necrosis
499 exhibits significant genetic variation and is not genetically correlated with
500 harvest weight in rainbow trout (Oncorhynchus mykiss). *Aquaculture, 479*,
501 155-160. doi:https://doi.org/10.1016/j.aquaculture.2017.05.042

502 Garver, K. A., Batts, W. N., & Kurath, G. (2006). Virulence Comparisons of
503 Infectious Hematopoietic Necrosis Virus U and M Genogroups in Sockeye
504 Salmon and Rainbow Trout. *J Aquat Anim Health, 18*(4), 232-243.
505 doi:10.1577/H05-038.1

506 Garver, K. A., Troyer, R. M., & Kurath, G. (2003). Two distinct phylogenetic clades
507 of infectious hematopoietic necrosis virus overlap within the Columbia
508 River basin. *Diseases of aquatic organisms, 55*(3), 187-
509 203. doi:10.3354/dao055187

510 Hill, B. J., & Way, K. (1995). Serological classification of infectious pancreatic
511 necrosis (IPN) virus and other aquatic birnaviruses. *Annual Review of Fish*
512 *Diseases, 5*(0), 55-77. doi:http://dx.doi.org/10.1016/0959-8030(95)00011-
513 9

514 Houston, R.D., Haley, C.S., Hamilton, A., Guy, D.R., Tinch, A.E., Taggart, J.B.,
515 Mcandrew, B.J., & Bishop, S.C. (2008). Major quantitative trait loci affect
516 resistance to infectious pancreatic necrosis in Atlantic salmon (Salmo
517 salar). *Genetics, 178*(2), 1109-1115. doi:10.1534/genetics.107.082974

518 Jarp, J., Gjevre, A. G., Olsen, A. B., & Bruheim, T. (1995). Risk-Factors for
519 Furunculosis, Infectious Pancreatic Necrosis and Mortality in Post-Smolt of
520 Atlantic Salmon, Salmo-Salar L. *Journal of Fish Diseases, 18*(1), 67-78.
521 doi:10.1111/j.1365-2761.1995.tb01267.x

522 Jorquera, E., Morales, P., Tapia, D., Torres, P., Eissler, Y., Espinoza, J.C., Conejeros,
523 P., & Kuznar, J. (2016). Chilean IPNV isolates: Robustness analysis of PCR
524 detection. *Electronic Journal of Biotechnology, 20*, 28-32. doi:
525 https://doi.org/10.1016/j.ejbt.2016.01.001

526 Macdonald, R. D., & Gower, D. A. (1981). Genomic and phenotypic divergence
527 among three serotypes of aquatic birnaviruses (infectious pancreatic
528 necrosis virus). *Virology, 114*(1), 187-195.
529 doi:http://dx.doi.org/10.1016/0042-6822(81)90264-6

530 Manríquez, R. A., Vera, T., Villalba, M. V., Mancilla, A., Vakharia, V. N., Yañez, A. J.,
531 & Cárcamo, J. G. (2017). Molecular characterization of infectious pancreatic
532 necrosis virus strains isolated from the three types of salmonids farmed in
533 Chile. *Virology Journal, 14*(1), 17. doi:10.1186/s12985-017-0684-x

534 McAllister, P. E., & Reyes, X. (1984). Infectious pancreatic necrosis virus: isolation
535 from rainbow trout, *Salmo gairdneri* Richardson, imported into Chile.
536 *Journal of Fish Diseases, 7*, 4. doi:https://doi.org/10.1111/j.1365-
537 2761.1984.tb00938.x

McCowan, C., Motha, J., Crane, M. S., Moody, N. J., Crameri, S., Hyatt, A. D., & Bradley, T. (2015). Isolation of a novel aquatic birnavirus from rainbow trout *Oncorhynchus mykiss* in Australia. *Dis Aquat Organ, 114*(2), 117-125. doi:10.3354/dao02858

Moen, T., Baranski, M., Sonesson, A. K., & Kjoglum, S. (2009). Confirmation and fine-mapping of a major QTL for resistance to infectious pancreatic necrosis in Atlantic salmon (*Salmo salar*): population-level associations between markers and trait. *BMC Genomics, 10*, 368. doi:10.1186/1471-2164-10-368

Moen, T., Torgersen, J., Santi, N., Davidson, W.S., Baranski, M., Odegard, J., Kjoglum, S., Velle, B., Kent, M., Lubieniecki, K.P., Isdal, E., & Lien, S. (2015). Epithelial Cadherin Determines Resistance to Infectious Pancreatic Necrosis Virus in Atlantic Salmon. *Genetics*, 200(4), 1313-1326. doi:10.1534/genetics.115.175406

Mohr, P. G., Moody, N. J., Williams, L. M., Hoad, J., & Crane, M. S. J. (2015). Molecular characterization of Tasmanian aquabirnaviruses from 1998 to 2013. *Diseases of aquatic organisms, 116*(1), 1-9. doi: 10.3354/dao02903

Munang'andu, H. M., Santi, N., Fredriksen, B. N., Lokling, K. E., & Evensen, O. (2016). A Systematic Approach towards Optimizing a Cohabitation Challenge Model for Infectious Pancreatic Necrosis Virus in Atlantic Salmon (Salmo salar L.). *PloS one, 11*(2), e0148467. doi:10.1371/journal.pone.0148467

Mutoloki, S., & Evensen, O. (2011). Sequence similarities of the capsid gene of Chilean and European isolates of infectious pancreatic necrosis virus point towards a common origin. *Journal of general virology, 92*(7), 1721-1726. doi:10.1099/vir.0.030270-0

Mutoloki, S., Jossund, T. B., Ritchie, G., Munang'andu, H. M., & Evensen, O. (2016). Infectious Pancreatic Necrosis Virus Causing Clinical and Subclinical Infections in Atlantic Salmon Have Different Genetic Fingerprints. *Front Microbiol, 7*, 1393. doi:10.3389/fmicb.2016.01393

Nishizawa, T., Kinoshita, S., & Yoshimizu, M. (2005). An approach for genogrouping of Japanese isolates of aquabirnaviruses in a new genogroup, VII, based on the VP2/NS junction region. *Journal of general virology, 86*(7), 1973-1978. doi:10.1099/vir.0.80438-0

OIE. (2006). *Manual of Diagnostic Tests for Aquatic Animals , 5th edition.* ed. Health World Organization for Animal, Paris.

Okamoto, N., Seno, Y., Taniguchi, N., & Sano, T. (1987). Relationship between inoculative quantity of infectious pancreatic necrosis virus and the mortality of rainbow trout [*Salmo gairdnerii*] fry. *Bulletin of the Japanese Society of Scientific Fisheries (Japan)*. doi: https://doi.org/10.2331/suisan.53.1975

Penaranda, M. M., Purcell, M. K., & Kurath, G. (2009). Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (Oncorhynchus mykiss) include host entry and virus replication kinetics. *J Gen Virol, 90*(Pt 9), 2172-2182. doi:10.1099/vir.0.012286-0

582 Penaranda, M. M., Wargo, A. R., & Kurath, G. (2011). In vivo fitness correlates
583 with host-specific virulence of Infectious hematopoietic necrosis virus
584 (IHNV) in sockeye salmon and rainbow trout. *Virology, 417*(2), 312-319.
585 doi:10.1016/j.virol.2011.06.014

586 Pfaffl, M. W. (2001). A new mathematical model for relative quantification in
587 real-time RT–PCR. *Nucleic acids research, 29*(9), e45-e45.
588 doi:10.1093/nar/29.9.e45

589 Purcell, M. K., Garver, K. A., Conway, C., Elliott, D. G., & Kurath, G. (2009).
590 Infectious haematopoietic necrosis virus genogroup-specific virulence
591 mechanisms in sockeye salmon, Oncorhynchus nerka (Walbaum), from
592 Redfish Lake, Idaho. *J Fish Dis, 32*(7), 619-631. doi:10.1111/j.1365-
593 2761.2009.01045.x

594 Purcell, M. K., Marjara, I. S., Batts, W., Kurath, G., & Hansen, J. D. (2011).
595 Transcriptome analysis of rainbow trout infected with high and low
596 virulence strains of infectious hematopoietic ecrosis virus. Fish Shellfish
597 Immunol, 30(1), 84-93. doi:10.1016/j.fsi.2010.09.007

598 Reed, L. J., & Muench, H. (1938). A simple method of estimating fifty per cent
599 endpoints. *American journal of epidemiology, 27*(3), 493-497. doi:
600 https://doi.org/10.1093/oxfordjournals.aje.a118408

601 Reyes-Lopez, F.E., Romeo, J.S., Vallejos-Vidal, E., Reyes-Cerpa, S., Sandino, A.M.,
602 Tort, L., Mackenzie, S., & Imarai, M. (2015). Differential immune gene
603 expression profiles in susceptible and resistant full-sibling families of
604 Atlantic salmon (Salmo salar) challenged with infectious pancreatic
605 necrosis virus (IPNV). *Dev Comp Immunol, 53*(1), 210-221.
606 doi:10.1016/j.dci.2015.06.017

607 Robledo, D., Taggart, J.B., Ireland, J.H., Mcandrew, B.J., Starkey, W.G., Haley, C.S.,
608 Hamilton, A., Guy, D.R., Mota-Velasco, J.C., Gheyas, A.A., Tinch, A.E., Verner-
609 Jeffreys, D.W., Paley, R.K., Rimmer, G.S., Tew, I.J., Bishop, S.C., Bron, J.E., &
610 Houston, R.D. (2016). Gene expression comparison of resistant and
611 susceptible Atlantic salmon fry challenged with Infectious Pancreatic
612 Necrosis virus reveals a marked contrast in immune response. *BMC*
613 *Genomics*, 17, 279. doi:10.1186/s12864-016-2600-y

614 Rodrigues, F., Flores-Mara, R., Yoshida, G., Barria, A., Jedlicki, A., Lhorente, J.P.,
615 Reyes, F., & Yanez, J.M. (2019). Genome-wide association analysis for
616 resistance to infectious pancreatic necrosis virus identifies candidate genes
617 involved in viral replication and immune response in rainbow trout
618 (*Oncorhynchus mykiss*). *bioRxiv*,
619 569632. doi:https://doi.org/10.1101/569632

620 Ruane, N. M., McCleary, S. J., McCarthy, L. J., & Henshilwood, K. (2015).
621 Phylogenetic analysis of infectious pancreatic necrosis virus in Ireland
622 reveals the spread of a virulent genogroup 5 subtype previously associated
623 with imports. *Archives of virology, 160*(3), 817-824. doi: 10.1007/s00705-
624 014-2307-9

Santi, N., Vakharia, V. N., & Evensen, Ø. (2004). Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. *Virology, 322*(1), 31-40. doi:10.1016/j.virol.2003.12.016

Skjesol, A., Skjæveland, I., Elnæs, M., Timmerhaus, G., Fredriksen, B.N., Jørgensen, S., Krasnov, A., & Jørgensen, J.B. (2011). IPNV with high and low virulence: host immune responses and viral mutations during infection. *Virology Journal, 8*(1), 396. doi:10.1186/1743-422x-8-396

Song, H., Santi, N., Evensen, O., & Vakharia, V. N. (2005). Molecular Determinants of Infectious Pancreatic Necrosis Virus Virulence and Cell Culture Adaptation. *Journal of Virology, 79*(16), 10289-10299. doi:10.1128/jvi.79.16.10289-10299.2005

Storset, A., Strand, C., Wetten, M., Kjøglum, S., & Ramstad, A. (2007). Response to selection for resistance against infectious pancreatic necrosis in Atlantic salmon (*Salmo salar* L.). *Aquaculture, 272*, S62-S68. doi:10.1016/j.aquaculture.2007.08.011

Tapia, D., Eissler, Y., Torres, P., Jorquera, E., Espinoza, J., & Kuznar, J. (2015). Detection and phylogenetic analysis of infectious pancreatic necrosis virus in Chile. *Diseases of aquatic organisms, 116*(3), 173-184. doi: 10.3354/dao02912.

Torres, P., Eissler, Y., Tapia, D., Espinoza, J. C., & Kuznar, J. (2016). Genotipificarión y relación hospedador-específica del virus de la necrosis pancreática infecciosa en Chile. *Latin american journal of aquatic research, 44*, 860-868. doi:http://dx.doi.org/10.3856/vol44-issue4-fulltext-23

Viera AJ, Garrett JM (2005) Understanding interobserver agreement: the kappa statistic. Fam Med 37:360–363.

Yoshida, G. M., Carvalheiro, R., Rodriguez, F. H., Lhorente, J. P., & Yanez, J. M. (2019). Single-step genomic evaluation improves accuracy of breeding value predictions for resistance to infectious pancreatic necrosis virus in rainbow trout. *Genomics, 111*(2), 127-132. doi:10.1016/j.ygeno.2018.01.008

TABLES

Table 1. Primers and probes used for the RT-qPCR assays to identify and quantify IPNV in samples from challenged individuals.

Assay name	Primer/Probe name	Primer sequence (5´-3´)	Target
IPNV VP1	IPNV VP1	56FAM-TACATAGGC-ZEN-AAAACCAAAGGAGACAC-3IABkFQ	IPNV Segment B: 668–820
	VP1F	GTTGATMMASTACACCGGAG	
	VP1R	AGGTCHCKTATGAAGGAGTC	
ELF4	ELF4 F	GTATGATCGTCACCTTCGCCC	Salmonid ELF 1α: 884-963
	ELF4 R	CGATTCCAGGGTCTCGTGGT	

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696 FIGURES

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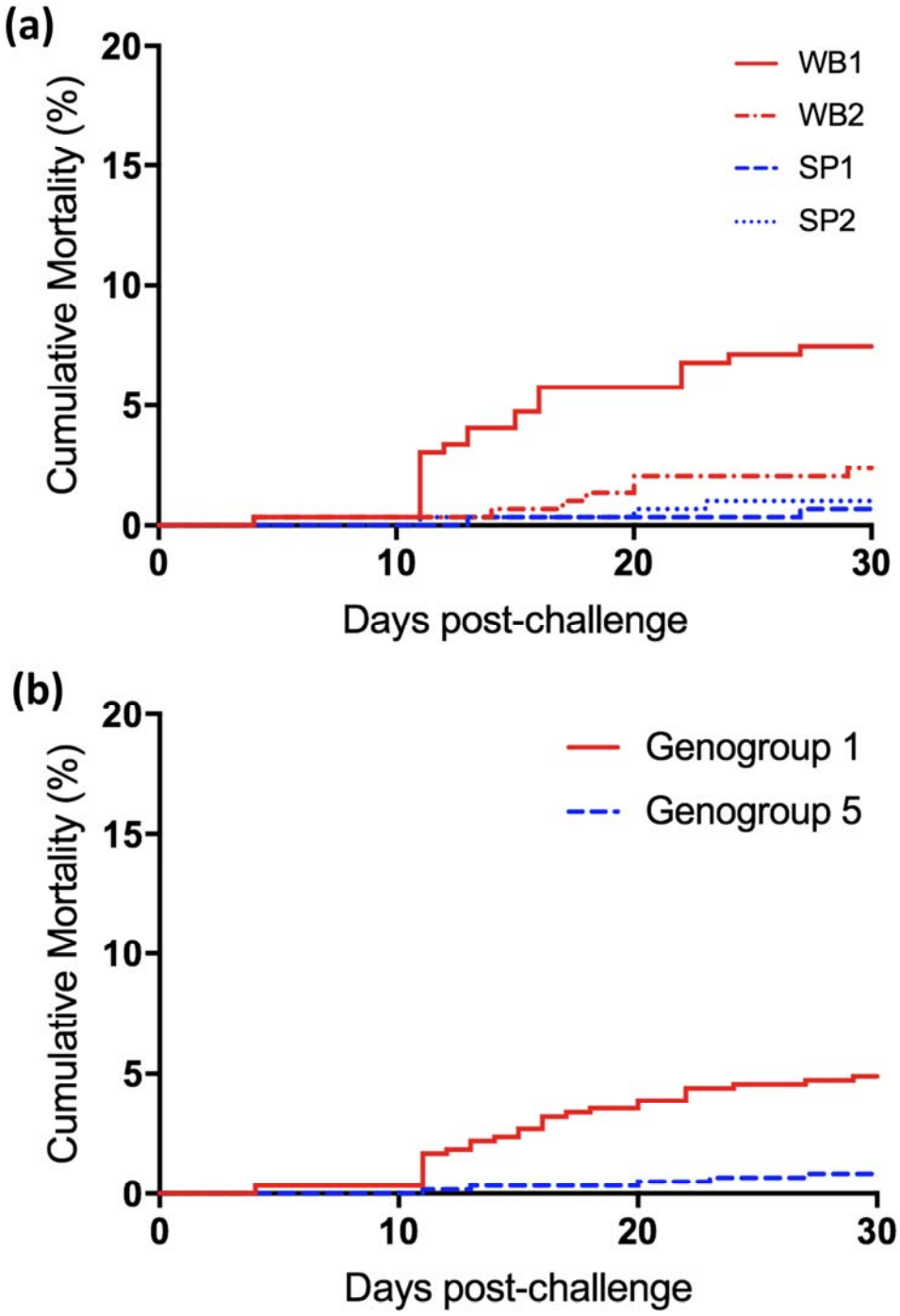


Figure 1. Kaplan-Meier mortality curves for rainbow trout infected with four isolates of IPNV from Genogroup 1 and 5. The cumulative mortality caused by each isolate is presented in a), and the combined mortality caused by isolates of each Genogroup is presented in b).

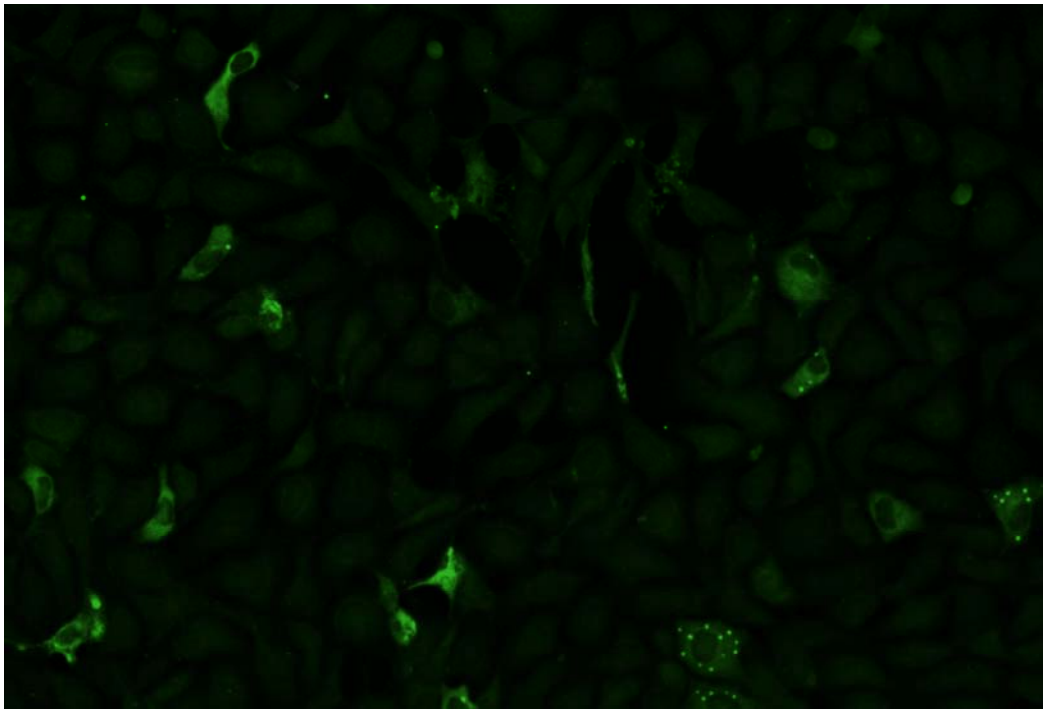
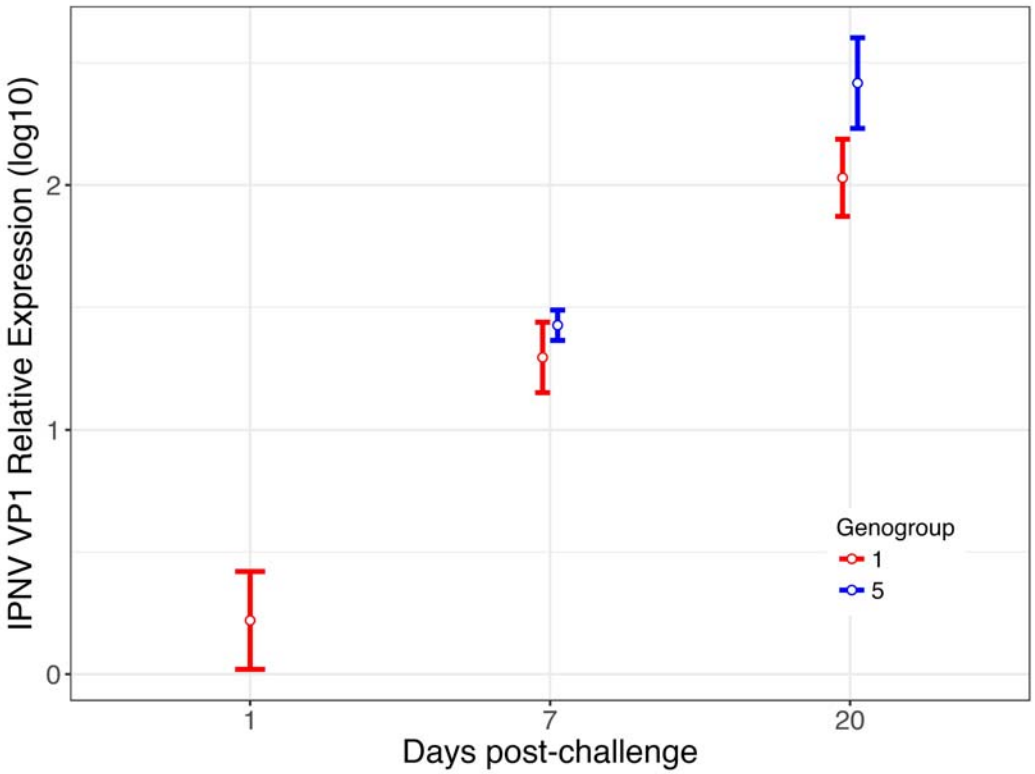


Figure 2. Indirect Fluorescent Antibody Test (IFAT) of IPNV positive mortality samples (10x). CHSE-214 cells infected with sample from fish challenged with Genogroup 5 isolate SP2 and stained with a polyclonal antibody against VP2 protein. Several infected (fluorescent) cells can be seen in the monolayer indicating a high viral titer.



715 Figure 3. IPNV virus load in survivor fish sampled at days 1, 7 and 20 post-
716 infection. Mean and standard error log10 VP1 transcript relative levels measured
717 by RT-qPCR using ELF1α as housekeeping gene. No IPNV was detected in
718 samples from genogroup 5 at day 1 post-infection.
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