Author's personal copy

Veterinary Immunology and Immunopathology 141 (2011) 190–200

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Evaluation of the level of Mx3 protein synthesis induced by infectious pancreatic necrosis virus (IPNV) strains of different infectivity

César Ortega[a,*], Sylvia Rodríguez[b], Ana I. de las Heras[b], Alex Romero[c], Mónica Monrás[c], Ricardo Enríquez[c]

[a] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, AP 4-56, Toluca, Mexico
[b] Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas), c/Ramiro de Maeztu 9, Madrid 28040, Spain
[c] Instituto de Patología Animal, Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Casilla 567, Valdivia, Chile

ARTICLE INFO

Article history:
Received 2 December 2009
Received in revised form 14 February 2011
Accepted 24 February 2011

Keywords:
IPNV
Mx
Infectivity
Virulence

ABSTRACT

The *in vitro* infectivity and genotype of three IPNV strains (V70, V112 and V98) was linked to the level of transcript synthesis for the Mx3 protein in RTG-2 (Rainbow trout gonad) cells and in *Salmo salar*. The V70 and V98 strains corresponded to the Sp genotype, whilst the V112 corresponded to VR-299; the presence of Pro-217 and Ala-221 in VP2 identified V70 as a strain of medium virulence level whilst V112 (Ala-217 and Thr-221) and V98 (Pro-217 and Thr-221) were of low virulence. This is concurrent with several *in vitro* tests which showed V70 to be a strain with highly infectivity ($P < 0.05$). In both the *in vitro* and *in vivo* trials, the strains demonstrated the induction of the Mx transcript, although no differences were detected, and the level always were significantly lesser that observed in poly I:C samples. The results suggest that the infectivity observed is related to the presence of certain specific residues in VP2, and that neither the infectivity nor the genotype appears to bear any relation to Mx induction capacity.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Infectious pancreatic necrosis virus (IPNV) causes an acute systemic and contagious disease which leads to high mortality in salmonids below 1500 day degrees, and in Atlantic salmon post-smolts (*Salmo salar*) after being moved from freshwater to saltwater. Fish that survive the clinical disease and those that are infected without developing any clinical signs act as carriers, representing a serious risk for their offspring and for other susceptible fish (McKnight and Roberts, 1976; Wolf, 1988). IPNV presents high antigenic and genotypic variability, and can infect a wide range of fish, mollusc and crustacean species in fresh and saltwater (Dobos, 1995; Smail et al., 2006). However, the cellular elements and mechanisms involved in the virus–cell interaction, virulence and outcome of the infection have not been clearly determined (Sano et al., 1992; Santi et al., 2005; Song et al., 2005).

The pathogenicity and virulence of IPNV has been linked to its ability to affect cell signalling, either by activating or inhibiting certain pathways and genes that on the one hand favour its replication or on the other affect defence mechanisms (Hong and Wu, 2002). The structural protein VP2, which forms the viral capsid and is linked to cell tropism, is considered to be one of the major determinants of virulence, and is associated with the sequence of certain residues in its central variable domain (Bruslind and Reno, 2000; Frost et al., 1995; Shivappa et al., 2004; Song et al., 2005); Song et al. (2005) identified that residues 217 and 221 determine virulence in Sp strains and that in certain combinations they present variation.

* Corresponding author. Tel.: +52 722 2965555; fax: +52 722 2965555.
E-mail addresses: cortegas@uaemex.mx, cos_mx@hotmail.com (C. Ortega).

0165-2427/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetimm.2011.02.022

Author's personal copy

In the cells of vertebrates the viral infection induces secretion of interferon-type (IFN) cytokines, which, as a mechanism for protecting non-infected cells, activate intracellular signalling pathways that are capable of interfering with some of the viral replication processes, develop immunomodulating functions which promote specific immune responses, and recruit and activate natural cytotoxic cells (NK cells) in order to destroy infected cells (Larsen et al., 2004; Robertsen, 2006). The antiviral effect is triggered by the binding of IFN-α/β with the receptor of IFN-α/β present in nucleated cells. This activates a signal transduction through the JAK-STAT pathway, which leads to transcription of the genes that encode proteins involved in antiviral activity, such as dsRNA-dependent protein kinase (PKR), 2′-5′ oligoadenylate synthetase (OAS) and GTPases like the Mx protein (Haller et al., 1998; Robertsen, 2006). However, there is little information concerning their specific action against certain viral components (Rodríguez and Pérez-Prieto, 2006).

IPNV is inhibited in *S. salar* cells which express high levels of Mx3 protein after stimulation with IFN-I or the synthetic oligonucleotide poly I-C (Jensen and Robertsen, 2000; Jensen et al., 2002b). *In vivo* the injection of IPNV into *S. salar* post-smolt induces expression of the Mx3 mRNA from day 1 up until 11 days after injection, although the response is not able to eliminate the infection. Despite the correlation between inhibition of replication and Mx expression in cells stimulated with IFN, the type of action has not been determined. Direct interaction between the protein and the target viruses (Caipang et al., 2003; Jensen and Robertsen, 2002; Jensen et al., 2002a) has been suggested. Whether or not induction of IFN and Mx3 expression is altered by different strains of IPNV, however, has not been determined, and neither have the viral proteins involved. This study was carried out to determine whether the infectivity of IPNV strains expressed *in vitro* is related to the synthesis induction level of the Mx3 protein transcript as an antiviral mechanism in cultured cells and *in vivo*.

2. Materials and methods

2.1. Cultured cells, viruses and fish

2.1.1. Cells

Two lines obtained from the American Type Culture Collection (ATCC) were used. Epithelial-type *Chinook salmon embryo* (CHSE-214) cells derived from *Oncorhynchus tshawytscha* embryonic tissue were used for viral replication and virulence tests *in vitro*, whilst *Rainbow trout gonad* (RTG-2) cells, derived from rainbow trout (*Oncorhyncus mykiss*) gonadal tissue, were used in the tests to assess Mx3 expression *in vitro*.

2.1.2. Viruses

Three IPNV isolates which were considered to be of interest, owing to clinico-pathological differences in affected fish on salmon farms in southern Chile, were used. The V*98* virus corresponded to an isolate that in 1998 caused 80% mortality in young *S. salar*. The *V70* virus was collected from the kidneys of young, imported *S. salar* which presented high initial mortality that later evolved into low mortality and chronic symptoms. The *V112* virus was isolated in young rainbow trout that presented 40% mortality during the clinical disease. The viruses were reactivated by inoculation in CHSE-214 cells with 90% confluence maintained in *minimal essential medium* (MEM) supplemented with 100 μg/ml of streptomycin, 60 μg/of penicillin and 2% of *foetal bovine serum* (FBS). When the cultures presented an extensive cytopathic effect (CPE) they were frozen for 24 h to enable maximum liberation of viral particles (OIE, 2005). They were then defrosted and centrifuged at 4000 rpm for 10 min at 4 °C. The supernatant was collected and assessed using the Reed and Muench method (Reed and Muench, 1938). Confirmation was carried out by immunological and genetic identification through indirect immunofluorescence (IFAT) using an anti-VP3 monoclonal antibody (Espinoza et al., 2000) and RT-PCR (Blake et al., 1995), respectively.

2.1.3. Fish

The *in vivo* trial was performed on 8.0 g Atlantic salmon, which were obtained from fish farming that was free of viral agents and kept acclimatised and at rest for 30 days (d) in a 200 l pond with a partial water recirculation system at a temperature of 15 °C. During this period the fish were given commercial feed (34% protein, 16% fat) and health checks were carried out using routine diagnostic techniques (OIE, 2005).

2.2. Identifying IPNV and obtaining the VP2 sequence

The viral RNA present in the cell culture supernatant was collected by column extraction in accordance with Viral RNA mini Kit Q1Aamp® (Cat. No. 52904, QiAGEN, Germany) specifications. After reverse transcription, amplification of a 1180 pb DNA genetic product corresponding to part of the VP2 coding sequence was carried out, as in Blake et al. (1995), using the forward primers PRA-1: 5′-TGA GAT CCA TTA TGC TTC CAG A-3′ (25 pmol) and reverse primers PRA-2: 5′-GAC AGG ATC ATC TTG GCA TAG T-3′. The product obtained was subjected to 1% agarose gel electrophoresis containing ethidium bromide for 30 min at a current of 70 mA. The sample was visualised using a transilluminator (TFX-20.M®, VILBER LOURMAT) and an image was obtained with *Kodak Digital Science 1D®* (Kodak) software, showing a 1180 pb product in the three strains used.

The PCR product was purified from the agarose gel using the *E.Z.N.A.® Gel Extraction Kit* (OMEGA BIO-TEK) and bound to the pGEMT-Easy® (PROMEGA) plasmid vector according to the supplier's instructions. Ten μl of binding solution (vector DNA) were mixed with 100 μl of competent cells (*E. coli* JM109) and incubated in ice for 30 min. These were then subjected to a thermal shock at 42 °C for 45 s. The tube was frozen for 2 min in ice and 0.9 ml of sterile S.O.C. medium (2% triptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl) were added, supplemented with Mg^2 (20 mM final concentration) and glucose (20 mM final concentration). The mixture was incubated at 37 °C for 60 min and agitated at 225 rpm. Then aliquots of 200 μl were incubated at 37 °C in a solid LB/ampicillin (50 μg/ml) medium for 16 h. Ampicillin-resistant colonies carrying the plasmid were

Author's personal copy

incubated for 16 h at 37 °C in 2 ml of LB/ampicillin liquid medium and agitated. 1.5 ml was centrifuged at 12,000 × *g* for 5 min at ambient temperature and the sediment was used for heat-induced extraction of DNA (Sambrook et al., 1989). Finally, the cDNA was eluted in 30 μl of sterile EPC water, and its concentration was determined by obtaining its optical density (O.D.) at 260 nm. The presence of VP2 insert was confirmed through universal PCR electrophoresis, showing the presence of the insert and the poly-cloning region or purely the latter in samples with no insert.

The VP2 cloning products were analyzed using the automatic sequencer (ABI PRISM 310, Applied Biosystems). The nucleotide sequences obtained were translated using the http://www.expasy.ch/software and the deduced aminoacid sequence was compared using *MEGA4*: Molecular Evolutionary Genetics Analysis 4.0 (http://www.megasoftware.net/) software, with VP2 sequences obtained from the NCBI (http://www.ncbi.nlm.nih.gov/), access codes: AJ829474 IPNV/Sp; AF342729 IPNV/Ab; L40584 IPNV/VR299. The GenBank access codes for VP2 of V122, V98 (as V33) and V70 were GU072914, GU072915 and GU072916, respectively.

2.3. Evaluating IPNV infectivity in vitro

The cell damage induced by the strains was evaluated using a modified cytotoxicity test. This determined the percentage of cells surviving after 72 hpi (Johansen et al., 2004; Rodríguez and Pérez-Prieto, 2006). CHSE-214 cells with 90% confluence were inoculated in sextuplicate in a 96-well plate at a multiplicity of infection (MOI) of 0.01, leaving 6 wells uninfected as a control. The plate was sealed and incubated at 15 °C. At 3 dpi, when the CPE was evident, the cells were washed once with PBS pH 7.2. Then they were stained for 10 min with 1% of crystal violet in 20% ethanol. The cells were washed three times with distilled water before the dye was dissolved in 100 μl of 50% ethanol containing 0.05 M of sodium citrate and 0.05 M of citric acid. The absorbency of the dye was determined using 550 nm spectrophotometry. The result was indicated as the percentage of cells surviving the viral infection, where 100% corresponded to absorbency in uninfected cells.

Possible alteration differences in the permeability of the membrane in CHSE-214 cells exposed to the 3 viruses were determined by evaluating the liberation level of the cytosolic enzyme lactate dehydrogenase (LDH) in the cell supernatant using the commercial *CytoTox 96® Assay* (Promega, Madison, WI, USA) kit. Cells with 90% confluence were infected at MOI = 1.0 in 96-well plates and after incubation at 15 °C for 6, 12, 20, 30, 36 and 48 hpi the LDH liberation level in 50 μl of supernatant was obtained in accordance with the supplier's instructions. As a control indicating 100% maximum LDH liberation, non-infected cells were treated with lysis solution and incubated for 45 min at 20 °C. After this time 50 μl received the same treatment as the infected samples. The result was obtained using the equation: % of cytotoxicity = [(LDH treated cells-control cells)/(maximum LDH liberation-control cells)] × 100 (Santi et al., 2005).

2.4. Determining viral titre in infected cells

To determine possible differences in the replication index of different strains, CHSE-214 cells were inoculated at MOI = 0.1 in 25 cm^2 culture bottles and at 12, 24, 36 and 45 hpi the supernatant was collected and assessed using the Reed and Muench method (Reed and Muench, 1938). As a control and to compare the replication levels of the three viruses during a simple viral replication cycle, 25 cm^2 plates were infected at MOI = 10. The supernatant was collected and assessed at 5, 10, 15 and 24 hpi.

3. Evaluating transcript induction for Mx3 protein

3.1. Infection in vivo

For the induction of *Mx* expression, groups of 15 fish were injected (i.p.) with one of the following: (A) 0.1 ml PBS containing poly I:C (50 $\mu g\,ml^{-1}$); (B) 0.1 ml PBS containing $10^{5.5}$ $TCID_{50}$/ml of the V112 IPNV strain; (C) 0.1 ml PBS containing $10^{5.5}$ $TCID_{50}$/ml of the V70 IPNV strain; (D) 0.1 ml PBS containing $10^{5.5}$ $TCID_{50}$/ml of the V98 IPNV strain or (E) 0.1 ml PBS (non-stimulated control); three salmon from each group were sacrificed at 1, 2 and 7 days infection, head kidney tissue was removed aseptically and processed to isolate the total RNA and to synthesise cDNAs, in order to determine the expression of Mx mRNA by real-time PCR.

3.2. Infection in vitro and extracting total RNA

Sub-confluent RTG-2 cells in 6-well plates were infected in triplicate at MOI = 0.1 in 300 μl of viral solution. After adsorption for 1 h at 15 °C the solution was replaced with 3 ml of L-15 medium with 2% FBS per well. The incubation continued at the same temperature until it was interrupted at 12, 24, 36 and 45 hpi when the supernatant was eliminated and replaced with 1 ml of Trizol® LS Reagent (Gibco) to initiate total RNA extraction. The RNA obtained was diluted in 25 μl of water containing no RNAses. Its concentration was quantified using 260 nm spectrophotometry (Collet et al., 2007). A similar procedure was carried out on cells inoculated with Poly I:C and non-inoculated control cells.

3.3. Real-time PCR

Amplifications were carried out in an iCycler iQ Real-Time PCR Detection System (Bio-Rad, Madrid, Spain) using TaqMan probes and primers designed to amplify the salmon actin (as an endogenous control), and Mx gene. Probes and primers designed to amplify the VP2 target gene were also developed. The probes were dual labelled with a reporter dye at the 5′end (FAM, 6-carboxy fluorescein) and a quencher dye at the 3′end (TAMRA, 6-carboxytetramthyrhodamine). Primers and probes were synthesised in Applied Biosystems and they are shown in Table 1.

PCR amplification was performed in a final volume of 20 μl and 1 μl of cDNA was added to the following mix in individual wells of a 96-well optical plate (Bio Rad): 10 μl of iQ supermix (Bio Rad), 8 μl of dH_2O and 1 μl of

Author's personal copy

Table 1
Primers and probes used in this study for real time RT-PCR analyses.

Gene	Accession no.	Name	Primers	Product size (bp)	Cycles	Cycling conditions
Mx1	U30253	Mx-5	F: 5′-GCTCCCTTGGCGTAGAGAAG-3′	65	1×	50 °C, 2 min
		Mx	P: 5′-CTGCCAGCCATCGCC-3′			95 °C, 10 min
		Mx-6	R: 5′-CGAACTCTGGTCCCCTATCAC-3′		40×	95 °C, 15s
						60 °C, 1 min
Actin	AJ438158	Actin-3	F: 5′-GGCCGTGTTGTCCCTGTAC-3′	57	1×	50 °C, 2 min
		Actin	P: 5′-CCTCTGGCCGTACCACC-3′			95 °C, 10 min
		Actin-4	R: 5′-CCGGAGTCCATGACGATACC-3′		40×	95 °C, 15 s
						60 °C, 1 min
IFN	AJ580911	IFN-F	F: 5′-GCGAAGTTATTAGCAGTTGAAAGCA-3′	68	1×	50 °C, 2 min
		IFN-P	P: 5′-AAGCTCGCGAATAGC-3′			95 °C 10 min
		IFN-R	R: 5′-CGGCTAGACTATTACTACAGCGAGAA-3′		40×	95 °C, 15 s
						60 °C, 1 min

Sequences for the Forward (F), Reverse (R) and Taqman probe (P).

a 20× mix containing the forward primer (18 μM), reverse primer (18 μM) and probe (5 μM, assay-by design service PE Applied Biosystem). The standard cycling conditions were 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The fluorescence output of each cycle was measured and recorded upon completion of the entire run, and a relative quantification of transcripts was performed. The efficiencies of the primers and probes were tested using a serial dilution of a known initial template (60–2 μg total RNA). The data were analyzed using the iQ5 Optical System Software (Version 2.0) and the relative quantification of the amplified gene products was calculated by the comparative Ct method. Fish injected with PBS were the calibrator group for the evaluation of Mx and VP2 gene expression. All samples were analyzed in triplicate and the results were expressed as the relative fold increase.

The value of the target normalised to the endogenous control (β-actin) was expressed as $2^{-\Delta\Delta Ct}$, where ΔCt is determined by subtracting the average β-actin Ct value from the average target Ct. The standard deviation of the difference is calculated from the standard deviation of the target and β-actin values, and ΔΔCt = ΔCt of samples of target gene −ΔCt of the calibrator.

3.4. Statistical analysis

Statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL, USA). Kruskal–Wallis test and Mann–Whitney test was used to evaluate statistical differences between groups. Values of $P < 0.05$ were considered statistically significant.

4. Results

4.1. Isolating and identifying IPNV

The presence and intracytoplasmic distribution of IPNV in CHSE-214 cells was confirmed from 24 hpi through IFAT using an anti-VP3 monoclonal antibody. The viruses were also identified by amplification of a 1180 pb cDNA gene product, corresponding to a fragment of the VP2 structural protein (Fig. 1). The bands obtained from the three isolates demonstrated the same migration pattern.

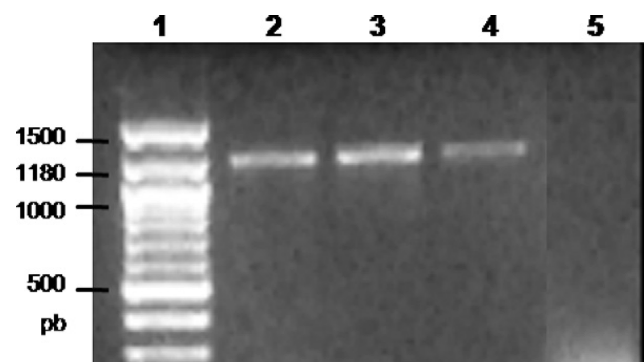

Fig. 1. Electrophoretic analysis of the specific PCR product of the IPNV VP2 protein. Detection of a 1180 pb gene product corresponding to the VP2 encoding region of the V70, V112 and V98 strains in lines 2, 3 and 4, respectively. Line 1 demarcates molecular weight (bench top 100 pb DNA ladder); line 5 appears blank for all reagents, except the sample.

Table 2
Similarity percentages for a 270-aminoacid segment of the IPNV VP2 protein among reference strains and the V70, V112 and V98 isolates.

Strain	Vs	Strain	aa similarity (%)
Sp		V7 0	98
Sp		V98	96
Sp		VR-299	88
Sp		V112	88
V7 0		V98	97
V7 0		VR-299	88
V7 0		V112	87
V7 0		Ab	87
V98		VR-299	87
V98		V112	87
VR-299		V112	96
VR-299		Ab	89
V112		Ab	89

GenBank access codes to obtain VP2 sequence in reference strains: L40584 (VR-299), AJ829474 (Sp), and AF342729 (Ab).

4.2. Sequencing and comparing VP2

The VP2 PCR reaction products were cloned (data not shown) in order to compare their aminoacid sequence with that of the reference strains. The identity percentage of V70, V98 and V112 with the Sp reference strain (GEnBank AJ829474) was 98%, 96% and 88%, respectively. For the VR-299 (L40584) reference strain, the identity values obtained

Author's personal copy

in the same order were 88%, 87% and 96%, whilst for the Ab (AF342729) strain percentages of 87%, 86% and 89%, respectively, were observed. Among the strains studied, V70 and V98 demonstrated 97% similarity and both demonstrated 87% similarity with V112 (Table 2).

Three genogroups were generated as a result of the comparisons. The first corresponded to Sp genotype strains, including the sequence of the V70 and V98 strains. The second genogroup contained only the Ab reference strain. The third contained the VR-299 and V112 strains (Fig. 2).

Table 3 indicates the position of those aa substitutes in a 270 residue VP2 segment of the analyzed strains and reference strains. It also shows the sequence in the 217 and 221 residues, which were previously implicated in IPNV virulence (Bruslind and Reno, 2000; Shivappa et al., 2004; Song et al., 2005). In the V70 strain these residues corresponded to Pro-217 and Ala-221. In V112 they corresponded to Ala-217 and Thr-221 and in the V98 strain they corresponded to Pro-217 and Thr-221. According to

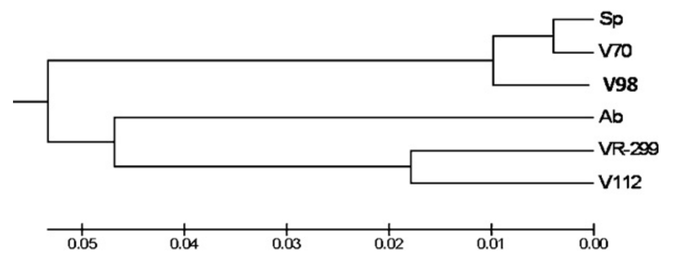

Fig. 2. Dendrogram, similarity of deduced aminoacid sequence in a 270 aa VP2 segment among the isolates V70, V112 and V98 and the Sp reference strains, VR-299 and Ab. Access codes for VP2 of reference strains: L40584 (VR-299), AJ829474 (Sp), and AF342729 (Ab).

this, V70 would be considered as one of the most virulent strains and the last two strains would be considered to have low virulence (Santi et al., 2004). The table also indicates those residues which are thought to be conserved between genogroups. The 72, 76, 243 and 261 residues can in fact

Table 3
Multiple alignment of the deduced aminoacid sequence of 270 residues in the VP2 of the IPNV strains V70, V112, V98 and reference strains VR-299, Sp and Ab.

Strain							
Sp	MLPETGPASI	PDDITERHIL	KQETSSYNLE	VSDSGSGILV	CFPGAPGSRI	GAHYRWNANQ	[74]
V70	....	....	....	..E.......	....	....	[74]
V98	....	....	....	..E....L..	....	....	[74]
VR-299	....N.....	....	....	..E....L..	V	.......L..	[74]
V112	....N.....	....	....	.......L..	V	.......L..	[74]
Ab	.......S..	....	....	.......V..	V	.......V..	[74]
Sp	TGLEFDQWLE	TSQDLKKAFN	YGRLISRKYD	IQSSTLPAGL	YALNGTLNAA	TFEGSLSEVE	[134]
V70	....	....	....	....	....	....	[134]
V98	....	....	....	....	....	....	[134]
VR-299	.A........	....	....	....	....	....	[134]
V112	.E....R...	....	....	....	....	....	[134]
Ab	.E........	....	....	V.........	......I...	....	[134]
Sp	SLTYNSLMSL	TTNPQDKVNN	QLVTKGVTVL	NLPTGFDKPY	VRLEDETPQG	LQSMNGAKMR	[194]
V70	....	....	....	....	....	....	[194]
V98	....	....	....	....	....	....	[194]
VR-299	....	....	......I...	....	....	P......R..	[194]
V112	....	....	......I...	....	....	S......R..	[194]
Ab	....	....	....	....	....	P......R..	[194]
Sp	CTAAIAPRRY	EIDLPSQRLP	PVPATGALTT	LYEGNADIVN	STTVTGDINF	SLAEQPAIET	[254]
V70	....	....	....	....	....	.......V..	[254]
V98	....T.....	....	......T...	....	....	.......V..	[254]
VR-299	....	......E...	T.A...TP..	I.........	..A.....T.	Q.EAE.VN..	[254]
V112	....	......E...	T.A...TP..	I.........	.......VT.	Q..AE..N..	[254]
Ab	....	.?....E...	T.A...TP..	I.?.?G....	........S.	...NN.TADI	[254]
Sp	KFDFQLDFMG	LDNDVPVVTV	[274]				
V70	....	....	[274]				
V98	....	...H......	[274]				
VR-299	R...I.Q.L.	....	[274]				
V112	R...I.Q.L.	........S.	[274]				
Ab	........L.	....	[274]				

Author's personal copy

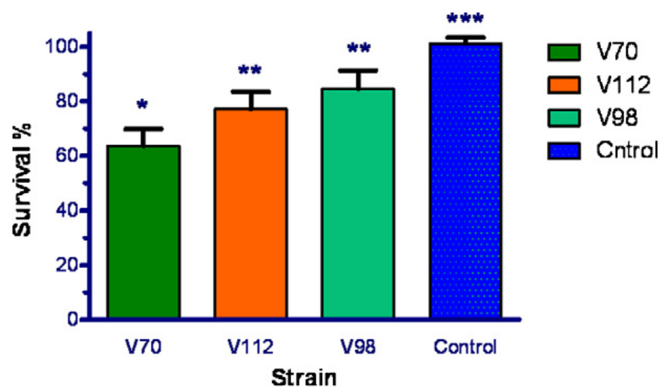

Fig. 3. Survival percentage of CHSE-214 cells infected at MOI = 0.01 with the V70, V112, V98 viruses and of non-infected CHSE-214 cells. The data shows the averages from 6 parallel samples taken at 72 hpi. Means values with the same asterisk inidicates no differences.

be seen to correspond among genogroups obtained in the alignment.

5. Evaluating IPNV infectivity *in vitro*

5.1. Cytotoxicity test

Fig. 3 shows the results from the cytotoxicity test to evaluate the virulence of the three strains. The survival percentage in cells inoculated with V70, V112 and V98 was 64%, 78% and 83%, respectively. Non-infected cells, however, demonstrated 100% survival. The experiment was repeated on three occasions without any significant variation. The result indicates that with an infection at MOI = 0.01, V70 had more infectivity than V112 and V98 for CHSE-214 cells.

5.2. Lactate dehydrogenase liberation

On evaluating the level of LDH liberation in CHSE-214 cells inoculated with the three viruses, it was observed that between 6 and 12 hpi the three strains presented a minimum increase in liberation up until around 20 hpi, after which a moderate decrease was recorded at 30 hpi. After this time, the three viruses demonstrated a very significant increase, but the cells infected with V70 showed the greatest level of liberation. The increase in V112 and V98 was similar but varied from that in V70 after 30 hpi (Fig. 4).

5.3. Viral replication kinetics

As Fig. 5 shows, the three viruses demonstrated similar replication behaviour. However, it is noticeable that at 10 hpi the V98 strain presented a higher titre than the other two strains. However, later in the incubation period the three viruses presented high titres from 15 hpi and the same viral titre at 24 hpi.

In an infection at MOI = 0.1, at 12 hpi the V98 supernatant presented the highest titre. However, at 24 hpi the three viruses presented a similar value of $1 \times 10^{5.5}$. At 36 hpi the V98 strain presented a similar titre to that obtained for V70, whereas the V98 titre was slightly higher than the V70 titre at 45 hpi. It is noticeable that V112 presented lower titres at 12 and 36 hpi (Fig. 6).

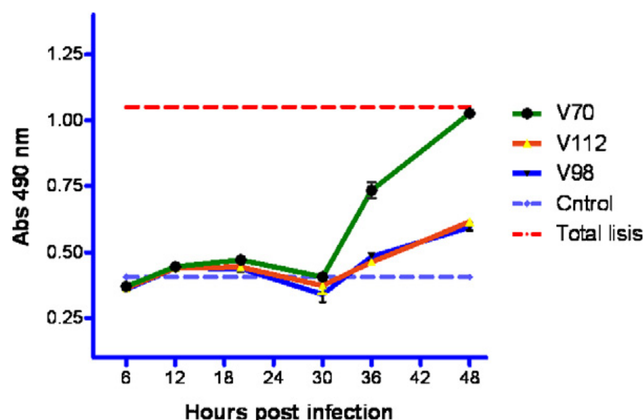

Fig. 4. LDH liberation in CHSE-214 cells infected at MOI = 1 with the V70, V112 and V98 viruses. Average data from three parallel samples.

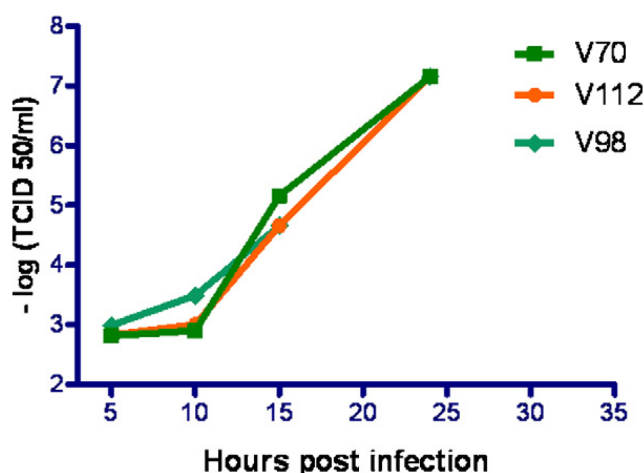

Fig. 5. Viral titres obtained in CHSE-214 cell cultures infected with the V70, V112 and V98 IPNV strains at MOI = 10 expressed as $TCID_{50}$/ml.

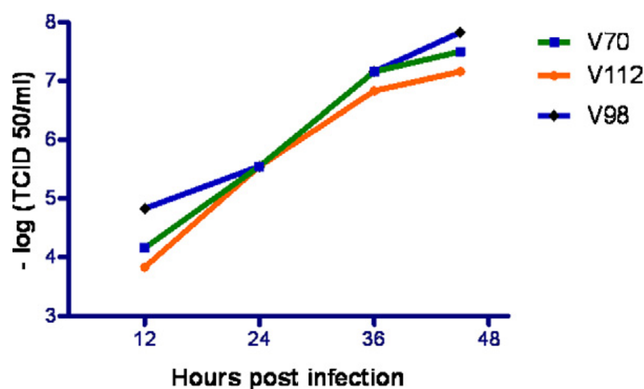

Fig. 6. Viral titres in supernatant from CHSE-214 cell cultures infected with IPNV strains at MOI = 0.1 expressed as $TCID_{50}$/ml.

5.4. Morphology of viral particles by EM

The electron microscopy images of CHSE-214 cells infected with the three viruses revealed the presence of icosahedral particles approximately 60 nm in diameter, distributed throughout the cell cytoplasm. The formation of tubular structures was not evident. In the cells infected with V112 the particles were rarer and isolated, unrelated to compartments and did not form clusters. In the other

Author's personal copy

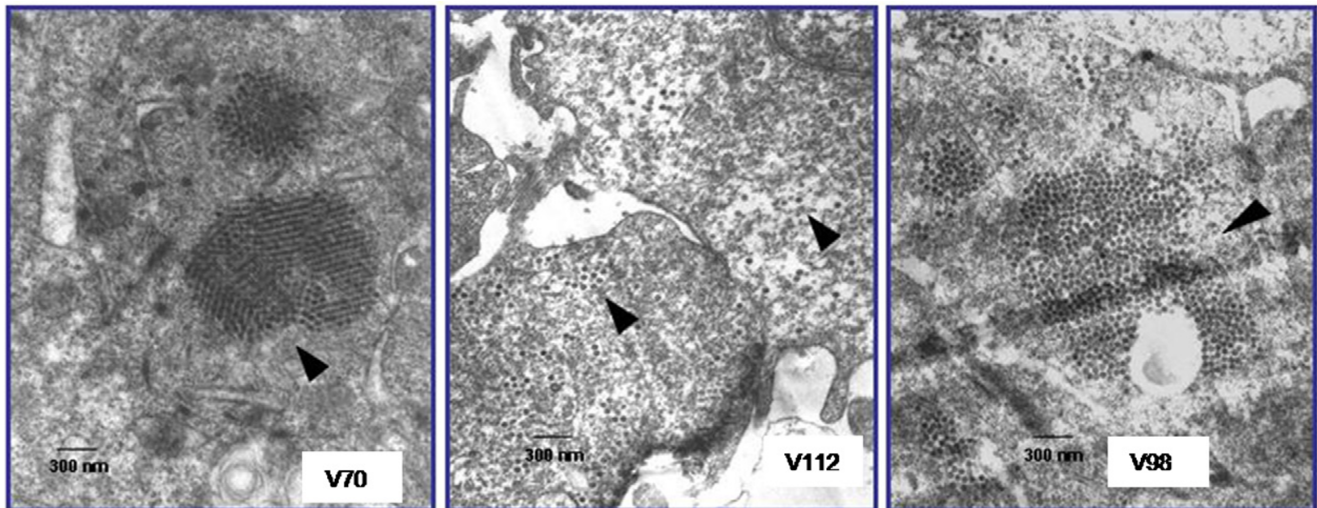

Fig. 7. Electron microscopy of the transmission of CHSE-214 cells infected with the V70, V112 and V98 viruses. The particles distributed throughout the cytoplasm present the typically hexagonal form of IPNV. They are more disperse in V112 (arrows). Actual magnification: 7×.

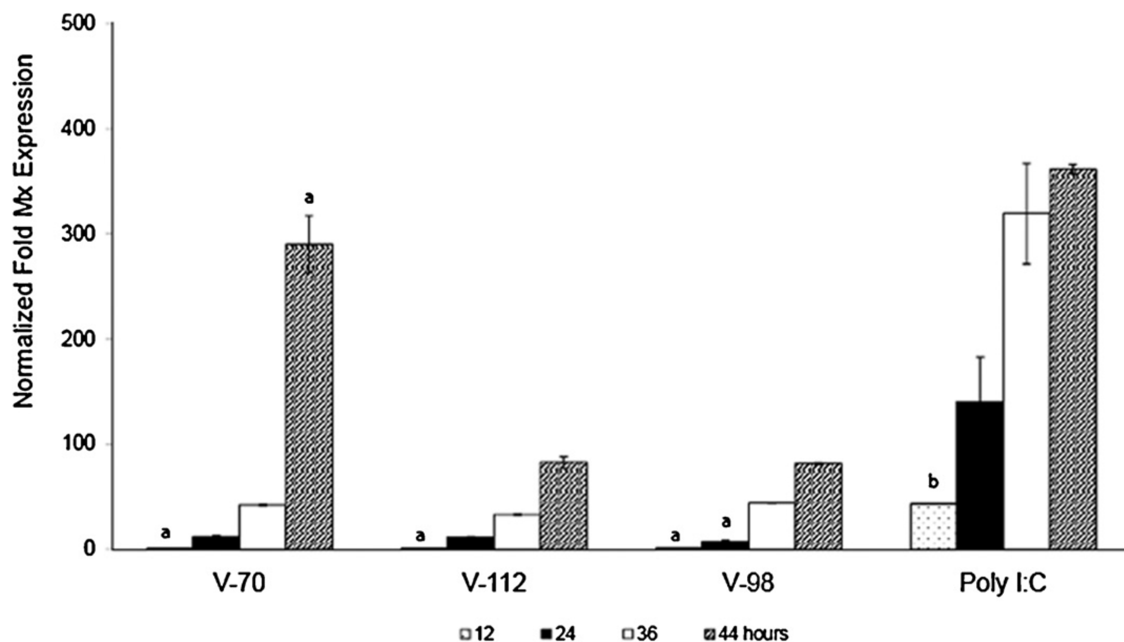

Fig. 8. Levels of Mx expression in RTG-2 cell line in response to infection with V112, V70, V98 at MOI = 0.1 or inoculation with Poly I:C to 12, 24, 36 and 44 h. Data are presented as mean relative expression ± standard deviation. Means values with an "a" are significantly different than data for the same isolate, whilst means values with "b" are significantly different than levels for the other isolates at the same hour. Kruskal–Wallis test and Mann–Whitney test was used to evaluate statistical differences between groups. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

two viruses the particles were abundant, forming clusters or accumulations that might correspond to virus factories or virus deposits (Fig. 7).

5.5. Expression of Mx3/β-actin in RTG-2 cells infected with IPNV

On evaluating the level of Mx3 synthesis in RTG-2 cells infected with the three viruses, in RTG-2 cells incubated with Poly I:C and in non-infected RTG-2 cells, it was observed that IPNV stimulates the transcription of this gene from 24 hpi with higher expression values being observed at 44 hpi, however, the expression always was lesser that poly I:C stimulated cells. Interesting, at the 44 hpi the Mx expression in V70 strain was higher at the another ones strains ($P > 0.05$), but lower at those cells incubated with poly I:C (Fig. 8).

5.6. Mx3 expression in head kidney of S. salar infected with IPNV

Kidney samples from the fish infected with the three viruses demonstrated a rapid induction in the synthesis of the Mx transcript, and in general the levels were significantly lower that poly I:C; however, the expression with V112 after 7 days is 5-fold higher than V70 and 3-fold

Author's personal copy

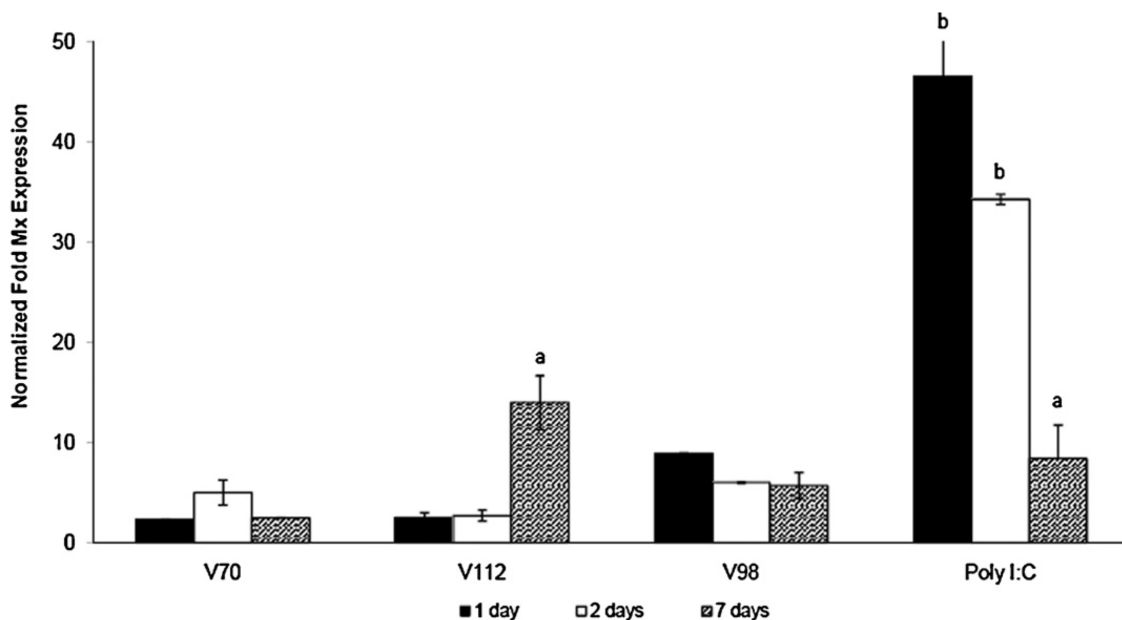

Fig. 9. Levels of Mx expression in head kidney in response to infection with V112, V70, V89 or inoculation with Poly I:C to 1, 2 and 7 days. Data are presented as mean relative expression ± standard deviation. Means values with an "a" are significantly different than data for the same isolate, whilst means values with "b" are significantly different than levels for the other isolates at the same date. Kruskal–Wallis test and Mann–Whitney test was used to evaluate statistical differences between groups. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using SPSS 17.0 (SPSS, Chicago, IL, USA).

than V98 and higher than poly I:C effect at the same time. The fishes inoculated with the synthetic oligonucleotide presented rapid expression on day 1, with values that gradually decreased up until the seventh dpi (Fig. 9).

6. Discussion

Owing to its wide antigenic and genotypic variability, the infectious pancreatic necrosis virus (IPNV) infects a huge number of aquatic organism species in fresh and saltwater. However, the viral and cellular elements that participate in the infection and pathogenesis are not fully known. In this study, the infectivity of three IPNV strains that caused different mortality pictures in the farms where they were obtained was linked to the aminoacid sequence of the VP2 protein and the induction level of mRNA synthesis for Mx3 as an antiviral defence mechanism.

The genotyping, through the deduced aminoacid sequence of a 1180 pb fragment of the VP2 protein, indicates that the V70 and V98 strains present molecular characteristics that link them to the Sp serotype, whilst V112 demonstrated a great similarity to VR-299. In support of this, the sequence also presented a link with the highly retained residues among genotypes: 72, 76, 243 and 261, commonly used to classify IPNV (Blake et al., 2001; Heppell et al., 1993). The result may be the reason for the antigenic differences observed whilst identifying the strains using IFAT, where cells infected with V70 and V98 presented a more intense signal and greater formation of inclusion bodies than V112, which may relate to the fact that during IPNV replication VP2 and VP3 are manifested in different stages (Espinoza et al., 2000). Given that in the immune reaction anti-VP3 was only used at one time, the expression kinetics of both proteins may confirm possible differences between strains, relating to the lower replication index in V112.

The VP2 protein is considered to be one of the main determinants in IPNV virulence (Sano et al., 1992; Song et al., 2005), through a domain which holds the residues that participate in the adsorption and epitopes of cell recognition (Coulibaly et al., 2005). Song et al. (2005) defined residues 217 and 221 as the main determinants of virulence and stated that in certain combinations they show variation. Strains that encode Thr-217 and Ala-221 are more virulent. Those which encode Pro-217 and Ala-221 are moderately virulent, and the strains encoding Thr-221, irrespective of the amino acid residue at position 217, are avirulent, ruling out the Thr/Ala-247 residue as a significant virulence factor. Consequently, V70 corresponds to a moderately virulent strain and both V112 and V98 to avirulent strains. This contravenes statements by various other authors who claim that Sp strains are more virulent than VR-299 (Blake et al., 2001). However, in the studies *in vitro* V112 and V98 showed differences which suggest that other factors may affect virulence, as was proposed by Smail et al. (2006), on observing that strains with Pro-217 and Ala-221 residues in VP2 have a similar virulence to Thr-217 and Ala-221 strains, as analyzed by Song et al. (2005). In this sense, it has been noted that polypeptides derived from VP2 maturation may cause deformation, instability and perforation of the cell membrane, through conformational changes suffered by the peptide, owing to the presence of proline in its structure (Chevalier et al., 2005; Galloux et al., 2007).

The strains were chosen for their clinical history and studies *in vitro* were carried out to establish differences in virulence. The cytotoxicity test, carried out at a low multiplicity of infection, indicates the greater infectivity of V70 on generating greater cell mortality. Moreover, despite the

Author's personal copy

LDH level in cells infected with the three strains not presenting differences prior to 30 hpi, after this time V70 was differentiated from V112 and V98 by its greater liberation of the enzyme, owing to greater damage of the cell membrane (Santi et al., 2005). This suggests that in addition to having the capacity to establish the infectious cycle and overcome cell barriers, this virus may also have a greater replication index and consequent liberation of viral particles with infective capacity (Song et al., 2005).

As regards the replication index, in general the V112 virus presented lower titres than V70 and V98, a result which is concurrent with the cell survival and LDH liberation tests. It was noted that at 12 hpi, V98 presented higher titres than the other two strains. However, when the final readings were taken the titre of both Sp strains was similar. This result may indicate that V98 presents a higher adsorption rate or a higher initial replication index. It was noted that the 217, 221 and 247 residues of VP2 influence the IPNV replication index. The strains that encode Pro-217 and Ala-247, as was the case with V70 and V98, present higher titres in comparison with those presented by Thr-217 and Thr-247 (Song et al., 2005). If the sequence of these residues does indeed affect replication, this could be a possible explanation for the result obtained.

Viruses that establish rapid specific binding to the cell surfaces have greater pathogenic potential if they later manage to enter the cell, replicate and mature (Kuznar et al., 1995; Granzow et al., 1997), having to overcome the cell response (Brandt et al., 2001). Adsorption is a process which depends on the cell type (Imajoh et al., 2003) and is also related to the VP2 sequence (Song et al., 2005; Smail et al., 2006). In this study, both V112 and V98 presented Thr-221. However, in accordance with the observations of Bruslind and Reno (2000), strains that present Ala-217 and Thr-221, as is the case with V112, would be less virulent as they present a lower ability to interact with cell receptors. In spite of this, whether other residues may be involved in this process and the type of interaction with the viral receptor remains to be determined. In this sense, in the case of IBDV it has still not been confirmed whether virulence is related to the capacity to bind to a 40–46 kDa polypeptide of the B lymphocytes, which is considered to be the receptor for serotype I or virulent virus strains (Nieper and Muller, 1996).

Structural or functional differences between the isolates may also be responsible for V70 and V98 potentially being more infective than V112. The electron microscope photographs taken to establish whether the viral particles present structural differences either individually or in aggregation, show that the virons of the three strains presented the typical pentagonal or hexagonal form of approximately 60 nm, but they did not present abnormal or elongated tubular forms. It was noticeable that the V112 particles were rarer and more isolated, and did not form clusters or accumulations that might correspond to virus factories or deposits (Espinoza et al., 2000). Generally, this finding relates directly to the remainder of the tests.

IPNV may be inhibited in cells induced to express Mx (Trobridge and Leong, 1995; Larsen et al., 2004); this protein would delay the synthesis and maturing of viral proteins when they are pre-treated with IFN or receive the cytokine at the very outset of the infection; however, in cases where the infection occurs without prior stimulation, even when Mx expression is evident, the virus continues to replicate, presumably by blocking the cell's ability to establish antiviral activity (Skjesol et al., 2009). However, the mechanism, and viral and cellular components involved are not clearly defined. It is also not known where there are differences in the level of induction between different strains.

Although differences in the induction levels among IPNV and other viruses have been reported (Rodríguez and Pérez-Prieto, 2007), there is no record of previous direct studies *in vitro* and *in vivo* using different strains. In this study, irrespective of the infectivity demonstrated and its aminoacid sequence in VP2, the three strains induced Mx transcript synthesis in RTG-2 cells and *S. salar* kidneys, which is concurrent with previous studies that have reported on their expression during IPNV infection (Trobridge and Leong, 1995; Jensen et al., 2002b; Collet et al., 2004; McBeath et al., 2007; Rodríguez and Pérez-Prieto, 2007); however, in both models in general the level of Mx induction for the three strains was less than that of poly I:C, and the probable differences between strains were observed only in the last record evaluated. The result suggests that though IPNV induces the Mx synthesis, the response is late and poor; however, it is difficult to establish the reason for the increased late induction of V70 *in vitro* and V112 *in vivo*, which showed different infectivity and sequence levels in this study. This could be linked to what Skjesol et al. (2009) report, which is that despite Mx expression being a response induced by IFN, in other models, alternate means of antiviral gene induction have been observed; this is a condition that has yet to be defined in the case of infection with IPNV and of possible differences between strains.

Certain viruses have developed strategies to evade the antiviral response; the hepatitis B virus (HBV), for example, through the capsid protein (HBc), is able to suppress MxA induction from its promotor, without affecting the synthesis of other IFN-induced proteins. In this sense, the differences in the VP2 sequence as a protein are not significant, given that IPNV replicates in the cell cytoplasm and its proteins or subproducts do not arrive in the nucleus. Nevertheless, one probability to be determined could be the inhibiting of the JAK-STAT signalling pathway, through the blocking of Tyk2 receptor activation, as was seen with the Japanese encephalitis virus (JEV) (Lin et al., 2004); in this case, Hong and Wu (2002) suggested that IPNV might alter cell signalling through its proteins or subproducts derived from processing, affecting the cell defence response to favour its propagation; however, with the exception of VP5, which has a questionable capacity to evade the apoptosis process, the way in which other viral proteins interact with cell elements is not known. Recently, Skjesol et al. (2009), reported that the VP4 protease and the non-structural VP5 protein inhibit IFN signalling by means of mechanisms which have yet to be determined, given that during the infection levels of STAT1 and STAT2 would seem to remain constant. The fact that some strains lack VP5 expression raises the issue of determining whether these strains affect IFN expression. It is worth noting that the V70 and V98

Author's personal copy

strains presented a truncated BH2 domain in VP5, whilst the domain was complete in V112 (data not shown).

In this study, the V112 strain, which showed a lower rate of infectivity and was associated with low virulence strains in its gene sequence (Song et al., 2005), showed increased late induction of Mx at 7 dpi, which could be linked to the hypothesis that less virulent strains induce a greater antiviral response; however, the study enables no definitive conclusion to be reached in this respect.

The fishes used in the study correspond to the stage susceptible to clinical IPNV infection, where the antiviral response against IPNV via Mx is more rapid and intense than in older fish (Rodríguez and Pérez-Prieto, 2007), because the nonspecific immune system's need to protect fish through natural limitation prevents an efficient specific rapid response (Lockhart et al., 2007). The late appearance of Mx mRNA in animals which are not susceptible is also linked to low levels of virus; this indicates low replication and therefore low initial stimulation. Despite this, there were no observed cases of mortality linked to the disease, although the virus was able to be reisolated. In this sense, it must be remembered that the clinical disease begins at approximately 7 dpi, when the third and final sample was taken, and it is possible that some fish may have been sacrificed before developing any clinical signs. Lockhart et al. (2004), establish that the rapid induction of IFN probably delays presentation of the disease. However, in other cases it has been observed that IFN expression by poly I:C is not capable of protecting against the infection. It is therefore difficult to establish whether the fish actually achieved some level of protection.

In other ways, the fishes inoculated with poly I:C showed a higher Mx induction level in the first dpi with lower levels at two dpi and minor levels at 7 dpi, when the level was lower that V112; Rodríguez and Pérez-Prieto (2007) comment that this fact could be associated because the poly I:C effect is not active in the time as it is on the viral infection.

The strains used in this study showed differences in genotype and infectivity together with certain variations in the sequence of residues considered significant in VP2, and both *in vivo* and *in vitro* the three viruses induced Mx mRNA expression. However, the induction level observed among the strains suggest that in the early infection the genotype and infectivity differences found in the viruses used do not translate into clear differences in the level of antiviral responses caused by Mx.

Acknowledgements

This study was supported in part by the National Council of Science and technology (Conacyt) project 99736, and PROMEP 103.5/09/4196 at Secretaría de Educación Pública from México; and Spanish Ministerio de Ciencia y Tecnología MICIIN, grants AGL2004-0382-E and PIE 20102OE084 (CSIC). And the contributions of Escuela de Graduados, Facultad de Ciencias Veterinarias and Laboratorio de Biotecnología y Patología Acuática, Instituto de Patología Animal of the Universidad Austral de Chile as well.

References

Blake, S.L., Schill, W.B., McAllister, P.E., Lee, M.K., Singer, J.T., Nicholson, B.L., 1995. Detection and identification of aquatic birnaviruses by PCR assay. J. Clin. Microbiol. 33, 835–839.

Blake, S.L., Ma, J.Y., Caporale, D.A., Jairath, S., Nicholson, B.L., 2001. Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. Dis. Aquat. Org. 45, 89–102.

Brandt, M., Yao, K., Liu, M., Heckert, R.A., Vakharia, V.N., 2001. Molecular determinants of virulence, cell tropism, and pathogenic phenotype of infectious bursal disease virus. J. Virol. 75, 11974–11982.

Bruslind, L.D., Reno, P.W., 2000. Virulence comparison of three Buhl-subtype isolates of infectious pancreatic necrosis virus in brook trout fry. JAAH 12, 301–315.

Caipang, C.M., Hirono, I., Aoki, T., 2003. In vitro inhibition of fish rhabdoviruses by Japanese flounder. *Paralichthys olivaceus* Mx. Virology 317, 373–382.

Chevalier, C., Galloux, M., Pous, P., Henry, C., Denis, J., Da Costa, B., Navaza, J., Lepault, J., Delmas, B., 2005. Structural peptides of a nonenveloped virus are involved in assembly and membrane translocation. J. Virol. 79, 12253–12263.

Collet, B., Boudinot, P., Benmansour, A., Secombes, C.J., 2004. A luciferase reporter system based on Mx1 promoter to study interferon pathways in rainbow trout. Dev. Comp. Immunol. 28, 793–801.

Collet, B., Munro, E.S., Gahlawat, G., Acosta, F., Garcia, J., Roemelt, C., Zou, J., Secombes, C.J., Ellis, A.E., 2007. Infectious pancreatic necrosis virus suppresses type I interferon signalling in rainbow trout gonad cell line but not in Atlantic salmon macrophages. Fish Shellfish Immunol. 22, 44–56.

Coulibaly, F., Chevalier, C., Gutsche, I., Pous, P., Navaza, J., Bressanelli, S., Delmas, B., Ray, F.A., 2005. The birnavirus crystal structure reveals structural relationships among icosahedral viruses. Cell 120, 761–772.

Dobos, P., 1995. The molecular biology of infectious pancreatic necrosis virus. Annu. Rev. Fish Dis. 5, 25–54.

Espinoza, J.C., Hjalmarsson, A., Everitt, E., Kuznar, J., 2000. Temporal and subcellular localization of Infectious pancreatic necrosis virus structural proteins. Arch. Virol. 145, 739–748.

Frost, P., Håvardstein, L.S., Lygrenm, B., Ståhl, S., Endresen, C., Christie, K.E., 1995. Mapping of neutralization epitopes on infectious pancreatic necrosis viruses. J. Gen. Virol. 76, 1165–1172.

Galloux, M., Libersou, S., Morellet, N., Bouaziz, S., Da Costa, B., Ouldali, M., Lepault, J., Delmas, B., 2007. Infectious bursal disease virus, a non-enveloped virus, possesses a capsid-associated peptide that deforms and perforates biological membranes. J. Biol. Chem. 282, 20774–20784.

Granzow, H., Weiland, F., Fichtner, D., Enzmann, J., 1997. Studies of the ultrastructure and morphogenesis of fish pathogenic viruses grown in cell culture. J. Fish Dis. 20, 1–10.

Haller, O., Frese, M., Kochs, G., 1998. Mx proteins: mediators of innate resistance to RNA viruses. Rev. Sci. Technol. 17, 220–230.

Heppell, J., Berthiaume, L., Corbin, F., Tarrab, E., Lecomte, J., Arella, M., 1993. Comparison of amino acid sequences deduced from a cDNA fragment obtained from infectious pancreatic necrosis virus strains of different serotypes. Virology 195, 840–844.

Hong, J.R., Wu, J.L., 2002. Induction of apoptotic death in cells via *Bad* gene expression by Infectious pancreatic necrosis virus infection. Cell Death Differ. 9, 113–124.

Imajoh, M., Yagyu, K., Oshima, S., 2003. Early interaction of marine birnavirus infection in several cell lines. J. Gen. Virol. 84, 1809–1816.

Jensen, V., Robertsen, B., 2000. Cloning of a Mx cDNA from Atlantic halibut (*Hippoglossus hipoglossus*) and characterization of Mx mRNA expression in response to double-stranded RNA or infectious pancreatic necrosis virus. J. Interferon Cytokine Res. 20, 701–710.

Jensen, I., Robertsen, B., 2002. Effect of double-stranded RNA and interferon on the antiviral activity of Atlantic salmon cells against infectious salmon anaemia virus and infectious pancreatic necrosis virus. Fish Shellfish Immunol. 13, 221–224.

Jensen, I., Larsen, R., Robertsen, B., 2002a. An antiviral state induced in Chinook salmon embryo cells (CHSE-214) by transfection with the double-stranded RNA poly I:C. Fish Shellfish Immunol. 13, 367–378.

Jensen, I., Albuquerque, A., Sommer, A., Robertsen, B., 2002b. Effect of poly I:C on the expression of Mx proteins and resistance against infection by infectious salmon anaemia virus in Atlantic salmon. Fish Shellfish Immunol. 13, 311–326.

Johansen, A., Collet, B., Elin, S., Secombes, C.J., Jorgensen, J.B., 2004. Quantification of Atlantic salmon type-I interferon using and Mx1 promoter reporter gene assay. Fish Shellfish Immunol. 16, 173–184.

Author's personal copy

Kuznar, J., Soler, M., Farias, G., Espinoza, J.C., 1995. Attachment and entry of Infectious pancreatic necrosis virus (IPNV) into CHSE-214 cells. Arch. Virol. 140, 1833–1840.
Larsen, R., Røkenes, T.P., Robertsen, B., 2004. Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein. J. Virol. 78, 7938–7944.
Lin, R.J., Liao, C.L., Lin, E., Lin, Y.L., 2004. Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. J. Virol. 78, 9285–9294.
Lockhart, K., Gahlawat, S.K., Soto-Mosquera, D., Bowden, T.J., Ellis, A.E., 2004. IPNV carrier Atlantic salmon growers do not express Mx mRNA and poly I:C induced Mx response does not cure the carrier state. Fish Shellfish Immunol. 17, 347–352.
Lockhart, K., McBeath, A.J., Collet, B., Snow, M., Ellis, A.E., 2007. Expression of Mx mRNA following infection with IPNV is greater in IPN-susceptible Atlantic salmon post-smolts than in IPN-resistant Atlantic salmon parr. Fish Shellfish Immunol. 22, 151–156.
McBeath, A.J., Snow, M., Secombes, C.J., Ellis, A.E., Collet, B., 2007. Expression kinetics of interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection with infectious pancreatic necrosis virus and infectious salmon anaemia virus. Fish Shellfish Immunol. 22, 230–241.
McKnight, I.J., Roberts, R.J., 1976. The pathology of infectious pancreatic necrosis. 1. The sequential pathology of the naturally occurring condition. Br. Vet. J. 132, 78–86.
Nieper, H., Muller, H., 1996. Susceptibility of chicken lymphoid cells to infectious bursal disease virus does not correlate with the presence of specific sites. J. Gen. Virol. 77, 1229–1237.
OIE, Oficina Internacional de Epizootias, 2005. Aquatic Animal Health Code, 8th ed. Paris, France.
Reed, J.L., Muench, H., 1938. A simple method for estimating fifty percent end points. Am. J. Hyg. 27, 493–497.
Robertsen, B., 2006. The interferon system of teleost fish. Fish Shellfish Immunol. 20, 172–191.
Rodríguez, S., Pérez-Prieto, S.I., 2006. Interferon mediated antiviral activity against salmonid fish viruses in BF-2 and other cell lines. Vet. Immunol. Immunopathol. 110, 1–10.
Rodríguez, S., Pérez-Prieto, S.I., 2007. Effects of salmonid fish viruses on Mx gene expression and resistance to single or dual viral infections. Fish Shellfish Immunol. 23, 390–400.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, USA.
Sano, M.N., Okamoto, H., Saneyoshi, M., Sano, T., 1992. Virulence of infectious pancreatic necrosis virus is associated with the larger RNA segment (RNA segment A). J. Fish Dis. 15, 283–293.
Santi, N., Vakharia, V.N., Evensen, Ø., 2004. Identification of putative motifs involved in the virulence of infectious pancreatic necrosis virus. Virology 322, 31–40.
Santi, N., Song, H., Vakharia, V.N., Evensen, Ø., 2005. Infectious pancreatic necrosis virus VP5 Is dispensable for virulence and persistence. J. Virol. 79, 9206–9216.
Shivappa, R.V., Song, H., Yao, K., Aas-Eng, A., Evensen, Ø., Vakharia, V.N., 2004. Molecular characterization of Sp serotype strains of infectious pancreatic necrosis virus exhibiting differences in virulence. Dis. Aquat. Org. 61, 23–32.
Skjesol, A., Aamo, T., Hegseth, M.N., Robertsen, B., Jorgensen, J.B., 2009. The interplay between infectious pancreatic necrosis virus (IPNV) and the IFN system: IFN signalling is inhibited by IPNV infection. Virus Res. 143, 53–60.
Smail, D.A., Bain, N., Bruno, D.W., King, J.A., Thompson, F., Pendrey, D.J., Morrice, S., Cunningham, C.O., 2006. Infectious pancreatic necrosis virus in Atlantic salmon, *Salmo salar* L., post-smolts in the Shetland Isles. Scotland: virus identification, histopathology, immuno histochemistry and genetic comparison with Scottish mainland isolates. J. Fish Dis. 29, 31–41.
Song, H., Santi, N., Evensen, Ø., Vakharia, V.N., 2005. Molecular determinants of infectious pancreatic necrosis virus virulence and cell culture adaptation. J. Virol. 79, 10289–10299.
Trobridge, G.D., Leong, J.A., 1995. Characterization of a rainbow trout Mx gene. J. Interferon Cytokine Res. 15, 691–702.
Wolf, K., 1988. Fish Viruses and Fish Viral Diseases. Canstock Publishing Associates-Cornell University Press, Ithaca, NY.