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Comparison of two birnavirus–rhabdovirus coinfections in fish cell lines

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ABSTRACT: Aquabirnaviruses, such as the infectious pancreatic necrosis virus (IPNV), Novirhabdoviruses, such as the infectious hematopoiteic necrosis virus (IHNV) and the viral hemorrhagic septicemia virus (VHSV), cause considerable losses to the salmonid industry worldwide. Coinfections of 2 viruses have been described, but the interactions between rhabdoviruses and birnaviruses have not been examined closely. Using virus titration, flow cytometry and RT-PCR assays, we compared the effect of IPNV on the replication of IHNV and VHSV in tissue culture cells. RT-PCR assays indicated that simultaneous infection of IPNV with VHSV does not affect the replication of the rhabdovirus either in the first or successive passages; the infective titers were similar in single and double infections. In contrast, coinfection of IPNV with IHNV induced a fall in infectivity, with reduced expression of IHNV viral antigens in BF-2 cells from *Lepomis macrochirus* and a loss of 4.5 $\log_{10}$ units of the infective titer after 3 successive passages. It was possible to stimulate BF-2 cells to produce significant interferon-like activity against IHNV but not against VHSV.

KEY WORDS: Infectious pancreatic necrosis virus · Infectious hematopoiteic necrosis virus · Viral hemorrhagic septicemia virus · Coinfections · Fish viruses

INTRODUCTION

Viral diseases have affected the development of aquaculture worldwide. Aquabirnaviruses, such as the infectious pancreatic necrosis virus (IPNV), and Novirhabdoviruses, such as the infectious haematopoiteic necrosis virus (IHNV) and the viral hemorrhagic septicemia virus (VHSV), have a severe impact on the industry's economy. All cause systemic infections with high mortality in young farmed rainbow trout *Oncorhynchus mykiss* and other salmonids, while the fish that survive a viral outbreak may, nevertheless, be asymptomatic carriers (Kim et al. 1999).

Originally enzootic on the west coast of North America, IHNV has been detected in Japan, Taiwan, China, Korea and Europe, where it has become established among several populations of reared rainbow trout (Winton 1991, LaPatra 1996). VHSV has historically affected farmed fishes in Europe (Wolf 1988), where until recently it was considered to cause a disease confined to freshwater salmonids. However, the presence of the virus in a wider range of marine fish species has been confirmed by numerous reports (Meyers et al. 1992, Mortensen et al. 1999).

IPNV is distributed worldwide and has also been isolated from other species of freshwater and marine fishes, molluscs and rotifers (Wolf 1988, Reno 1999, Rodriguez et al. 2003).

The broad distribution of IPNV, IHNV and VHSV among salmonids may favor the presence of several viruses in a single population. Some viral coinfections in fishes have been described: Schlotfeld & Frost (1974) showed that rainbow trout can be infected with IPNV and VHSV; naturally occurring coinfections of IPNV and IHNV were reported in rainbow trout by Mulcahy & Fryer (1976), LaPatra et al. (1993), Vilas et al. (1994) and Rodríguez et al. (1995)

Other coinfections in Atlantic salmon *Salmo salar* with IPNV and infectious salmon anemia virus (ISAV) have also been reported, with evidence that IPNV

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provides some protection against the secondary ISAV infection (Johansen & Sommer 2001). Melby & Falk (1995) infected an IPNV carrier stock of Atlantic salmon with tissue homogenates containing the infectious salmon anemia virus (ISA), and demonstrated that ISA infection did not activate the latent IPNV infections. More recently, Pakingking et al. (2003) explored experimental dual infections in flounder *Paralichthys olivaceus* with an aquabirnavirus and the VHSV on the one hand, and bacteria on the other. They concluded that the primary infection suppresses the secondary viral infection, but facilitates the bacterial infection. Further, the studies by Brudesth et al. (2002) on interactions between VHSV and IHNV during an experimental coinfection of rainbow trout demonstrated a reduced systemic distribution of IHNV.

All these reports indicate that interactions between viruses during simultaneous infections is as interesting a line of research in fishes, as it is proving to be in mammals. In previous studies we reported that IPNV interferes with the replication of IHNV in tissue culture cells and in fishes (Alonso et al. 1999a, 2003). The aim of the present study was to determine whether coinfections of VHSV or IHNV with IPNV present similar features; i.e. whether VHSV growth is affected in a similar way to that of IHNV and whether its infectivity is reduced. This is relevant to diagnoses, since the detection methods for viral coinfections would need to be improved, as rapid identification could prevent further spread. In addition, little is known about interactions between viruses during simultaneous infections in fishes. In this study, virus titration, flow cytometry and RT-PCR assays were performed to examine the effect of IPNV on the replication of IHNV and VHSV in fish cell lines.

MATERIALS AND METHODS

Cell lines and virus propagation. BF-2 from bluegill *Lepomis macrochirus* (ATCC CCL 91) and *Epithelioma papulosum cyprini* (EPC) cell lines were maintained in Leibovitz medium (L15, Gibco) supplemented with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS).

The Sp serotype of IPNV and the VR714 strain of IHNV were obtained from the American Type Culture Collection (ATCC VR714 and ATCC VR1318, respectively). VHSV Strain D was kindly provided by Dr. J. L. Barja, University of Santiago de Compostela. The IPNV–IHNV and IPNV–VHSV samples were a mix of IPNV with either IHNV or VHSV strains, and contained equal infectious titers of each virus. To prepare stocks, confluent BF-2 cells were infected at a multiplicity of infection (MOI) of 0.01 with the IPNV–IHNV mix, the IPNV–VHSV mix or with IPNV, IHNV or VHSV reference viruses. After adsorption for 60 min at 15°C, L15 supplemented with 2% FBS medium (L15 2%) was added to the monolayers. When a complete viral cytopathic effect (CPE) was evident, the tissue-culture supernatant was harvested and centrifuged at 2500 × *g* for 10 min at 4°C to remove cell debris. The 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) of the resulting supernatant was determined.

Virus titration. Subconfluent BF-2 cell monolayers growing in 25 cm^2 flasks were inoculated with 2×10^5 $TCID_{50}$ ml^{-1} of IPNV, IHNV or VHSV reference strains. We inoculated 2 additional flasks with mixed samples of either IPNV–IHNV or IPN–VHSV, respectively. The samples were diluted to contain the same proportion of the reference strains. The infected cultures were incubated at 15°C and samples of cells plus medium were processed at 48 and 120 h post infection (p.i.), by freezing and thawing the cells 3 times to determine the total yield (intracellular and extracellular) of the infectious virus. The viruses in coinfection were evaluated following a treatment that suppressed the activity of one virus without affecting the infectivity of the other, and the infective titers were determined. IPNV was neutralized by a 60 min incubation at 15°C with rabbit anti-IPNV serum. IHNV or VHSV were inactivated by the addition to the culture medium of an equal volume of chloroform for 1 h, agitation and separation of the mixtures by centrifugation for 10 min at 4000 × *g*. The concentrations of infectious virus were estimated in BF-2 cells and EPC cells by the $TCID_{50}$ ml^{-1} assay.

Flow cytometry. BF-2 cell monolayers were processed for flow cytometry as described elsewhere (Pérez et al. 1994, Rodriguez et al. 2001). After the trypsin treatment, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 min, washed twice in PBS, and permeabilized with 0.01% Triton X-100 in PBS for 1 min. The pelleted cells were incubated for 30 min at room temperature with a 1:100 dilution of either IPNV polyclonal antiserum or VHSV polyclonal antiserum (Hill et al. 1981, Vilas et al. 1990) or with a 1:10 dilution of IHNV monoclonal antibody (MAb 1NDW14D, DiagXotics). After several washes in PBS supplemented with 2% FBS (PBS 2%), the cells were incubated for 30 min at room temperature with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC) conjugate or anti-mouse IgG-FITC conjugate (Sigma) and washed again. Finally, the fluorescence of the cell suspensions was determined using an EPICS XL flow cytometer equipped with an argon ion laser (Coulter). As controls, mock-infected cells were stained with the first and second antibodies; 5000 cells from each sample were analyzed.

Oligonucleotide primers. The oligonucleotide primers were designed using the available sequences of the IPNV VP2, IHNV G or VHSV G genes (Koener et al. 1987, Thiéry et al. 1991, Pryde et al. 1993). To ensure specificity, the primers were compared with the sequences deposited in the GenBank using FASTA software; the primers G4/G5 were used to amplify a 1512 bp segment of the IHN viral cDNA encoding the G protein; the G4 primer (5′-ATGATCACCACTCC-GCTCATT-3′) was hybridized to Positions 1–21 (sense orientation) in the open reading frame (ORF) of the IHNV G gene; the G5 primer (5′-CCGGTTTGCC-AGGTGATACAT-3′) was 21 nucleotides long and hybridized to Positions 1492–1512 of the ORF (antisense orientation). The primers VG1/VGR (Bruchof et al. 1995) directed the synthesis of a 1522 bp segment of the VHSV viral cDNA encoding the G protein; the primer G1 (5′-ATGGAATGGAACACTTTTTTC-3′) hybridized to Positions 1–21 (sense orientation) in the ORF of the G gene; the primer GR (TCAGACCGTCT-GACTTCTGGA-3′) hybridized to Positions 1505–1524 (antisense orientation) in the ORF of the G gene. The primers V1/V2 bracketed a 613 bp fragment within the VP2 coding region of the IPNV cDNA; the primer V1 (5′-GAACCCCCAGGACAAAGT-3′) was 18 nucleotides long and hybridized to Positions 568–585 (sense orientation) and the primer V2 (5′-TGATTGGTCT-GAGCACGC-3′) hybridized to Positions 1164–1181 (antisense orientation) of the ORF (Pryde et al. 1993) The oligonucleotides were synthesized at the Laboratorio de Química de Proteínas, Centro de Investigaciones Biológicas (CSIC, Spain).

RNA extraction and cDNA synthesis. BF-2 cell monolayers were infected with an 0.01 MOI of IPNV, IHNV or VHSV reference strains or with either the IPNV–IHNV or IPNV–VHSV coinfected samples. After 2 to 3 d, total RNA was extracted following the guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sachi 1987). The RNA pellets were washed in 70% ethanol, dried, and resuspended in diethyl pyrocarbonate-treated water. Total RNA was incubated at 42°C for 1 h in a reverse transcription reaction mixture containing 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 80 mM sodium pyrophosphate, 10 mM of each dATP, dGTP and dTTP, and 5 mM dCTP, 20 U of human placental ribonuclease inhibitor, 70 µM of random hexanucleotide primer and 5 U of reverse transcriptase. Following reverse transcription, the RNA–DNA hybrids were denatured at 100°C for 5 min and 2 µl of the reaction mixture was used as a template for each of the subsequent PCR amplifications.

Polymerase chain reaction (PCR). Amplifications were performed in a 100 µl reaction containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8), 50 mM KCl, 50 pmol each of sense and antisense primers, 2 mM dNTP mix, 1 U *Taq*-polymerase and 2 µl of c-DNA. Amplifications were performed on an automatic thermocycler (Perkin Elmer Cetus) as follows: (1) 30 cycles of 1 min at 94°C, 1.10 min at 55°C and 1 min at 72°C for the G4-G5 primers; (2) 35 cycles of 1 min at 94°C, 1 min at 53°C and 1 min at 72°C for the V1-V2 primers; (3) 25 cycles of 45 s at 94°C, 1 min at 55°C, and 1.5 min at 72°C for the VG-VGR primers. The amplified products were analyzed by electrophoresis in 1.2% agarose gels stained with ethidium bromide.

Interference assay. The BF-2 cells were examined for their ability to induce anti-viral factors or interferon-like activity that could be secreted from the cells, stimulate new cell monolayers, and contribute to the loss of infectivity of the virus. The method used to assay BF-2 cell supernatants was adapted from that described by Jensen et al. (2002a). Briefly, cells growning in plastic flasks of 25 cm^2 were left untreated or stimulated with 500 µl polyinosinic:cytidylic acid (poly I:C, 10 µg ml^{-1}) and 50 µl of DEAE-dextran (100 µg ml^{-1}) for 48 h. Supernatants of the cultures were removed and used to stimulate subconfluent cell monolayers growing in 48-well plates. After 1 h adsorption, the medium was removed, the cells washed once in PBS, infected with IPNV, IHNV and VHSV (0.05 MOI and 4 wells per sample) and incubated at 15°C. After 48 h, pooled medium and cells from the 4 wells of each sample were harvested, frozen and thawed 3 times to disrupt the cells, and inoculated (serial decimal dilutions) in 96-well plates for viral titration.

RESULTS

Viral replication

The relative amounts of infectious particles of each of the viruses present in either the IPNV–IHNV or IPNV–VHSV mixed samples were evaluated at 48 h p.i. For IPNV–IHNV samples, titrations were also performed at 120 h p.i., as IHNV growth is slower. The IPNV and VHSV showed infective titers of 1×10^8 $TCID_{50}$ ml^{-1} as early as 48 h p.i., while at this time the IHNV displayed titers of 1×10^6 $TCID_{50}$ ml^{-1} (Table 1 P_1). When BF-2 cells were infected with the IPNV– IHNV mixed sample, the infective titer of IHNV decreased by 2 log_{10} units at 120 h p.i., compared to the IHNV in the single infection. No differences between the infective titers of IPNV were observed in BF-2 cells infected with IPNV in single or double infections with either IHNV or VHSV (IPNV infective titers were always around 1×10^8). The EPC cells did not support IPNV replication, which allowed us to observe both IHNV and VHSV replication. In these cells, IHNV and VHSV in coinfection

Table 1. Infectivity of IPNV and VHSV in single and double infections in 2 fish cell lines (EPC, BF-2). P_1: Infectivity at first passage; P_3: infectivity after 3 successive passages; cytopathic effects were evaluated at 2 to 10 d post-infection

Virus		Infective titer ($\log_{10}$ $TCID_{50}$ ml^{-1}) EPC		BF-2	
		48 h	120 h	48 h	120 h
Coinfection IPNV–IHNV	P_1	1×10^8	1×10^8	1×10^6	$1 \times 10^{7.5}$
IPNV control	P_1	1×10^8	1×10^8	0	–
IHNV control	P_1	$1 \times 10^{6.5}$	1×10^8	1×10^6	1×10^7
IHNV control[a]		–	0	–	0
IPNV control[b]		0	–	0	–
IPNV coinfected[a]	P_1	1×10^8	1×10^8	0	0
IHNV coinfected[b]	P_1	1×10^5	1×10^6	1×10^6	1×10^7
Coinfection IPNV–IHNV	P_3	–	1×10^9	–	$1 \times 10^{7.5}$
IPNV control	P_3	1×10^9	–	0	–
IHNV control	P_3	–	1×10^8	–	1×10^8
IPNV coinfected[a]	P_3	$1 \times 10^{8.5}$	–	0	–
IHNV coinfected[b]	P_3	–	$1 \times 10^{3.5}$	–	$1 \times 10^{7.5}$
Coinfection IPNV–VHSV	P_1	1×10^8	–	1×10^8	–
VHSV control	P_1	1×10^8	–	1×10^8	–
VHSV control[a]		0	–	0	–
IPNV coinfected[a]	P_1	$1 \times 10^{7.5}$	–	0	–
VHSV coinfected[b]	P_1	1×10^8	–	$1 \times 10^{7.5}$	–
Coinfection IPNV–VHSV	P_3	1×10^9	–	1×10^9	–
VHSV control	P_3	$1 \times 10^{8.5}$	–	1×10^8	–
IPNV coinfected[a]	P_3	1×10^9	–	0	–
VHSV coinfected[b]	P_3	1×10^9	1×10^9	–	

[a]Infective medium treated with chloroform to inactivate IHNV or VHSV and evaluate IPNV
[b]Infective medium treated with anti-IPNV Sp antiserum to inactivate IPNV and evaluate IHNV or VHSV

with IPNV showed no decrease, or differences of only 0.5 $\log_{10}$ units, in the infective titer when evaluated (after neutralizing the IPNV) at 48 h p.i.

To detect the mixed virus after 3 successive simultaneous replications (1:100 virus dilution), an additional titration experiment was performed (Table 1: P_3). The IHNV from the IPNV–IHNV coinfected sample was evident after neutralization of the IPNV and showed a marked reduction in its infective titer (4.5 $\log_{10}$ units) compared to that of the IHNV in the single infection. In contrast, titrations of the IPNV–VHSV mixed infection rendered similar infective titers of both viruses in the BF-2 or EPC cells.

Flow cytometry analysis of coinfected cells

Flow cytometry was used to quantify IPNV-VP2, IHNV-G or VHSV-G viral antigens in culture cells infected with a virus or a mixture of 2 viruses. The histograms in Fig. 1 represent the quantitative analysis of fluorescence in infected cells stained by immunofluorescence with specific antisera. At 48 h p.i., 88 and 95% of the cells were positive for IPNV in single and in IPNV–IHNV mixed infections, respectively. Similar results were obtained for mixed infection with VHSV. The relative number of cells expressing VHSV viral antigens at 48 h p.i. was 81.4% in single infection and 72.6% in a IPNV–VHSV coinfection (Fig. 1, VHSV). In contrast, of the cells coinfected with IPNV–IHNV, few expressed IHNV antigens at 48 h p.i. (IHNV, 16.5%) compared with the single infection (IHNV, 53% fluorescent cells). The 10% decrease in VHSV-positive fluorescent cells in cultures coinfected with IPNV–VHSV is also consistent with the slight decrease in the infective titer found in these samples.

Analysis of RT-PCR products from IPNV–IHNV or IPNV–VHSV coinfections

The V1/V2 primer pair was used to amplify an IPNV-VP2 gene fragment from culture cells infected with IPNV alone or with mixed infections. As expected, in all cases the reaction yielded a fragment of 613 bp (Fig. 2A). For VHSV, a product of 1522 bp was always obtained from cell cultures infected with either VHSV or coinfected with IPNV–VHSV. The IPNV and VHSV were detected by RT-PCR at all successive IPNV–VHSV passages (Fig. 2A).

A fragment of 1512 bp corresponding to the IHNV gene was obtained from cell cultures infected with either IHNV or IPNV–IHNV mix. However, after several passages of the IPNV–IHNV coinfected sample, no IHNV PCR product was detected (Fig. 2B).

Interference assay

After stimulation of the BF-2 cells, inteferon-like activity was demonstrated. As shown in Table 2, the medium of supernatants from poly I:C-DEAE-dextran treated cells was able to provide different levels of

Table 2. Virus yield ($TCID_{50}$ ml^{-1}) of untreated and poly I:C-DEAE dextran-treated BF-2 cells (from bluegill *Lepomis macrochirus*) infected with IPNV, IHNV or VHSV

Virus	Treatment	Infective titer	Fold reduction in virus yield
IPNV	Untreated	1×10^8	0
	Poly I:C-DEAE	1×10^8	
VHSV	Untreated	$1 \times 10^{7.5}$	5
	Poly I:C-DEAE	1×10^7	
IHNV	Untreated	1×10^8	200
	Poly I:C-DEAE	1×10^6	

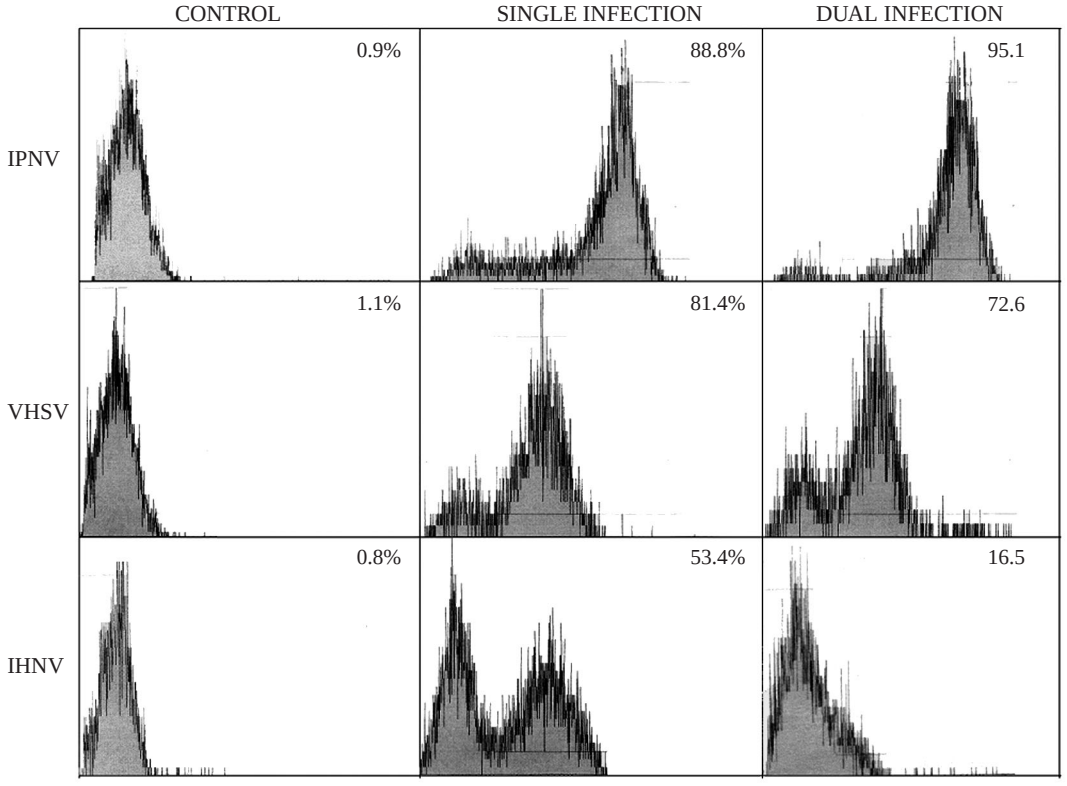

Fig. 1. Flow cytometry histograms for samples of BF-2 cells (from bluegill *Lepomis macrochirus*) infected singly with IPNV, VHSV or IHNV and with IPNV–VHSV or with IPNV–IHNV dual infections. (*y*-axis: % fluorescent cells; *x*-axis: intensity of fluorescence). Cells stained by indirect immunofluorescence at 48 h post-infection with polyclonal anti-IPNV anti-VHSV serum or IHNV monoclonal antibody and a commercial anti-species IgG-FITC conjugate. Background fluorescence measured by negative controls (mock-infected cells stained with corresponding reactives to each virus: control histograms; 5×10^3 cells per sample were analyzed. Dual infection panels represent percentages of cells expressing IPNV after coinfection with VHSV (upper histogram), percentages of cells expressing VHSV antigens after coinfection with IPNV (middle histogram) and the same for IHNV from a coinfection with IPNV (bottom histogram). The single infection column represents percentages of cells expressing indicated viral antigens, in conventional infections

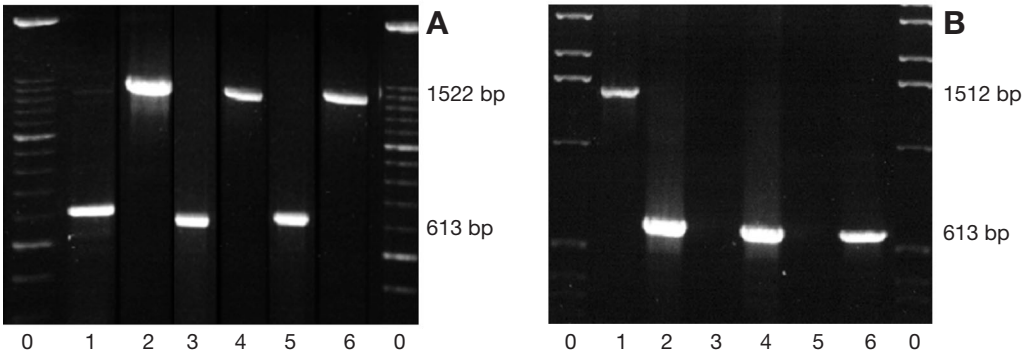

Fig. 2. Analysis of RT-PCR products from samples of experimental coinfections of IPNV with IHNV or VHSV. (A) RT-PCR amplification of IPNV-VP2 gene fragment (613 bp) and the VHSV-G gene fragment (1522 bp), from the IPNV–VHSV coinfection of BF-2 cells (from bluegill *Lepomis macrochirus*), at 3 successive passages. Lanes 1, 3, 5: PCR products from samples of the IPNV fraction of the coinfection IPNV–VHSV at the 1st, 2nd and 3rd passages, respectively; Lanes 2, 4, 6: PCR products from samples of the VHSV fraction of the coinfection IPNV–VHSV for the same passages. Lanes 0: XIV Boehringer size marker. (B) Same experiment as (A), but with the IPNV–IHNV coinfection. Lanes 1, 3, 5: PCR products from samples of the IHNV fraction at the 1st, 2nd and 3rd passages, respectively; Lanes 2, 4, 6: PCR products from samples of the IPNV fraction for same passages. Lanes 0: XIV Boehringer size marker

protection against the tested viruses. The highest protection was observed against the IHNV, which exhibited differences of 2 $\log_{10}$ in the infective titer compared to the untreated control. The VHSV was only slightly affected (0.5 $\log_{10}$ less infective titer), while no protection against the IPNV was detected.

DISCUSSION

This study has demonstrated that viral coinfection of IPNV with VHSV does not affect the replication of any of the viruses in BF-2 cells or EPC, while the coinfection of IPNV with IHNV in BF-2 cells induces a decrease in the infective titer of this rhabdovirus, and this is more evident after 3 passages.

It was possible to detect both IPNV and VHSV by RT-PCR at each of the 3 successive passages in coinfection, while IHNV was only detected at the first passage. In previous studies, we demonstrated that IPNV interferes with the growth of IHNV in tissue culture cells and in fishes (Alonso et al. 1999a, 2003). Successive passages of the coinfected sample led to a decrease in IHNV mRNA and the absence of the specific PCR product for IHNV, with a nested-PCR assay being required to amplify the IHNV (Alonso et al. 1999b). We demonstrated that IPNV also interferes with IHNV replication *in vivo*. Rainbow trout populations infected with the IPNV–IHNV.S46 coinfected sample showed 50% less mortality than fish infected with the reference viruses alone (Alonso et al. 2003).

Herein, we have shown that VHSV, in simultaneous infection with IPNV, infected BF-2 cells with similar titers to those for single infections. In addition, the number of cells expressing VHSV-viral antigens at 48 h p.i. was similar in a VHSV infection and an IPNV–VHSV coinfection, as were the intensity and distribution of the fluorescence revealed by flow cytometry. An IPNV–IHNV coinfection decreases infectivity of the IHNV by 2 $\log_{10}$ in this cell line at the first passage, and more than 4 $\log_{10}$ after successive passages. Flow cytometry experiments showed that the number of cells expressing IHN viral antigens was 3.25-fold lower in a coinfection with IPNV than in a single infection. RT-PCR experiments confirmed this reduction.

The mechanisms of host–virus interaction are poorly understood in the case of rhabdovirus infection in fishes and there is little information indicating why IHNV and VHSV multiplication differ so markedly in the presence of IPNV. One explanation might be that the faster growing IPNV out-competes IHNV and replicates to a high titer, whereas IHNV replicates to a much lower titer. Thus, during passages, there is more IPNV, which again out-competes IHNV. As the VHSV replicates at a similar rate to IPNV, there is equal competition for cells and no difference is seen in the IPNV–VHSV system. This hypothesis would be supported by the results with the EPC cells, in which the IPNV did not replicate and the IHNV titer was not affected. Further, elsewhere (Alonso et al. 1999a), we demonstrated a MOI-dependent inhibition of IHNV infection by IPNV; however, a 1000-fold reduction of IPNV was necessary to balance the IHNV replication, and it is probable that other factors contribute to the loss of infectivity. Competition between viruses for a common receptor may be one such factor.

Brudesth et al. (2002) characterized the interactions between IHNV and VHSV during the experimental coinfection of rainbow trout in terms of viral distribution, histopathological changes and the extent of viral replication in the kidney. They concluded that both viruses establish an infection and that double infection does not result in a significantly lower titer of any of the viruses in kidney. However, the distribution of IHNV in internal organs was more restricted, and was not found in the brain, suggesting competition for the same cell receptor on the target cells in brain tissue. These results agree in part with our observations on the IPNV–VHSV coinfection and with the earlier study of Wolf & Vestergard-Jorgensen (1970) on RTG-2 cells: the viruses can infect cell cultures simultaneously, yielding similar infective titers but it seems that IPNV does not compete with VHSV for attachment sites; there are no data for IHNV, but in a previous work we demonstrated that the dominance of IPNV in dual infection with IHNV in BF-2 cells occurred even when IHNV infection began 24 h earlier (Alonso et al. 1999a).

There is another factor that could affect virus growth in dual infections: the production of interferons (IFN) and the establishment of an antiviral state by induction of antiviral proteins. IFN-like activity has been demonstrated in a number of fish species (Robersten 1998) and in several salmonid cell lines (Eaton 1990, Nygaard et al. 2000). Antiviral proteins are inducible by the double-stranded RNA (dsRNA) polyinosinic:polycytidylic acid (poly I:C), which is a well-known inducer of Type I IFN in higher vertebrates (Trobridge et al. 1997). Jensen et al. (2002b) demonstrated that CHSE-214 (Chinook salmon embryo) cells are able to produce Type I IFN in the presence of transfection agents.

Herein we have shown that BF-2 cells transfected with the dsRNA poly I:C are able to induce secretion of components with characteristics of Type I IFN-like activity which exhibit differential protection against the viruses tested. While the IPNV infective titer was not reduced in the treated cells and VHSV was only slightly affected, the IHNV yield was reduced 200-fold. Considering that double-stranded RNA viruses are inducers of Type I IFN(s) (Dorson et al. 1992), IPNV

might confer some protection and contribute to the loss of infectivity of IHNV in the coinfections. The role that innate immunity plays in protecting fish from virus infection is of great interest, but the interactions between viruses have yet to be investigated at this level. Our preliminary results (data not shown) suggest that *in vitro* induction of IFN-like activity is highly dependent on the cell cultures and the infection procedures selected, so comparative studies of several cell lines would be particularly interesting.

Previous reports by Chinchar et al. (1998) showed the ability of CCO cells to synthesize detectable levels of an interferon-like factor following infection with ultraviolet-inactivated catfish reovirus; this virus inhibits channel catfish herpesvirus replication by 2 different mechanisms—the induction of an antiviral factor and, as a result, the induction of its own replication. Similarly, Jensen et al. (2002a) studied the effect of interferon and poly I:C on the antiviral activity of SHK-1 and TO cells against infectious anemic salmon (ISA) virus and IPNV, and demonstrated that these viruses showed very different sensitivities to the antiviral activity of IFN in Atlantic salmon cells. Whereas IPNV infection was strongly inhibited in TO cells stimulated with poly I:C or AS-IFN, no inhibition of ISAV infection was observed.

In vivo, the state of partial resistance generated by a primary invader (IPNV) against a secondary infection with IHNV or VHSV was first described by de Kinkelin et al. (1992). Since then, other interesting approaches to viral interference mediated by interferon have been shown experimentally (Hedrick et al. 1994, LaPatra et al. 1995, Johansen & Sommer 2001). More recently, Pakinking et al. (2004) have demonstrated the essential role of an aquabirnavirus (ABV) in limiting the proliferation of VHSV in flounder in ABV–VHSV dual infection, and have documented the ability of the ABV to induce the synthesis of a potent IFN-like substance that possesses antiviral activity against VHSV.

In all these studies, the challenge with the second virus was conducted some days after the primary infection. No data are available on antiviral states induced in simultaneous coinfections. Thus, *in vitro* and *in vivo* studies of the correlation between viral inhibition and the expression of antiviral proteins in IPNV–IHNV and IPNV–VHSV systems would be of interest and we are currently undertaking such work in rainbow trout and brown trout.

The results of viral interactions in piscine dual infections depend on the type of viruses involved and are important for sanitary control. While samples from hypothetical coinfections of IPNV–IHNV need to be assayed using sensitive methods (nested-PCR, neutralization of IPNV) to reveal the presence of the rhabdovirus, we have demonstrated here that both standard and molecular methods detect the VHSV in single and double infections. This observation is useful for diagnostic programs, since VHSV is a matter of concern for marine aquaculture: increasing outbreaks of the virus are being reported among marine fish (Mortensen et al. 1999, Meyers et al. 1992, Meyers & Winton 1995) and the potential virulence of such strains has not yet been clearly determined.

More studies on viral coinfections are necessary, since they provide useful models for piscine virus studies either for practical purposes, such as the selection of optimal diagnostic methods for the accurate detection of all viruses present in a fish population, or for academic study, such as analyses of virus–virus and virus–cell interactions.

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