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Study of the viral interference between infectious pancreatic necrosis virus (IPNV) and infectious haematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*)

N. Byrne*, J. Castric, F. Lamour, J. Cabon, C. Quentel

Agence Française de Sécurité Sanitaire des Aliments, Site de Brest, Laboratoire d'Etude et de Recherches en Pathologie des Poissons, Technopôle Brest-Iroise, BP70, 29280 Plouzané, France

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Abstract The resistance of rainbow trout (*Oncorhynchus mykiss*) to an infectious haematopoietic necrosis virus (IHNV) challenge following a preceding non-lethal infection with infectious pancreatic necrosis virus (IPNV) was investigated through experimental dual infections. Trout initially infected with IPNV were inoculated 14 days later with IHNV. Single infections of trout with 1 of the 2 viruses or with cell culture supernatant were also carried out and constituted control groups. No mortality was noted in fish after a single infection with IPNV. This virus had no influence on the head kidney leucocyte phagocytic activity and plasma haemolytic complement activity. IHNV induced a high mortality (72%) and reduced the macrophage phagocytic activity and complement haemolytic activity. It also induced a late production of anti-IHNV antibodies which occurred after clearance of the virus in the fish. In trout co-infected with both viruses, a mortality rate of 2% occurred and the immune parameters were similar to those observed in the fish infected with IPNV only, demonstrating that in co-infected trout IPNV inhibits the effects of IHNV. The studied parameters did not allow us to define the mechanism of interference occurring between these 2 viruses, but some hypothesis are put forward to explain the interference between the 2 viruses.

Introduction

Infectious pancreatic necrosis virus (IPNV) and infectious hematopoietic necrosis virus (IHNV) are the agents of economically important diseases of farmed salmonids worldwide. IPNV, a member of the Birnaviridae family, is

* Corresponding author. Laboratoire de Microbiologie des Environnements Extrêmes, Institut Universitaire Européen de la Mer, Place Nicolas Copernic, 29280 Plouzané, France. Tel.: +33 2 98 49 86 28; fax: +33 2 98 49 87 05.
E-mail address: nathalie.byrne@univ-brest.fr (N. Byrne).

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a non-enveloped, double stranded RNA virus which may induce mortalities as high as 90% in rainbow trout fry *O. mykiss* [1]. The recovered fish frequently become carriers of IPNV [2,3]. IHNV, belonging to the Rhabdoviridae family, is a coated, single negative-stranded RNA virus [4]. The virus can cause devastating mortality in salmonid farms. Outbreaks of IHN may result in losses varying from 25–30% in large fish to 100% in fry [5].

Among viral co-infections occurring in fish farms, those due to Birnaviruses, like IPNV, and Novirhabdoviruses, like IHNV, are the most frequently reported in salmonids [6–8]. In marine species, the infection of Japanese flounder, *Paralichthys olivaceus*, by an aquatic Birnavirus (ABV) and a Novirhabdovirus, VHSV, has also been reported in Japan [9,10].

Interaction between Birnaviruses and Rhabdoviruses and the immunizing mechanisms associated with these double infections have only been investigated in a very specific way [11–13]. The primary infection of Salmonids by an Aquabirnavirus, a Picornavirus or an Aquareovirus involves a protection of fish to other viruses such as viral haemorrhagic septicaemia virus (VHSV), IHNV [14–16] or Nodavirus [17]. This protection, transient or partial, has been shown both *in vivo* and *in vitro* [11,13,18]. Various mechanisms have been demonstrated or suspected to explain this virus interference. Thus, Alonso et al. [13] showed that an inoculation with a mix of IPNV and IHNV induced a decrease in mortality rate of around 50% compared to an inoculation of 1 of the 2 viruses alone. They demonstrated that IPNV interferes with the replication of IHNV. However, they also observed a loss of virulence of their IHNV strain which could not be attributed to a specific change in the *glycoprotein* gene. A possible competition for the same cell receptor was considered during a VHSV–IHNV co-infection [19]. This study provides support for the conclusion that simultaneous infection with 2 piscine Rhabdovirus in a susceptible host results in some degree of interaction at the cell level, leading to a reduction in systemic distribution of IHN virus. An anti-VHSV activity was also demonstrated in the serum of Japanese flounder infected by an aquatic Birnavirus (ABV) and a high expression of a *Mx* gene, a molecular marker of type I interferon occurred in the head kidneys of the ABV-challenged flounder suggesting IFN activity [20]. It was also shown that ABV induced IFN activity against a secondary Betanodavirus infection [17].

In the present study, a more detailed investigation of the onset of the resistance in rainbow trout conferred by a preceding IPNV infection against a subsequent IHNV infection was conducted. Mortality, virus concentration in the organs and immunological parameters like antibodies, head kidney leucocyte phagocytic activity and plasma complement activity were studied in fish after single or dual infections.

Materials and methods

Fish

One year old trout, with a mean weight of 160 ± 10 g, produced from the AFSSA laboratory – Brest, and free of any virus as demonstrated by regular negative analysis on cell lines were randomly distributed in 2 experimental 200 l tanks. Each tank received 200 fish and was supplied with filtered well oxygenated and thermo-regulated fresh water (10 ± 1 °C). Fish were acclimated for 1 month prior to the experiment and a water renewal of 200 l h^{-1} was maintained throughout the experiment. Trout were fed with dry commercial pellets (Le Gouesssant) at 2% body weight per day.

Cell lines and viruses

RTG_2 (Rainbow Trout Gonad) and EPC (*Epithelioma Papulosum Cyprini*) cell lines were cultivated in an Eagle's modified Mac Pherson Stoker medium supplemented with 10% of foetal bovine serum and tryptose phosphate broth, 100 UI ml^{-1} of penicillin, 0.1 mg ml^{-1} of streptomycin and 200 mM of L-Glutamine (Eurobio®). The cell lines were cultivated in 25, 75-cm^2 flasks or in 96 wells microplates (Nunclon) with 3×10^6, 9×10^6 and 7×10^4 cells for RTG2 or 6×10^6, 20×10^6 and 2×10^5 cells for EPC, respectively.

IPNV used in the experiment was the strain I39P isolated from diseased rainbow trout fry displaying typical signs of IPN. A 60 ml production obtained in RTG_2 cells at 14 °C was divided into aliquots between 1 and 10 ml and conserved at −80 °C. IHNV (isolate N61 from diseased rainbow trout) was produced on EPC cells at 14 °C. The generated virus was divided into volumes of 1 and 5 ml and conserved at −80 °C. A small amount of VHSV (reference strain 07/71) was prepared on EPC cells at 14 °C, and stored in 1–10 ml aliquots at −80 °C.

Titration of the viruses was carried out according to the end point technique. Aliquots of neat and 10-fold dilutions (10^{-1} to 10^{-9}) of the cell supernatant were added to each well of a 96 well tissue culture plate. Twenty-five microliter of each dilution was then inoculated in each 4 wells of a 96 well microplate containing confluent cells (RTG2 for IPNV, EPC for IHNV and VSHV). A control cell with non-infectious cell culture medium supernatant was included on each plate. The plates were incubated for 4 days at 14 °C, then the cell monolayers were fixed and colored simultaneously by addition of 50 μl per well of a 0.26% violet crystal solution in 20% ethanol. The plates were rinsed with tap water and dried. The infectious titres of the viruses were calculated according to the method of Kärber [21] and expressed in $TCID_{50}$ ml^{-1}.

The infectious titres of the viral production were 1×10^7 $TCID_{50}$ ml^{-1} for IPNV, 4×10^6 $TCID_{50}$ ml^{-1} for IHNV and 1×10^8, $TCID_{50}$ ml^{-1} for VHSV, respectively.

Experimental infection

After being anaesthetized with 2-phenoxyethanol 0.02% (Merck), 200 trout were intraperitoneally (i.p) injected at day 0 with 200 μl of IPNV suspension containing an infectious dose of 1×10^5 $TCID_{50}$/fish (group N). The control group consisted of 200 fish i.p injected with the same volume of RTG_2 cell culture supernatant free from virus (group C). Three samples of 10 fish from each tank were sampled on days 4, 7 and 14 after this first infection for measuring immunological parameters.

Fourteen days after IPNV injection, 80 fish from each of the 2 groups C and N were i.p infected with 200 μl of IHNV containing an infectious dose of 1×10^4 $TCID_{50}$ $fish^{-1}$. These groups were named CN and NN, respectively, while 80 fish i.p