Entry and sequential distribution of an aquatic birnavirus in turbot (*Scophthalmus maximus*)

B. Novoa^a, J.L. Barja^b, A. Figueras^{a,*}

^aInstituto de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

^bDepartamento de Microbiología y Parasitología, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

* Corresponding author. Tel. (34-86)-231930; Fax (34-86)-292762.

Abstract

Experimental infections with a birnavirus strain isolated from turbot (*Scophthalmus maximus*) were conducted by immersion and intraperitoneal injection in turbot. Virus could be recovered in skin, mucus and intestine from fish infected by immersion, only up to 3 days post-infection. The distribution in internal organs was fast after intraperitoneal injection and virus was recovered with high titres from intestine, kidney, liver and spleen. The lowest titres were detected in brain and gills. Virus was isolated from plasma and blood fractions of the intraperitoneally infected turbot. Preliminary work on the influence on infection of turbot by intraperitoneal injection on the haematological parameters shows that there is no significant difference between infected and uninfected fish in the haematocrit value, number of leukocytes and concentration of plasma proteins.

Keywords: *Scophthalmus maximus*; Birnavirus; Infectious pancreatic necrosis virus; Haematology

1. Introduction

Infectious pancreatic necrosis virus (IPNV) is the causative agent of a serious salmonid disease which can produce high mortalities (more than 90%) in juveniles of less than 6 months. Adults can also be infected experiencing low mortalities. Most of the juveniles that survive a birnavirus infection and the adults develop a chronic infection and they become asymptomatic carriers which can spread the virus via faeces, urine and sexual products (Yamamoto, 1975; Reno et al., 1978; Wolf, 1988). Although in recent years

the virus has been isolated from non-salmonid fish (fresh water and marine species) (Wolf, 1988), it is only since 1987 that isolation of birnaviruses has been made from cultured turbot (*Scophthalmus maximus*) in France (Gastric et al., 1987), Spain (Ledo et al., 1990; Novoa et al., 1993a,b) and Norway (Mortensen et al., 1990). The presence of birnavirus in turbot did not always result in mortality or clinical disease as Roberts (1978) pointed out for salmonids. In fact, many of these isolations in Spain were made from apparently healthy fish (Ledo et al., 1990; Novoa et al., 1993a,b), which could transmit the infection to other susceptible species. However, IPNV was discovered to be the cause of high mortalities recorded in several populations of farmed turbot in Norway without pancreatic necrosis of the infected individuals (Mortensen et al., 1990, 1993). To date, there are few reports about the course of viral infections in turbot and only Gastric et al. (1987) reported the presence of neutralizing antibodies against a viral pathogen.

The study of the influence of birnavirus infection on the haematological parameters, as well as knowledge of the portals of entry and the replication sites can be a first approach to a better understanding of the relationship between virus and fish.

2. Materials and methods

Virus isolates

The TB-306 strain isolated from turbot (Novoa et al., 1993a) was used for infection studies. Virus stock was prepared in a chinook salmon embryo (CHSE-214) cell line as previously described. When a cytopathic effect (CPE) was extensive, cells were harvested and centrifuged to eliminate cell debris. The virus stock was titred in 96 multiwell plates in CHSE-214 cell line according to Reed and Muench (1938). Viral titres were expressed as 50% of the tissue culture infectious dose (TCID₅₀). Viral stock was stored at - 70°C until use.

Portals of entry

Turbot (3 g) were infected by immersion adding 1 ml of the virus suspension to 1 litre of sea water. The final titre was 6.3×10^6 TCID₅₀/ml. After 1 h, tanks were filled with 5

1 of sea water and the temperature was maintained at 15-18°C during the course of the experiment. A control group was treated as infected fish. At 3, 6, 12, 24 h and 2, 4, 6 days, 4 infected and 2 control fish were sacrificed. After two consecutive baths in sterile sea water, to eliminate superficial residual virus, the fish were sacrificed. Two pools were made from infected and uninfected turbot with blood, gills, skin, muscle, kidney, spleen and intestine, being processed for virological analysis following standard procedures (Amos, 1985). In addition, mucus from infected and uninfected fish was taken by gently scraping the surface with a coverslip and processed as previously described.

Sequential distribution of virus in turbot infected intraperitoneally

Sixty turbot of 15 g were inoculated intraperitoneally with 100 µl of viral suspension (2.1×10^9 TCID₅₀/fish) and 20 control fish were inoculated with Minimal Essential Medium (MEM). Five infected and 2 uninfected fish were sampled weekly, anaesthetized with MS-222 (Sandoz) and weighed. Blood was taken from the caudal vein of each turbot with heparinized capillary tubes, which were centrifuged at 2000 x g plasma. In one of the tubes, individual haematocrit value was measured. After that, blood from infected turbot was pooled and the same was done for the control fish. Tubes were broken and the blood cells were added to 1 ml of PBS which was layered on 1.5 ml of Ficoll-Histopaque (Sigma) and centrifuged at 400 x g for 30 min at room temperature (Swanson and Gillespie, 1982). The plasma-Ficoll interphase was removed with a Pasteur pipette and washed 3 times in PBS by centrifugation. The Ficoll phase and the pellet of erythrocytes were also taken and washed in the same way as the interphase. An aliquot of each blood fraction was taken and counted in a Neubauer chamber. Remaining blood fractions and the plasma taken initially were titred in the CHSE-214 cell line without freezing. In addition, several tissues of infected and uninfected turbot (kidney, spleen, brain, gills, intestine, muscle and liver) were aseptically taken weekly, processed for virological assays (Amos, 1985) and titred according to Reed and Muench (1938). Viral titers were expressed as 50% of the tissue culture infectious dose (TCID₅₀)

Determination of haematological parameters

The quantification of leukocyte number was made in the whole blood and in each of the cellular fractions obtained with the Histopaque centrifugation. A 1:100 dilution in Trypan blue (0.4% Sigma) was made and counted in a Neubauer chamber. In order to determine the number of different leukocyte types, a blood smear from each fish was made and stained with the Haemacolor-Kit (Merck).

The determination of plasma protein concentration was made using a protein kit (Biorad). Absorbances were measured in a Hewlett Packard 8452A spectrophotometer. The results of haematocrit values, number of leukocytes and concentration of plasma proteins were statistically analyzed using analysis of variance and Student's t-test.

3. Results

Portal(s) of entry

The birnavirus strain TB-306 was detected in the first 3 days after infection of turbot by immersion only from skin, mucus and intestine, but not from internal organs and blood. The viral titre ranged from 3.6×10^3 to 4.9×10^4 TCID₅₀/ml. After these 3 days the virus was not recovered from any challenged fish.

Sequential distribution of virus in turbot infected intraperitoneally

No mortality occurred during the experiment of intraperitoneal fish infection that lasted 42 days. Virus was recovered from all fractions of blood: the Ficoll-plasma interphase (composed essentially of leukocytes); the Ficoll phase (composed of leukocytes and erythrocytes) and the pellet of erythrocytes. The birnavirus strain was recovered from plasma 21 days after injection; however, after that date, it could not be detected (Fig. 1A). In addition, virus was reisolated from cellular fractions of blood. There was an initial replication and then a gradual diminution of viral titre. This fact was observed later in the case of leukocytes (Ficoll-interphase) (Fig. 1B).

The evolution of viral titre in internal organs is illustrated in Fig. 2. Virus could be recovered from every organ assayed 3 days after infection. The highest titre of TB-306 was obtained in intestine (6.3×10^9 TCID₅₀/g). After 21 days there was a low decrease

of viral titre (less than 2 logs). In liver, muscle, spleen and kidney there was also a high virus replication between 3 and 21 days. However, in brain, the virus was detected at low level and there was a progressive decrease of viral titre after the third day post-infection. There was a gradual decrease of viral titre in gills from infected turbot.

Haematological parameters in turbot infected intraperitoneally

The evolution of the haematocrit and the leukocytes number during the course of the experiment is represented in Fig. 3. No significant differences were observed between these parameters in infected and control turbot. However, 21 days after infection, the number of leukocytes of infected fish was significantly lower than in controls, and the haematocrit value was lower in infected fish at the end of the experiment than in controls.

Fig. 4 shows the evolution of the percentage of each type of white cell; a high individual variation was observed in these parameters. Lymphocytes were the more abundant cellular type and the number was relatively constant throughout the experiment. No significant differences were observed between infected and uninfected turbot. Although granulocytes were constant in control fish, an increase in the number of this cellular type was observed in turbot injected with the birnavirus strain 7 days post-inoculation. Monocytes and thrombocytes were present in low numbers and the variations could not be attributed to the viral infection.

The concentration of plasma proteins did not show any significant variations during the course of the experiment in either infected or uninfected fish.

4. Discussion

This study represents a new contribution to our limited knowledge of the kinetics of viral infections in turbot (*Scophthalmus maximus*). Since by immersion (water challenge) the virus was detected in skin mucus and intestine, these could be interpreted as portals of entry of this virus in turbot. However, the lack of virus recovery, in the water challenge, from internal organs could indicate that they might also act as first

barriers against the infection, in which host defense mechanisms are involved. These findings are similar to what Roberts (1989) postulated for salmonids.

The evolution of the viral titre in intraperitoneally injected fish shows that kidney is a preferential replication site for birnavirus since highest titres were obtained in this organ. This is well associated with the necrosis of the haematopoietic tissue of kidney previously reported (Gastric et al., 1987; Novoa et al., 1993a). However, Mortensen et al. (1993) could not find this lesion in experimentally infected turbot. These differences may be due to a variation in the pathogenicity of the viral strain or to different susceptibilities of the turbot populations. The brain of salmonids has been reported to be a reservoir of viral particles in carrier fish (Dorson, 1983; McAllister, 1983); however, in turbot, the titres found in brain were the lowest of the organs assayed. Viral strain TB-306 was recovered from gills and muscle, but titres decreased throughout the experiment, especially in the gills. We think that although this organ is not a viral replication zone, it can be the way through which the virus is eliminated to external water or alternatively that they are organs with a high immune response against viral infection. No cellular response was detected in any of the histological slides examined in other experimental infection carried out (Novoa et al. 1993a). The possibility of a localized humoral response has not been investigated.

Our data suggest that kidney and spleen are the best organs for detecting birnavirus in turbot and should be preferentially used to diagnose the infection. Although liver and intestine yield a high virus titre, samples often produce toxicity in cell cultures (unpublished data). These results are in agreement with those reported for salmonids (McAllister et al., 1993).

IPNV distributes in every organ of brook trout (*Salvelinus fontinalis*) after inoculation with the virus, which can be explained by blood diffusion (Bootland et al., 1991). In our work, viraemia was also observed in turbot injected intraperitoneally, since virus was reisolated from different blood fractions. Virus was not recovered from plasma after 21 days post-infection. This fact can be attributed to the presence of antiviral substances (neutralizing antibodies, interferon etc.). Interferon production was demonstrated in fish (Graham and Secombes, 1990; Eaton, 1990) and, recently, Dorson et al. (1992) detected interferon synthesis when rainbow trout fry were inoculated with Sp strain of IPNV. We

have not determined this activity in turbot, which will be a further study to carry out in the future; however, low titres of neutralizing antibodies were detected previously in turbot (Gastric et al., 1987; Novoa et al., 1994). This humoral response does not seem sufficient to avoid the infection and it is possible that a cellular response or a combined system could play a role in the total neutralization of virus infectivity. The birnavirus IPN is able to persist in its hosts in a carrier state and can remain for a period of time up to 3 years. Virus may persist also in a non-infectious form in leukocytes becoming infective as a response to an stimulus such as cell division (Roberts, 1989). Although the virus may not be pathogenic, it may predispose its host, like other viruses, to a secondary bacterial or parasitic infection by immunosuppression (Wheeler and Abramson, 1993).

There are not many references on the influence of viral infection in blood parameters. Alvarez-Pellitero and Pintó (1987) reported that haematological parameters can be altered in diseased fish infected with different pathogenic agents. Although our results are preliminary, we can suspect that viral infection is related to an increase in granulocytes. At the end of the experiment the haematocrit value seemed to be lower in infected turbot than in controls, which is in agreement with that reported for salmonids (Wolf, 1988). However, the other parameters analyzed did not suffer significant variations.

Further research is currently being carried out to clarify the turbot-birnavirus relationship at the immune response level and to understand how the virus can survive in fish by escaping the host immune mechanisms.

Acknowledgements

We thank Isabel Caride and Hilda Alvarez their technical assistance and A.E. Toranzo for reviewing the manuscript. This work was supported by Grant AGF93-0769-C02-02 from the Comisión Interministerial de Ciencia y Tecnología (CICYT). Beatriz Novoa acknowledges the Ministerio de Educación y Ciencia (Spain) for a research fellowship.

References

- Alvarez-Pellitero, P. and Pintó R., 1987. Some blood parameters in sea bass, *Dicentrarchus labrax*, infected by bacteria, virus and parasites. *J. Fish. Biol.*, 31 (Suppl. A): 259-261.
- Amos, A.H., 1985. Procedures for the Detection and Identification of Certain Fish Pathogens, 3rd edn. Fish Health Section, American Fisheries Society, Corvallis, OR, 114 pp.
- Bootland, L.M., Dobos, P. and Stevenson, R.M.W., 1991. The IPNV carrier state and demonstration of vertical transmission in experimentally infected brook trout. *Dis. Aquat. Org.*, 10: 13-21.
- Gastric, J., Baudin-Laurencin, F., Coustans, M.F. and Auffret, M., 1987. Isolation of infectious pancreatic necrosis virus Ab serotype from epizootic in farmed turbot, *Scophthalmus maximus*. *Aquaculture*, 67: 117-126.
- Dorson, M., 1983. Infectious pancreatic necrosis of salmonids. Overview of current problems. In: D.P. Anderson, M.M. Dorson and Douborget P. (Editors), *Antigens of Fish Pathogens*. Fondation Marciel Merieux, Lyon, pp. 7-32.
- Dorson, M., De Kinkelin, P. and Torchy, C., 1992. Interferon synthesis in rainbow trout fry following infection with infectious pancreatic necrosis virus. *Fish Shellfish Immunol.*, 2: 311-313.
- Eaton, W.D., 1990. Anti-viral activity in four species of salmonids following exposure to polyinosinic:cytidylic acid. *Dis. Aquat. Org.*, 9: 193-198.
- Graham, S. and Secombes, C.J., 1990. Do fish lymphocytes secrete interferon- γ ? *J. Fish Biol.*, 36: 563-573.
- Ledo, A., Lupiani, B., Dopazo, C.P., Toranzo, A.E. and Baja, J.L., 1990. Fish viral infections in Northwest of Spain. *Microbial SEM*, 6: 21-29.
- McAllister, P.E., 1983. Infectious pancreatic necrosis (IPN) of salmonid fishes. *Fish Diseases leaflet 65*, U.S. Fish and Wildlife Service National Fish Health Research Laboratory, Kearneysville, WV, pp. 1-12.
- McAllister, P.E., Schill, W.B., Owens, W.J. and Hodge, D.L., 1993. Determining the prevalence of infectious pancreatic necrosis virus in asymptomatic brook trout *Salvelinus fontinalis*: a study of clinical samples and processing methods, *Dis. Aquat. Org.*, 15: 157-162.
- Mortensen, S.H., Hjeltne, B.K., Rodseth, O.M., Krogsrud, J. and Christie, K.E., 1990. Infectious pancreatic necrosis virus, serotype N 1, isolated from Norwegian turbot

- (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and scallops (*Pecten maximus*). Bull. Eur. Assoc. Fish Pathol., 10(2): 42-43.
- Mortensen, S.H., Evensen, O., Rodseth, O.M. and Hjeltnes, B.K., 1993. The relevance of infectious pancreatic necrosis virus (IPNV) in farmed Norwegian turbot (*Scophthalmus maximus*). Aquaculture, 115: 243-252.
- Novoa, B., Figueras, A., Puentes, C.F., Ledo, A., Barja, J.L. and Toranzo, A.E., 1993a. Characterization of a birnavirus isolated from diseased turbot cultured in Spain. Dis. Aquat. Org., 15: 163-169.
- Novoa, B., Toranzo, A.E., Dopazo, C.P., Barja, J.L. and Figueras, A., 1993b. Isolation in Europe of the VR-299 serotype of IPNV from turbot. Dis. Aquat. Org., 17: 61-65.
- Novoa, B., Figueras, A., Rivas, C. and Toranzo, A.E., 1994. Pathogenicity of birnaviruses isolated from turbot (*Scophthalmus maximus*). Comparison with reference serotypes of IPNV. Aquaculture, in press.
- Reed, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent endpoints, Am. J. Hyg., 27: 493-497.
- Reno, P.W., Darley, S. and Savan, M., 1978. Infectious pancreatic necrosis: experimental induction of a carrier state in trout. J. Fish. Res. Board Can., 35: 1451-1456.
- Roberts, R.J., 1978. Fish Pathology, 1st edn. Bailliere Tindall, London, 232 pp.
- Roberts, R.J., 1989. Fish Pathology, 2nd edn. Bailliere Tindall, London, 467 pp.
- Swanson, R.N. and Gillespie, J.H., 1982. Isolation of infectious pancreatic necrosis virus from the blood and blood components of experimentally infected trout. Can. J. Fish. Aquat. Sci., 39: 225-228.
- Wheeler, J.G. and Abramson, J.S., 1993. Microbial and pharmacological induction of neutrophil dysfunction. In: J.S. Abramson and J.G. Wheeler (Editors), The Neutrophil. IRL Press, Oxford, pp. 139-166.
- Wolf, K., 1988. Fish Viruses and Fish Viral Diseases. Cornell University Press, Ithaca, NY, 476 pp.
- Yamamoto, T., 1975. Frequency of detection and survival of infectious pancreatic necrosis virus in a carrier population of Brook trout (*Salvelinus fontinalis*) in a lake. J. Fish. Res. Board Can., 32: 568-570.

Fig. 1. Evolution of viral titre in blood from infected fish by intraperitoneal injection with the birnavirus strain TB-306. (A) Viral titres in plasma of infected fish. (B) Viral titre in cellular fractions obtained after centrifugation in Ficoll-Histopaque.

Fig. 2. Evolution of viral replication in internal organs from infected turbot by intraperitoneal injection.

Fig. 3. Evolution of haematocrit values (A) and number of leukocytes (B) in infected and uninfected turbot corresponding to the experiment of intraperitoneal infection. Bars represent standard deviation of the values.

Fig. 4. White cells in blood from infected and uninfected turbot along 42 days. Bars represent standard deviation of the percentages of each type of leukocyte.