Pathogenicity of birnaviruses isolated from turbot (Scophthalmus maximus) : comparison with reference serotypes of IPNV

B. Novoa[a], C. Rivas[b], A.E. Toranzo[b], A. Figueras[a],*

[a]Institute de Investigaciones Marinas, CSIC, Eduardo Cabello 6, 36208 Vigo, Spain

[b]Departamento Microbiologia y Parasitologia, Facultad de Biologia, Universidad de Santiago, 19706 Santiago de Compostela, Spain

*Corresponding author.

Abstract

The pathogenicity of different birnavirus strains to turbot was determined by challenge experiments. The reference strains of IPNV (Sp, Ab and VR-299) were not pathogenic for turbot (Scophthalmus maximus). The Norwegian serotype (N1) and two selected birnavirus strains isolated from turbot and related to the Ab serotype of IPNV (TB-306 and TB-390) were virulent to different degrees for turbot and the virus could be recovered from survivor fish. Other turbot birnavirus strains (TB-3 11, TB-231,TB-160 andTB-460) wereless virulent for this species. Neutralizing antibodies were detected with low titer in 30 g turbot inoculated with a virulent strain (TB-306). No pancreatic necrosis was found in turbot inoculated with this viral strain (TB-306)) but this lesion was present in inoculated rainbow trout (Oncorhynchus mykiss).

Keywords: Diseases and their control - fish; Birnavirus; Infectious pancreatic necrosis virus; Scophthalmus maximus; Antibodies

1. Introduction

Infectious pancreatic necrosis (IPN) is a viral disease of salmonids, often causing high mortality in fry. This virus has been isolated from several non-salmonid fishes, including freshwater and marine species (Wolf, 1988). IPNV, serotype Ab, was isolated from several estuarine fishes in the United States (McAllister et al., 1984) and from farmed turbot (Scophthalmus markus) in France (Gastric et al., 1987). In both cases, high mortalities were reported, associated with the isolation of this virus.

Since 1989, several IPN-like virus have been isolated in Galicia (NW of Spain), from salmon (Salmo salar) and turbot (Ledo et al., 1990; Novoa et al., 1991). Most of the viruses were detected in routine microbiological surveys on fish without disease signs. A particular strain from turbot (TB-306)) an atypical birnavirus related to the Ab serotype of IPNV, has been recently isolated (Novoa et al., 1993a) and preliminary virulence characteristics were reported. In addition, the isolation of two viral strains similar to the VR-299 serotype was also reported (Novoa et al., 1993b).

In the present work we present the results of further pathogenicity assays conducted on the turbot isolates previously reported as well as on other birnavirus strains recently isolated from turbot (TB-390, TB-231 and TB-460) in our laboratory. A comparison of virulence of the reference strains of IPNV (Sp, Ab, VR-299 and N1) was done. Experimental infections were carried out in turbot of 0.3-1 g using two routes of inoculation (immersion and intraperitoneal injection) and in rainbow trout (Oncorhynchus mykiss) using intraperitoneal injection. In addition, turbot response was determined by neutralizing antibody assays.

2. Materials and methods

Virus and virological assays

The reference serotypes of IPNV Sp, Ab and VR-299, the Norwegian strain Nl (Christie et al., 1988) and the turbot birnavirus strains TB-306 (Novoa et al., 1993a), TB-311 and TB-160 (Novoa et al., 1993b), TB-390, TB-231 and TB-460 (unpublished data) were grown in the Chinook salmon cell line (CHSE-214) and the supernatant of lysed cell culture was used as inoculum in the challenge experiments. No heavy mortalities were detected in the fish stocks where these viruses were isolated.

For virus detection, organs or whole fish were proccessed using standard virological procedures (Amos, 1985). Titrations of the virus were made in 96-well plates of CHSE-214 according to Reed and Müench (1938).

Experimental infections

In all the experiments, fish, previously tested for virus, were maintained at 15°C in 25-liter tanks using recirculated sea water with aeration. Four different infection trials were conducted:

(1) In order to compare turbot susceptibility to the strains isolated from turbot (TB-306, TB-390, TB-311, TB-231, TB-160 and TB-460), the Norwegian serotype (N1) as well as the reference strains from trout (Sp, Ab and VR-299)) 8 turbot were injected intraperitoneally with 50 µ1 of several doses of each viral suspension (8×10^7, 8×10^6, 8×10^5 $TCID_{50}$/ml). The controls were treated as the infected fish and were inoculated with culture medium. Dead and survivor fish were collected for viral titrations during a 30-day period. The lethal 50% dose (LD_{50}) was calculated by the method of Reed and Müench (1938).

(2) Eight turbot of 0.3 g were exposed to each virus mentioned above by immersion for 30 min in 100 ml of water containing 10^6 $TCID_{50}$/ml. A similar volume of culture medium was added to the control flask. After infection, water was added to 1.5 liters in each flask; 75% of the water was changed daily. Dead and survivor fish were removed for viral assays after 10 days post-infection.

(3) Trout susceptibility to the turbot birnaviruses strains cited above was assayed. Ten rainbow trout of 2 g were inoculated intraperitoneally with 100 µ1 of the viral suspension of each strain containing 10^6 $TCID_{50}$/ml and 10 trout were inoculated with culture medium and used as controls. Moribund fish were collected for histology and dead fish for virus assays.

(4) To determine the immune response of inoculated turbot against a virulent strain of birnavirus (TB-306), neutralizing antibodies were quantified for 30 days. Sixty turbot of 30 g, previously tested for virus, were injected intraperitoneally with 100 µ1 of a virus suspension containing 10^6 $TCID_{50}$/ml of the virus TB-306. The controls were treated as the infected fish and were inoculated with culture medium. Serum from 7 inoculated and 7 uninoculated fish were taken weekly and used for detection of neutralizing antibodies. To determine the distribution of virus in different organs, survivor fish were

sacrificed and pools of brain, kidney, spleen and liver were collected and processed for virus assays and histology.

Detection of neutralizing antibodies

Blood was drawn from the caudal vein from turbot that had been challenged as described above in (4) and kept in heparinized Eppendorf vials. Plasma was obtained by centrifugation of whole fish blood for 5 min at 2000 x g in a refrigerated centrifuge and stored at -20°C until use. An initial 1: 5 dilution of the serum was heated at 45°C for 30 min to inactivate the complement and doubling dilutions in MEM were made up to a 1: 640 dilution (Agius et al., 1982). An equal volume of virus suspension was added to 0.2 ml from each dilution in order to contain 1000 $TCID_{50}$/ml. The mixture was allowed to react for 30 min at 20°C and 100 µ1 aliquots were inoculated on triplicate wells of CHSE-214 microtiter plates. The neutralizing titer was expressed as the reciprocal of the dilution observed to prevent the appearance of a cytopathic effect (CPE).

Histology

Transverse sections approximately 5 mm thick of brain, kidney, spleen and liver from trout and turbot were excised, fixed in 4% buffered formalin, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin.

3. Results

Experimental infections

(1) Turbot of 1 g inoculated intraperitoneally with the 4 reference serotypes and the turbot birnaviruses were susceptible to different degrees, under the experimental conditions, to these strains (Table 1). Viruses of the Sp and Ab reference strains of IPNV had a similar pattern of virulence for turbot. However, the Ab serotype replicated more efficiently in turbot tissues than Sp viruses since it could be recovered in higher titer from dead fish. The VR-299 virus was less pathogenic for turbot than the other reference IPNV strains and the virus could not be recovered from survivor fish. N1 caused a milder mortality, but the virus could be recovered from dead and survivor fish.

In the case of the birnaviruses isolated from turbot, several differences were observed among the isolates. Whereas the TB-311 and TB-160 viral isolates, both similar to the VR-299 serotype of IPNV (Novoa et al., 1993b) and TB-231 and TB-460, non-typable strains (unpublished data) were avirulent for turbot, the TB-306 and TB-390 isolates, similar to the Ab reference serotype (Novoa et al., 1993a), were more pathogenic for this species. Although the degree of virulence was not high, some (TB-306, 311, 390) of the strains from turbot, as well as the Norwegian strain N1, were present after 30 days post-inoculation in fish without disease symptons, reaching titers of 10^3-10^4 $TCID_{50}$/g of fish.

(2) Similar results were obtained in the immersion challenge (Table 2). Turbot strains TB-306 and TB-390 were more virulent than the other birnaviruses assayed. The highest mortality rate was caused by TB-306 (87.5%). TB-390 produced a mild mortality similar to Sp and Ab. This virus was recovered from dead fish with higher titers than in the case of Sp and Ab reference strains. The virus was also recovered from survivor fish, which was not the case with Sp and Ab. Although N1 caused a mild mortality, the virus was recovered with a high titer from dead fish and moderately high from survivor fish. The rest of the viruses assayed caused even lower mortality rates and the viruses were recovered with a low virus titer or were not re-isolated.

(3) Trout were susceptible to the birnaviruses isolated from turbot. Necrosis was found in pancreas of moribund fish, but no intestinal mucosal damage was observed. Virus was recovered with a titer ranging between 1.1 and 3.2 x 10^6 $TCID_{50}$/g of fish tissue.

4) In fish weighing 30 g no mortality occurred after 2 months. Externally the fish appeared normal, but the virus was recovered from all the fish. The virus titer recovered in different organs of survivor fish is illustrated in Fig. 1. The highest titer was found in the kidney, and the brain displayed the lowest virus recovery ranging from 5 x 10^3 to 3 x 10^4 $TCID_{50}$/g.

The neutralizing antibody titers were low. In the first week after inoculation neutralizing activity was not detected. The mean antibody titer and the percentage of fish with this activity increased with time (Fig. 2). Neutralizing activity was not detected in turbot uninoculated or inoculated with MEM used as controls.

4. Discussion

At present, most of the IPN-like viruses isolated in our area from turbot were associated with apparently healthy fish. Only on a few occasions were they associated with diseased fish (Ledo et al., 1990; Novoa et al., 1991).

The pattern of virulence of the reference strains Sp, Ab and VR-299 was opposite to that reported for salmonids. In rainbow trout, the Sp serotype is more virulent than the Ab, but in turbot no marked differences were detected between these two serotypes. The VR-299 virus was not highly pathogenic for turbot and virus was only recovered from dead fish infected by immersion. The viral strains N1, TB-306 and TB-390 were the most virulent for turbot and were present after 30 days post-inoculation in fish without disease symptoms in both routes of infection assayed. This is important because they can act as carriers of the virus.

Some turbot birnaviruses (TB-311, TB-160, TB-460) were not virulent for this species, similar to other isolates reported in striped bass (Morone saxatilis) (Wechsler et al., 1986) and northern pike (Esox lucius) (Ahne, 1978), but their importance as vectors for other susceptible species should be kept in mind.

The different degrees of virulence of these viral isolates for turbot show a wide diversity among the viral strains used in these challenge experiments. Wolf (1972), at the beginning of research on IPNV, pointed out the great heterogeneity of 15 different isolates with a cumulative mortality ranging from 10 to more than 90%.

The results obtained for the TB-306 isolate are in agreement with the preliminary virulence characteristics of this viral strain previously reported (Novoa et al., 1993a). Moreover, another isolate (TB-390) with a close resemblance to the reference strain Ab had the same virulence pattern.

The kidney proved to be the best organ to recover the virus (TB-306) in survivor turbot, similarly to that reported in salmonids. It is interesting to note that in these experimental

challenges, mortality appeared only in the 1 g animals, which could be explained by the fact that the immune system is not formed completely at this age.

The neutralizing antibody titers were low and in agreement with those obtained by other authors in rainbow and brook trout (Reno et al., 1978; Agius et al., 1982). Mangunwiryo and Agius (1987) reported for tilapia (Oreochromis spilurus) that circulating antibody levels peaked at week 2 and dropped rapidly so that by week 3 they were undetectable. In turbot, however, the antibody titer increased with time at least during the first 4 weeks.

The birnaviruses isolated from turbot did not produce necrosis of the pancreatic tissue, but they were virulent for small turbot and were harbored in a carrier state in older fish. In addition, as previously reported (Novoa et al., 1993a) it produced necrosis of the hematopoietic tissue of kidney and spleen. However, pancreatic necrosis was found in inoculated rainbow trout, so these isolates could be attributed to the infectious pancreatic necrosis virus group. Sano et al. (1981) reported a viral epizootic of Anguilla japonica caused by the European eel virus (EVE). This birnavirus produced histopathological alterations in kidney from infected eels. However, in comparison with turbot birnaviruses, it did not cause pancreatic necrosis when trout were experimentally infected.

In Spain, rainbow trout farming is an important industrial activity and IPNV is usually found associated with this species (Ledo et al., 1990)) most of the isolated birnavirus being related to the Sp serotype. This could be the source of turbot infection, but, interestingly, a variety of birnaviruses related to the three reference strains were isolated from turbot farms.

Knowledge of the pathology and transmission of this virus in turbot may be important for epizootiological and ecological studies.

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Table 1

lntraperitoneal infection conducted in turbot with birnavirus strains from trout and turbot

Virus	LD_{50}[a]	Time to death (days)	Re-isolation from dead fish ($TCID_{50}$/g)	Recovery from survival fish after 30 days
Reference strains				
SP	8.0×10^6	4-6	1.1×10^5	-
Ab	8.0×10^6	4-5	1.1×10^6	-
VR-299	$>10^7$	-	NA	-
N1	8.0×10^5	4-25	6.3×10^5	$\sim 10^3$
Turbot strains				
TB-306	1.1×10^6	4-7	6.3×10^6	$\sim 10^4$
TB-311	$>10^7$	-	-	$\sim 10^3$
TB-390	8.0×10^5	4-20	1.1×10^6	$\sim 10^3$
TB-231	$>10^7$	-	NA	-
TB-160	$>10^7$	-	NA	-
TB-460	$>10^7$	-	NA	-

[a] Mean lethal dose 50% (LD_{50}) is expressed as the titer of virus needed to kill 50% of inoculated animals.

NA = not applicable.

Table 2

Infectivity trials conducted in turbot infected by immersion with biiavirus strains from trout and turbot

Virus	Percentage of dead fish (%)	Time to death (days)	Re-isolation from dead fish ($TCID_{50}$/g)	Recovery from survival fish after 10 days
Reference strains				
SP	50	3-4	1.1×10^3	-
Ab 5	0	4-7	3.6×10^4	-
VR-	37.5	2-10	2×10^4	-

299				
N1	25	8-10	3.6×10^8	1.1×10^3-3.6×10^6
Turbot strains				
TB-306	87.5	7-10	$1.16.6 \times 10^6$	NT
TB-311	0	-	NA	-
TB-390	50	7-10	1.1×10^6 -6.3×10^8	6.3×10^5-6.3×10^6
TB-231	12.5	2-3	1.1×10^8	-
TB-160	37.5	9-10	-	-
TB-460	12.5	9-10	-	-
Control	12.5	10	-	-

NA = not applicable; NT = not tested.

Fig. 1. Comparison of the virus titer in the internal organs of infected turbot after 30 days post-infection.

Fig. 2. Evolution of neutralizing antibodies production against the birnavirus strain TB-306. (A) Mean titer of neutralizing antibodies found in turbot plasma. (B) Percentage of fish containing neutralizing antibodies.