Heterogeneity of Marine Birnaviruses Isolated from Turbot
(Scophthalmus maximus)

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Six marine birnaviruses isolated from turbot (Scophthalmus maximus) were compared with the infectious pancreatic necrosis virus reference strains Sp, Ab and WB from salmonid fish. This comparison was carried out by PAGE analysis of viral RNA and polypeptides and comparison of the reaction pattern with 4 monoclonal antibodies against the reference strains. High heterogeneity was found among the 6 isolates when comparing the RNA and polypeptide electrophoretic patterns and their reactions with the monoclonal antibodies. Most of the strains isolated from turbot could not be ascribed to any established serotype.

Key words: birnavirus, IPNV, turbot, heterogeneity, marine birnavirus

The family Birnaviridae includes icosahedral unenveloped viruses with double stranded and bisegmented RNA genome. Aquatic birnaviruses are the largest group in this family, causing diseases in a variety of fish and shellfish species all around the world. The prototype strain, the infectious pancreatic necrosis virus (IPNV), is an important pathological agent for salmonids, causing high mortalities and economic losses in trout and salmon cultures (Wolf, 1988).

Most of the aquatic birnaviruses are antigenically related, however, three major serotypes have been traditionally distinguished (Sp, Ab and WB) (Wolf, 1988). Using reciprocal cross-neutralization with polyclonal antibodies, Hill and Way (1983) identified six additional serotypes (Hetch, Tellina, Jasper, Canada 1, Canada 2, Canada 3). All these serotypes constitute a major serogroup, named the serogroup A of aquatic birnaviruses. Although some years later, Christie et al. (1988) characterized a tenth serotype: N1, this was later assimilated to the Sp serotype (Melby and Christie, 1994; Melby et al., 1994). Recently, monoclonal antibodies have been developed to determine the antigenic relationship among different isolates from all over the world (Caswell-Reno et al., 1986, 1989; Lipipun et al., 1989; Chi et al., 1991) and RNA and polypeptides patterns have also been compared (Hedrick et al., 1985; Hsu et al., 1989). However, the classification of this group is a complex and continuously changing field with many controversies among research groups (Dorson, 1983; Novoa et al., 1993a, 1993b).

As a result of a five year survey of the health status of several turbot farms in Galicia (Spain), several marine birnavirus strains were isolated using standard procedures (Novoa et al., 1993a, 1993b). In addition, several turbot juvenile importations from different countries were analyzed before their transfer to Spanish turbot farms. Studies on the variability of marine birnaviruses, which were isolated from marine fish or marine environment, are not only important for aquaculture activities but also for the marine ecosystem. The pathogenicity and virulence of one strain will depend on the viral properties of one particular isolate and on the susceptibility of the host. Marine birnaviruses have been isolated from sediment and natural populations of fish and shellfish in the vicinity of aquaculture plants (Rivas et al., 1993) with the consequent risk of spreading the virus to other susceptible populations of the ecosystem. In the present study we characterize these marine birnaviruses comparing them with the freshwater reference strains using several techniques.

Materials and Methods

Cell culture and media

The chinook salmon embryo (CHSE-214) cell line
was used for virus assays. Cells were cultivated at 15°C in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum, containing 100 I.U. penicillin and 100 μg streptomycin per ml.

**Virus**

Viral isolations from turbot were performed following standard procedures (Amos, 1985). Prototype strains of aquatic birnavirus, belonging to the three major serotypes of IPNV, (Sp, Ab and WB) were used in this study. In addition, 6 strains isolated from turbot (Scophthalmus maximus) were selected for this study.

All the isolated strains were identified as aquatic birnaviruses by the distinctive morphology of the unenveloped virions, the characteristic CPE and by the double-stranded character of its RNA (Novoa et al., 1993a). Strains 160 and 311 were isolated in samples that came directly from a Norwegian turbot farm, while strains 306, 390, 460 and 231 were isolated from turbot grown in Spanish farms (Galicia, northwest of Spain). The fish weight ranged from 3–4 g (strain 160) to brood stock (strain 311). Type of sampling was also variable. While 3 isolates (390, 160, 460) were obtained from routine surveys of apparently healthy fish, other isolates (306, 311, 231) were associated to mortalities.

All strains were grown in the chinook salmon embryo (CHSE-214) cell line at 15°C. When cytopathic effect was extensive, supernatant was harvested and centrifuged to eliminate cell debris. Clarified supernatants without purification were used for RNA extraction.

**Viral RNA electrophoresis**

Concentrated virus (pelleted at 100,000 × g for 1 h) was treated with proteinase K (200 μg/ml) at 37°C for 60 min. Samples were mixed with sample buffer, boiled for 5 min and analyzed in SDS-PAGE (Laemmli, 1970), using 7.5% polyacrylamide gels, that were run for 3 h at 100 volts in a Mini Protean Gel. The RNA bands were visualized by staining with silver nitrate (Blum et al., 1987). The RNA segments of a characterized rotavirus from turbot (TRV) (Dopazo, 1991) were used as molecular weight markers.

**35S-methionine labelling and SDS-PAGE analysis of virion polypeptides**

CHSE-214 cells grown on 24-well plates were infected at a multiplicity of infection of 1 TCID₅₀/cell. After 1 h of adsorption, MEM with 2% FBS was added and the uncovered plates were irradiated with UV light (44 ergs/s/mm² at 25 cm) as Dobos and Rowe described (1977). Five hours later, 10 μCi/ml of (³⁵S)-methionine (sp. act. 1,000 Ci/mmol) in methionine-free medium were added for 3 h. Then the cells were washed 3 times with PBS and suspended in 200 μl of 0.1 N NaOH, 1% SDS and 200 μl of sample buffer. The samples were boiled for 2 min and then subjected to SDS-PAGE on 12% polyacrylamide gel. The gel was run for 5 h at 11 mA. The gel was treated twice with dimethylsulfoxide (DMSO) for 30 min, once with 20% of 2,5-diphenyloxazole (PPO) in DMSO during 3 h and then washed with water. After this treatment, the gel was dried and autoradiography was done on X ray film (Kodak).

**Immunodot with monoclonal antibodies**

The interaction of 6 turbot isolates and 3 reference strains with 4 monoclonal antibodies (E2, W3, W5 and AS-1) was analyzed by an immunodot assay following Caswell-Reno et al. (1989). The monoclonal antibodies had different specificities and their pattern of reactions with aquatic birnaviruses, in comparison with those obtained with established serotypes, gives a presumptive serotyping of this viral group (Caswell-Reno et al., 1986, 1989). Epitopes recognized by MAb AS-1 appear to be highly conserved among these viruses, since these epitopes were present on all aquatic birnaviruses tested from the three established serotypes. MAb E2 reacts with all serotype Ab viruses. MAb W3 reacts with Sp and WB and MAb W5 is specific for WB viruses.

**Results**

**Viral RNA electrophoresis**

Sp, Ab and WB strains could be distinguished by the different mobilities of their two genomic segments. All the turbot isolates showed, also, two RNA segments and could be assigned to the prototype IPNV strains by their mobility (Fig. 1). Isolates 231 and 460 showed similar RNA pattern than the Sp strain. RNA segments of the strains 311 and 160 had the same molecular weight that those of the
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American serotype WB (1.9 and 2.1×10^6 daltons), and the isolates 306 and 390 were similar to Ab serotype (Fig. 1).

**Virion polypeptides**

After PAGE-electrophoresis all turbot birnaviruses, as well as the three selected IPNV serotypes could be distinguished by the relative mobilities of their polypeptides (Fig. 2). Treatment with UV light decreased cellular protein synthesis, but did not inhibit this process totally. Differences between the polypeptide pattern of turbot birnaviruses and the reference strains were observed in VP-2 and minoritary proteins.

Turbot isolates 311 and 160 showed the same pattern as the prototype WB. The strain 306 was similar to Ab and the strain 390 to the Sp reference strain. However, the strains 460 and 231 showed a different pattern than the reference strains, while VP 2 polypeptides had the same mobility than those of Ab, minoritary polypeptides were similar to those from Sp related isolates.

**Immunodot with monoclonal antibodies**

The results of the immunodot assay using 4 monoclonal antibodies (MAbs) are summarized in Table 1. The MAbs reaction patterns of the reference strains (SP, Ab, WB) were similar to those reported by Caswell-Reno et al. (1986, 1989). All turbot isolates reacted with the MAb AS-1. The strains 231 and 460 isolated from turbot, did not react with E2, W3 and W5. Turbot strain 306 reacted with MAb W3 (which recognizes Sp and WB epitopes) and with the MAb E2 (which recognizes Ab strains). The strain 390 showed the same reaction pattern that Sp serotype. Finally, birnaviruses 311 and 160 reacted with MAb W3 and W5, which indicates a similarity with the american serotype WB.
Discussion

Aquaculture is a developing activity with a potential high impact in the marine environment. This activity could also play an important role in the spread of viruses that could derive from marine or freshwater cultured species since most of these viruses are highly stable in water with a widespread range of physical and chemical properties (Toranzo and Hetrick, 1982; Wolf, 1988). Farmed production of turbot is steadily increasing in Europe, mainly in France and Spain. Mortalities, attributed to marine birnavirus infections, have been reported in farmed turbot in Norway and Spain (Mortensen et al., 1990, 1993; Novoa et al., 1993a). The high dependence of some of these production plants on the importation of turbot juveniles from other geographical areas increases the risk of introducing “new” pathogens to unexposed marine populations. In spite of the widespread monitoring for aquatic birnaviruses in the freshwater and marine environments, only recently the IPNV American serotype (WB) has been isolated from turbot in Europe (Novoa et al., 1993b). Furthermore, birnaviruses have been isolated from marine bivalves (Underwood et al., 1977; Lo et al., 1988; Rivas et al., 1993) and even from several species used as food for cultured fish. This is the case of rotifer (Brachionus plicatilis), other cultured marine fish larvae, and the fish species used as fresh food for turbot such as raw sand-eel (Ammodytes sp) and blue whiting (Micromesistius potassou) (Rivas et al., 1993). Moreover, infectious IPNV was isolated from wild piscivorous birds faeces frequenting salmonid fish hatcheries (McAllister and Owens, 1992). Also, the persistence and stability of IPNV in commercial fish silage has been reported (Smail et al., 1993). The previous cases could be taken as examples of the danger that aquaculture has for natural populations, once a pathogen is introduced it is virtually impossible to eradicate. A full characterization of the viral isolates from a particular geographical area is particularly important, since the biochemical and molecular properties are usually related to the virulence of the different viral strains.

The characterization of these 6 turbot birnaviruses isolated in a five years survey of health status of several turbot farms (Scophthalmus maximus) revealed a high heterogeneity from such a restricted geographical area as Galicia (NW of Spain). This contrasts with the results obtained by Ledo et al. (1990) and Rivas et al. (1993), that reported that most of the isolates from trout and bivalve molluscs in the same area belonged only to the Sp serotype, in spite of the extensive samplings carried out. Considering that movements of rainbow trout and eggs are more important, the detection of Sp birnaviruses by these authors is even more striking.

Kusuda et al. (1993) compared the serological characteristics of several marine birnavirus strains isolated from different marine fish species. Their biochemical properties were similar, although different to those of the reference strains. This is opposite to our results, since several viral strains isolated from one marine fish (Scophthalmus maximus) from the same geographical area were highly heterogenous. All the strains analyzed showed the RNA and polypeptides pattern of aquatic birnavirus group and they had a positive reaction with the MAb AS-1 which recognizes all the aquatic birnavirus strains (Caswell-Reno et al., 1989). However, only the turbot isolates 311 and 160 could be clearly attributed to the reference serotype WB as Novoa et al. pointed out (1993b). The other strains (390, 306, 460, 231) could be attributed to one or other serotype depending on the technique used. This makes the classification of these strains quite difficult.

The increasing number of birnaviruses isolated from such a variety of aquatic environments is generating a situation of confusion that requires further study and a review of the actual methods being used for the classification of this viral group. As it has been previously reported (Novoa et al., 1995), all marine birnaviruses studied exhibit, as a common characteristic, a positive reaction with the monoclonal antibody N1B9 (produced against the Norwegian strain N1) (Christie et al., 1990), that distinguish them from the aquatic birnaviruses isolated from freshwater species. The neutralization tests currently being used to diagnose the presence of aquatic birnaviruses are often being carried out with non standard polyclonal antibodies. This, in our opinion, is not sure enough to adscribe one particular viral isolate to a certain reference serotype. For this reason, there is an urgent need for the standardization of the methodology applied to the serological characterization of the new strains. More attention should be paid to the introduction of viral pathogens in the marine environment as the consequence of freshwater or marine aquaculture since they may be the
cause of unexplained mortalities or changes in the structure of natural communities adjacent to fish farms.

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