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Isolation and Genetic Characterization of *Betanodavirus* from Wild Marine Fish from the Adriatic Sea

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Abbreviations: BFNNV, barfin flounder nervous necrosis virus; bp, base pair; RGNNV, redspotted grouper nervous necrosis virus; SJNNV, striped jack nervous necrosis virus; TPNNV, tigger puffer nervous necrosis virus

INTRODUCTION

Betanodavirus infection is widespread in a broad spectrum of fish species worldwide. In Italy, it is responsible for outbreaks of Viral Encephalo-Retinopathy (VER) that cause mortality and economic losses mainly in European sea bass (*Dicentrarchus labrax*) farming. *Betanodaviruses* are classified in the Nodaviridae family and 4 official species are present in the genus: BFNNV, Barfin Flounder Nervous Necrosis Virus, RGNNV, Redspotted Grouper Nervous Necrosis Virus, SJNNV, Striped Jack Nervous Necrosis Virus and TPNNV, Tigger Puffer Nervous Necrosis Virus. Recent studies have showed that the infection is also widespread in wildlife (Gagné *et al*., 2004; Guercio *et al*., 2004), where there are generally no observed clinical manifestations. Interspecies transmission has been demonstrated and the presence of asymptomatic carriers in wildlife is strongly suspected. The risk of horizontal transmission between wildlife and farmed fish is particularly high in fattening farming of marine fish that is generally conducted in sea cages or in brackish ponds where there is high possibility of contact with natural environment. As there is no efficient control system available for this infection, knowledge regarding the extent of its spread is fundamental for direct prophylaxis and control of the disease.

In this study we investigated the presence of *Betanodavirus* infection in wild fish population in Adriatic Sea and genetically characterised the strains isolated to compare them with strains isolated during outbreaks with mortality in farmed fish.

MATERIALS AND METHODS

In this study we analysed several wild fish species collected from the Fish Market of Cesenatico (Italy) and fished in the Adriatic Sea, in the area between Rimini and Ravenna. Weekly sampling was conducted between the 25th of May and the 6th of December 2005 with a suspension from the 6th of July to the 15th of September due to interruption of the fishing activity. The number of fish collected each time was variable, based on seasonal availability. In total 293 fish of 22 different species were collected: anchovy, common sea bream, gilthead sea bream, sole, flounder, garpike, shi drum, striped sea bream, Atlantic stargazer, round sardinella, black goby, flathead mullet, red mullet, bogue, gurnard, whiting, horse mackerel, pilchard, annular sea bream, scald fish, Atlantic mackerel and European hake. Samples were tested in pools when there was more than one fish of each species collected on the same days, giving a final number of 109 samples. Viral isolation was conducted from the brain on the SSN-1 cell line. Samples positive to isolation were subjected to viral RNA extraction with TriReagent™ (Sigma, USA), according to the manual instructions. RNA was then used for two step RT-PCR with primers S6 and S7 as previously described (Ciulli *et al*., 2005a). The PCR products were purified with the High Pure PCR Product Purification Kit (Roche, Germany) and sequenced with an automatic sequencer ABI 377 (Applied Biosystem, CA). Sequences obtained were aligned and compared using the Clustal W program (Thompson *et al*., 1994) of Lasargene Biocomputing software (DNASTAR Inc. Madison, USA) with sequences available from GenBank. Phylogenetic analysis was conducted with MEGA version 2.1 program (Kumar *et al*., 2004). The phylogenetic distance was calculated using the Jukes-Cantor method and the phylogenetic tree was built with the Neighbour-joining method. To evaluate the analysis reliability, bootstrap test was carried out on 1000 replicates.

RESULTS

In this study we isolated *Betanodavirus* from 13 samples. All samples positive to viral isolation were confirmed with RT-PCR. The 13 positive samples belong to 10 different fish species of which six (horse mackerel, gurnard, garpike, whiting, European hake and bogue), had never been identified as being susceptible to *Betanodavirus* infection and 4 (flathead mullet, pilchard, red mullet, black goby) had previously been found infected in studies on farmed or wild fish (Guercio *et al*., 2004). The percentages of positive samples were 30 % for gurnard, 28.6 % for black goby, 25 % for pilchard and European hake, 18.2 % for flathead mullet, 14.3 % for horse mackerel, 12.5 % for whiting and 9.1 % for red mullet. The positive garpike sample was the only sample tested for this species. In total 11.9 % of samples were positive for *Betanodavirus* infection.

Nine strains out of 13 were sequenced to obtain a fragment of 395 bp including the variable region of RNA2 of the viral genome. Sequences obtained were compared with reference strains and, on the base of nucleotide similarity and phylogenetic analysis, classified in the species RGNNV.

On the base of phylogenetic analysis the strains isolated were divided into 2 clusters. 100 % of the nucleotide identity was observed inside each cluster, while an identity of 98,2% was observed between the two groups.

Comparing our strains with sequences of virus isolated during VER outbreaks from European sea bass in the Mediterranean Sea and with sequences of virus isolated from other wild fish during a previous survey in Sicily (Ciulli *et al*., 2005b), we observed two different clusters in the phylogenetic tree in which we can identify virus isolated from both wild and farmed fish. Nucleotide identity between virus isolated from wild and farmed fish was in the range of 93.7–100 %. Considering strains isolated from the same fish species, 2 out of 3 viruses isolated from gurnard belong to a cluster, while the other one is clustered in a different group. Furthermore, comparing pilchard strains isolated in this and in a previous study (Ciulli *et al*., 2005b), we observed 98.2 % nucleotide identity.

DISCUSSION

In this survey we investigated the spread of *Betanodavirus* infection in wildlife present in the Adriatic Sea. Six species were found to be susceptible to the infection for the first time. Some of these belong to a family previously been found infected, as in the case of Gadidae, Sparidae and Carangidae, while no report had been made on susceptible species in the Beloniformi and Clupeiformi orders. *Betanodavirus* was found both in bentonic and pelagic (garpike, pilchard, bogue) species. These pelagic fish generally travel a large distance and could easily be in touch with farmed fish, bringing infection from a farm to others.

Aside from consideration of species, a high number of tested fish were positive in this study, showing a large diffusion of the *Betanodavirus* infection in the Adriatic Sea, although no symptoms or signs of this infection has ever been reported in wild fish in this area.

Phylogenetic analysis showed that more than one strain is widespread in wildlife, even if all viruses belong to RGNNV species. Furthermore in some wild fish we found the same strains previously isolated from farmed sea bass during ERV outbreaks.

Phylogenetic analysis revealed that there is no correlation between host species and genetic characteristics.

Further study should be conducted to clarify whether *Betanodavirus* infection in wild fish could be a possible source of infection for farmed fish or if they are, rather, the consequence of virus amplification that occurs during VER outbreaks in farmed fish.

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