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MONOGRAPH

Viral encephalopathy and retinopathy (VER) in Mediterranean wild and farmed fish species: the experience of the Istituto Zooprofilattico Sperimentale of Sicily (Italy)

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ABSTRACT *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 causes mortality and economic losses in sea fish farming. The infection is also widespread in wildlife, where there are generally no observed clinical manifestations. In this study we report the results obtained from the decennial activity of Istituto Zooprofilattico Sperimentale of Sicily on the research of *Betanodavirus* infection in wild fish of Mediterranean Sea and in farmed fish. Among the fish species analyzed, *Gobius niger* (Linnaeus, 1758), *Mullus barbatus* (Linnaeus, 1758), and *Trisopterus minutus capelanus* (Lacepède, 1800) were found positive and these could be a reservoir in which the virus can survive for long periods of time. The *Betanodavirus* isolation from pelagic species such as *Pagellus erythrinus* (Linnaeus, 1758), *Sardina pilchardus* (Walbaum, 1792), *Lepidopus caudatus* (Euphrasen, 1788), *Epinephelus marginatus* (Lowe, 1834), *Epinephelus aeneus* (Geoffroy Saint-Hilaire, 1817) resulted interesting because these species could play a more significant epidemiological role, being able to move even at distances.

KEY WORDS Viral encephalopathy and retinopathy (VER); *Betanodavirus*; sea fish; farmed fish.

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INTRODUCTION

Betanodavirus is non-enveloped, spherical and approximately 25 nm in diameter, RNA virus belonging to the family of Nodaviridae (Schneemann et al., 2005). The genome consists of two molecules of positive-sense ssRNA: RNA1 (3.1 kb) encodes the replicase (110 kDa) and RNA2 (1.4 kb) encodes the coat protein (42 kDa). Since 1995 the ERV has also been reported in Italy where the most affected species is European bass, *Dicentrarchus labrax* (Linnaeus, 1758). It is responsible for outbreaks of Viral Encephalo-Retinopathy (VER) otherwise known as viral nervous necrosis (VNN), considered to be a serious disease of several marine fish species, characterised by significant losses associated to vacuolating lesions of the central nervous system and the retina. To date, the disease has been reported in more than 50 fish species, mainly marine with the greatest impact being in striped jack, European sea bass (*Dicentrarchus labrax*), groupers, and flatfishes (Munday et al., 2002; Sano et al., 2011).

VER causes mortality and economic losses in sea fish farming; the infection is also widespread

in wildlife (Gagnè et al., 2004; Guercio et al., 2004), where there are generally no observed clinical manifestations. A few outbreaks have also been documented in freshwater farms (Bovo et al., 2011; Chi et al., 2003) suggesting that salinity is not a limiting factor and it is possible that the virus may spread to economically important freshwater species. 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 (Ciulli et al., 2007). Furthermore, the presence of *Betanodaviruses* was detected in bivalve molluscs. This invertebrates when reared in the same area as farmed and wild finfish could act as a reservoir of the virus. Current European regulations allow relaying activities and the sale of live bivalve molluscs, which could pose a real risk of spreading *Betanodaviruses* across different geographic regions (Volpe et al., 2017). Knowledge regarding the extent of disease spread is fundamental for its direct prophylaxis and control.

In this study, we reported the results obtained from the decennial activity of Istituto Zooprofilattico Sperimentale (IZS) of Sicily on the research of *Betanodavirus* infection in wild fish of Mediterranean Sea and in farmed fish (Ciulli et al., 2005; Ciulli et al., 2006a; Ciulli et al., 2006b; Guercio et al., 2004; Nishizawa et al.,1994; Purpari et al., 2007; Toffan et al., 2014).

MATERIAL AND METHODS

Between 2004 and 2016, a total of 1614 tests for *Betanodavirus* (RT-PCR and viral isolation on cell cultures) were performed on brain samples of wild-caught and farm-raised fishes along the coasts of Sicily (Italy).

Viral isolation on cell cultures

Brain samples were homogenised with 1:5–1:10 volumes of Hanks' balanced salt solution (HBSS, Sigma-Aldrich) containing antibiotics (penicillin - 800 International Units [IU]/ml and streptomycin - 800 µg/ml) to avoid bacterial contaminations. The antibiotic treatment was performed for 4 hours at 15°C or overnight at 4°C.

The antibiotic treated tissue suspension at two different dilution: the primary dilution in culture medium L-15 (Sigma-Aldrich) and a 1:10 dilution thereof, were inoculated on SSN-1 cells (cell line derived from striped snakehead (Frerichs et al., 1996) and incubated at a temperature of + 25°C. The cells were monitored by microscopy daily for 10 days in order to highlight the presence of a cytopathic effect (ECP) attributable to the presence of the virus. If no CPE occurred after the primary incubation period, subcultivation was performed on fresh cultures, using a similar cell growing area to that of the primary culture.

One Step RT-PCR

Viral RNA was extracted from brain samples and cell culture supernatants using the High Pure Isolation Kit (Roche) according the manufacturer's instructions. Total RNA was subjected to reverse transcription followed by PCR amplification performed by AccessQuickTM RT-PCR System (Promega) following the manufacturer's recommendations. A pair of primers, designated as OIEF2/ OIE-R3 (Table 1), specific for a 427 bp fragment of the T4 region of the RNA2 gene coding for the 42 KDa capsid protein (Nishizawa et al.,1994) was used. The thermocycling conditions were: + 54°C for 30 min, + 92°C for 2 min and 35 cycles of 30 s denaturation at + 94°C, 30 s annealing at + 55°C and 30 s elongation at + 72°C; the reaction was terminated with a 7 min elongation at + 72°C.

The methods used were consistent with what is described in the O.I.E. Manual (2013) and certified according to UNI EN ISO/IEC 17025:2018 quality standards.

PRIMERS	SEQUENCE
OIE-F2	5'-CGT GTC AGT CAT GTG TCG CT-3'
OIE-R3	5'-CGA GTC AAC ACG GGT GAA GA-3'

Table 1. Primers used in One Step RT-PCR.

RESULTS

Over a decade of activity, a total of 736 virus isolations on cell cultures (Table 2) and 878 RT PCR for *Betanodavirus* (Table 3) have been performed on brain samples of wild fish and breeding species from all over Sicily. A total of 88 samples were positive in RT-PCR and 126 by the culture method. The discrepancy in the results is due to the fact that it has not always been possible to analyze the samples with both methods; in particular, none of the 2004 samples and not all the 2005 samples were examined by the RT-PCR. SSN-1 cells inoculated with positive samples showed the presence of a cytopathic effect (ECP) attributable to the presence of the virus (Figs. 1, 2).

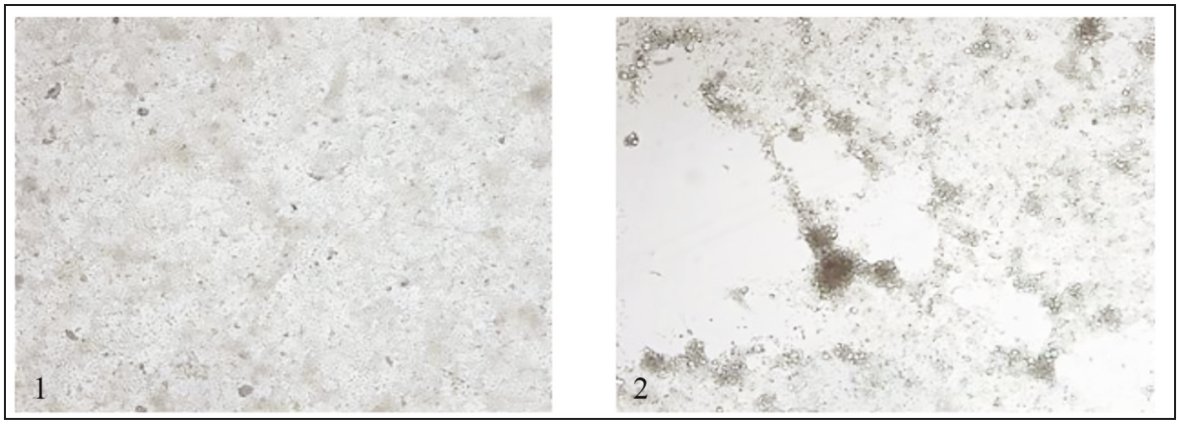
The "Fish, Crustaceans and Molluscs Disease Reference National Centre of the IZS Venezie" confirmed the positives. Among the species positive for *Betanodavirus* are commonly affected species such

YEAR	N°. TEST/YEAR	NEGATIVE	POSITIVE/FISH SPECIES	WILD/FARMED FISH
2004	5	3	*1 Pagellus erythrinus* *1 Trisopterus minutus capellanus*	wild
2005	369	307	50 *Gobius niger* 5 *Sardina pilchardus* 6 *Mullus barbatus* 1 *Trachiurus trachiurus*	wild
2006	97	97	0	
2007	99	99	0	
2008	97	97	0	
2009	11	3	8 *Epinephelus marginatus*	wild
2010	-	-	-	
2011	-	-	-	
2012	-	-	-	
2013	1	0	1 *Epinephelus aeneus*	wild
2014	57	4	2 *Epinephelus marginatus* 51 *Dicentrarchus labrax*	wild farmed
2015	-	-	-	-
2016	-	-	-	-
TOT	**736**	**610**	**126**	-

Table 2. Results of the VER isolation on SSN-1 cells from fish brains from 2004 to 2016.

YEAR	N°. TEST/YEAR	POSITIVE	NEGATIVE
2005	165	23	142
2006	58	0	58
2007	151	0	151
2008	225	2	223
2009	52	8	44
2010	15	0	15
2011	5	0	5
2012	3	0	3
2013	102	0	102
2014	58	53	5
2015	41	2	39
2016	3	0	3
TOT	878	88	790

Table 3. Results obtained in RT-PCR for VER performed on fish brains from 2005 to 2016.



Figures 1, 2. SSN-1 monolayer (Fig. 1) andECP induced by VER on SSN-1 cells (Fig. 2).

as *Dicentrarchus labrax*, wild benthic species such as *Gobius niger* (Linnaeus, 1758), *Mullus barbatus* (Linnaeus, 1758) and *Trisopterus minutus capelanus* (Lacepède, 1800) and wild pelagic species very common in the Mediterranean Sea such as *Pagellus erythrinus* (Linnaeus, 1758), *Sardina pilchardus* (Walbaum, 1792), *Lepidopus caudatus* (Euphrasen, 1788), *Epinephelus marginatus* (Lowe, 1834), *Epinephelus aeneus* (Geoffroy Saint-Hilaire, 1817). A total of 55 strains of *Betanodavirus* have been also cryo-preserved in liquid nitrogen.

DISCUSSION AND CONCLUSIONS

Since 2004, IZS of Sicily in collaboration with IZS delle Venezie is concerned with the research of *Betanodavirus*, highlighting its widespread diffusion in breeding species and in many wild species

of the Mediterranean Sea, in which, in most cases, the presence of virus had never been reported. Among the species that were positive, *Gobius niger*, *Mullus barbatus* and *Trisopterus minutus capellanus* can be reservoir in which the virus can survive for long periods of time. The isolation of *Betanodavirus* from pelagic species such as *Pagellus erythrinus*, *Sardina pilchardus*, *Trachiurus trachiurus*, *Epinephelus marginatus*, *Epinephelus aeneus* has aroused particular interest.

In fact, these species could play a more significant epidemiological role, being able to travel a large distance and these could easily be in touch with farmed fish, bringing infection from a farm to others. Such a wide spread of the pathogen, even in a natural environment, makes it necessary to investigate the existing epidemiological correlations between infection in farmed and wild species for the close contact between the two environments.

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