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Short communication

# Outbreak of Viral Nervous Necrosis in Endangered Fish species *Epinephelus costae* and *E. marginatus* in Northern Tunisian Coasts

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**ABSTRACT**—In this study, we report outbreak of viral nervous necrosis (VNN) in wild *Epinephelus* species, which are of an endangered fish group, in different Tunisian coastal areas in 2012. Seven fish of *E. marginatus* and *E. costae* caught at dead or moribund condition were investigated. Betanodavirus was detected in the brain and retinal tissues of all fish by RT-PCR and at high infective titers ($10^{6.0–8.8}$ $TCID_{50}$/g) in five of seven fish. Sequence and phylogenic analyses of the viral genes revealed that the viruses belonged to RGNNV genotype and were closely related to some previously reported Mediterranean betanodavirus strains, suggesting virus exchanges among different fish populations in the Mediterranean Sea.

**Key words:** betanodaviruses, disease outbreak, *Epinephelus* spp., phylogeny, RGNNV

Viral nervous necrosis (VNN) is a major concern for marine aquaculture (Munday *et al.*, 2002). Similarly, VNN represents a menace to wild fish populations, affecting numerous species of nine distinct orders (Panzarin *et al.*, 2012). Diseased fish present an uncoordinated swimming behaviour as a consequence of lesions occurring in the central nervous system. In larval and juvenile stages, this disease can cause up to 100% mortality and in adults, significant mortality was reported (Bovo *et al.*, 1999; Munday *et al.*, 2002 ).

VNN is caused by betanodaviruses, non-enveloped viruses belonging to the family *Nodaviridae.* Betanodaviruses have a single-stranded positive-sense RNA genome divided into two segments: RNA1 (3.1 kb) and RNA2 (1.4 kb) coding for a RNA-dependent RNA polymerase and a coat protein respectively (Mori *et al.*, 1992). On the base of phylogenetic analysis of the RNA2 segment, five genogroups have been described: red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV), tiger puffer nervous necrosis virus (TPNNV), striped jack nervous necrosis virus (SJNNV) and turbot nervous necrosis virus (TNNV) (Nishizawa *et al.*, 1997; Johansen *et al.*, 2004). However, NNV genetic diversity is more complex since many reassortant viruses bearing an RNA1 segment related to a specific genotype and an RNA2 segment from another genotype have been described. Thus the combined characterization of RNA1 and RNA2 segments is recommended (Panzarin *et al.*, 2012).

The genus *Epinephelus*, one of the most relevant fish genus, is highly susceptible to betanodavirus infection. Numerous VNN outbreaks have been reported in farmed and wild *Epinephelus* spp. in Asia (Munday *et al.*, 2002). In the Mediterranean sea, VNN outbreaks in *Epinephelus* spp. was first reported in 1999 in the natural marine reserve of Ustica, Italy (Marino and Azzurro, 2001) and recently in 2011 in the South of Italy (Vendramin *et al.*, 2013a) and the Northeastern of Algeria (Kara *et al.*, 2014). RGNNV isolates were also found in such groupers in 2001, 2002 and 2009 in Italy and Greece (Panzarin *et al.*, 2012).

In the Mediterranean Sea, the genus *Epinephelus* is of great interest from an ecological and economic point of view. Among these species, *E. marginatus,* which is heavily exploited, is safeguarded and included in the Red list of marine fishes (Zabala *et al.*, 1997). Particularly, in Tunisia, groupers are represented by six species: *E. aeneus, E. caninus, E. costae, E. haifensis, E. marginatus* and *Mycteroperca rubra* (Bradai *et al.*, 2004). Notably, standings and mortalities of wild groupers, such *Epinephelus* spp., were reported in the Northern regions during the last years. Betanodavirus RGNNV strains were isolated from farmed fishes since 1992 (Thiéry *et al*., 2004) and, recently in 2011, we demonstrated the spread of RGNNV strains among farmed fishes along the Tunisian coasts (Haddad-Boubaker *et al.*, 2013). However, the presence of betanodavirus in wild fish stocks has never been officially described so far.

The present study reports the occurrence of severe disease and mortality of wild *Epinephelus* species in association with betanodaviruses in northern coasts of Tunisia (Fig. 1).

## Material and Methods

In 2012, in the period of August to November, severe mortalities of wild dusky groupers *E. marginatus* and golden groupers *E. costae* in northern Tunisian coasts (Tabarka, Cap Negro, Cap Serrat, Kef Abed, Bizerte, Cap Bon and Kelibia were documented by fishermen and scuba divers (Fig. 1). More than 250 specimens presented neurologic signs or were recovered stranding on the beach. A total of eight samples were collected from September to November (Table 1):

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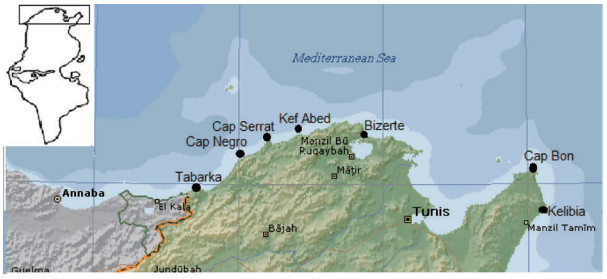


**Fig. 1.** Geographical distribution of sites where moribund groupers were observed.

seven wild *Epinephelus* specimens sampled in Tabarka, Bizerte, Kelibia regions (Fig. 1) and one farmed European seabass *Dicentrarchus labrax* specimen obtained during an outbreak in the Sahel region (Eastern Tunisian coast) (Table 1). *Epinephelus* specimens were identified as *E. marginatus* (n = 4) and *E. costae* (n = 3). Six of them were caught by fishermen and exhibited opaque eyes, while one moribund specimen was collected alive by scuba diver during an exploration of Tabarka region in the first of November (Table 1). The moribund fish (T2.12) presented erratic swimming behavior with loss of equilibrium and, sometimes, sank to the bottom and then floated to the surface again. The sea surface temperature was 24°C at the time of capture. The caught specimens were obtained in September (T1.12, B1.12, K1.12), October (T3.12, B2.12) and November (T4.12) at 26–27°C, 24–25.5°C and 22°C sea surface temperature respectively. Concerning the *D. labrax* specimen, the fish was collected at the first of September at 27°C sea surface temperature.

The total length and weight of each specimen were recorded. Examination and dissection of fish were focused on the observation of swim bladder and search of parasites in the gills and digestive tract. At autopsy, brain and retinal tissues were sampled. Portions of brain obtained from specimens T1.12, B2.12 and K1.12, caught respectively from Tabarka, Bizerte and Kelibia regions, were conserved for histopathological examination.

Virus isolation was assessed on striped snakehead fry cell line (SSN-1) (Frerichs *et al.*, 1996). Briefly, SSN-1 cells were propagated at 29°C in Leibovitz medium (L-15) (Sigma) supplemented with 10% of fetal bovine serum, penicillin (100 I.U./mL) and streptomycin (0.1 mg/mL). The samples of brain and retinal tissues were separately homogenized with a mortar and pestle and diluted 1:10 (w:v) in L-15 medium containing penicillin (200 I.U./mL), streptomycin (0.2 mg/mL) and kanamycin (0.2 mg/mL). The homogenates were centrifuged at 3,000 × *g* for 15 min (4°C) and the supernatants were either immediately used or stored at –80°C. Virus isolation was assessed at 22–27°C, according to the water temperature during fish collection. Infectivity viral titers, expressed as tissue-culture infective dose ($TCDI_{50}$/mL), were calculated as described by Reed and Muench (1938).

For molecular detection, RNA extraction was performed by the NucleoSpin RNA II kit (Macherey-Nagel) on the tissue homogenates. A segment of 170 bases in the RNA1 gene was amplified using oPVP154 and oPVP155 primers and the One-Step RT-PCR kit (QIAGEN) (Haddad-Boubaker *et al.*, 2013).

For genetic characterization, products of 680 bases in the RNA2 segment and 930 bases in the RNA1 segment were obtained using the oPVP88/oPVP111 (Bigarré *et al.*, 2010) and the VNNV5/VNNV6 primer sets (Toffolo *et al.*, 2007) respectively and the One-Step RT-PCR kit (QIAGEN).

The phylogenetic analysis of the obtained RNA1 and RNA2 sequences and relevant sequences available in GenBank was conducted using the maximum-likelihood method available in the MEGA 5.1 package. Robustness of individual nodes was confirmed with 1,000 bootstrap replicates. The sequences generated in this study were submitted to GenBank (Table 1).

## Results and Discussion

The examination of the digestive tract exhibited a total vacuity (100%) and no signs of parasitism. A hyperinflated swim bladder was observed in five samples (T1.12, T2.12, T3.12, B2.12 and K1.12).

Histopathological examination of the three samples of brain (T1.12, B2.12 and K1.12) revealed the presence of severe cytoplasmic vacuolation with consistent nuclear pyknosis suggesting the involvement of betanodavirus in the mortality.

Molecular investigation showed positive PCR results for all the samples confirming the presence of betanodavirus. For five samples, positive results were also confirmed by virus isolation (T4.12 and B1.12 samples were negative): virus infective titers in the brain

**Table 1.** Details of investigated fish specimens and related GenBank accession numbers

| Specimen | Species | Geographic origin | Weight (kg) | Total length (cm) | Clinical signs | Virus Titer ($TCID_{50}$/g) | | Accession number | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Brain | Retina | RNA1 | RNA2 |
| T1.12 | *E. marginatus* (w) | Tabarka | 5.3 | 65 | Opaque eyes | $10^{8.2}$ | $10^{7.5}$ | KF748953 | KF748945 |
| T2.12 | *E. costae* (w) | | 6.9 | 74 | Neurological signs, Superficial lesions | $10^{8.8}$ | $10^{7.9}$ | KF748949 | KF748941 |
| T3.12 | *E. marginatus* (w) | | 3.2 | 59 | Opaque eyes | $10^{6.9}$ | $10^{6.0}$ | KF748950 | KF748942 |
| T4.12 | *E. costae* (w) | | 7.2 | 69 | Opaque eyes | – | – | KF748951 | KF748943 |
| B1.12 | *E. costae* (w) | Bizerte | 4.6 | 64 | Opaque eyes | – | – | KF748952 | KF748944 |
| B2.12 | *E. marginatus* (w) | | 6.3 | 72 | Opaque eyes | $10^{7.0}$ | $10^{6.2}$ | KF748954 | KF748946 |
| K1.12 | *E. marginatus* (w) | Kelibia | 5.2 | 66 | Opaque eyes | $10^{6.4}$ | $10^{6.0}$ | KF748955 | KF748947 |
| S16.12 | *D. labrax* (f) | Sahel region | 0.15 | 11 | No | $10^{7.3}$ | $10^{7.0}$ | KF748956 | KF748948 |

(w): Wild fish; (f): Farmed Fish

and retinal tissues were $10^{6.4}$–$10^{8.8}$ $TCID_{50}$/g and $10^{6.0}$–$10^{79}$ $TCID_{50}$/g respectively (Table 1). Thus, high infective titers in nervous organs in addition to positive molecular results confirmed involvement of betanodavirus in the mortality for five samples (T1.12, T2.12, T3.12, B2.12 and K1.12) collected from the three Northern Tunisian regions. In the other hand, for samples T4.12 and B1.12, the absence of swim bladder inflation and cytopathic effect on SSN-1 cells may only suggest a carriage of betanodavirus.

The phylogenic analysis of the RNA1 and RNA2 sequences demonstrated that the studied viruses belong to the RGNNV genotype (Fig. 2). These viruses shared 95.2–100% and 96.2–100% nucleotide identity for the RNA1 and RNA2 segments respectively. Both topologies confirmed that strains T1.12 and B2.12 detected in *E. marginatus* specimens in two different regions are 100% identical, but differ from the other Tunisian strains herein characterized. On the base of the RNA1 and the RNA2 sequences, they were highly related to Italian strains (JX290521 and JX290531) isolated from wild *Mullus barbatus* (99.8% and 99.7% identity, respectively) and *E. costae* (99.7% and 99.5% identity, respectively) collected in 2011 as well as to a Tunisian strain (HE796793) (99.9% RNA2 identity) obtained during an outbreak in 2011. B1.12 strain originating from Bizerte showed to be highly related to samples T2.12, T3.12 and T4.12 collected in Tabarka (99.9% identity for RNA1 and RNA2, respectively).

Viruses S16.12 and K1.12 detected in farmed sea bass and wild dusky grouper respectively, were highly similar to each other (99.9% identity for RNA2). Overall, high nucleotide similarities were observed between the investigated viruses and Tunisian viruses previously detected in farmed fish in 2011 (HE796793, HE796785, HE796792 and HE796795) as well as with viral isolates originating from Italy (JX290521, JX290531, JN189799, JN189952) and Greece (JN189823, JN189828, JN189980, JN189975). Taken together, these data suggest the existence of epidemiological connections between different geographic areas of Tunisia and the Mediterranean, and the occurrence of viral exchanges between reared and feral fish.

In the Mediterranean basin, several reports described the presence of betanodavirus in wild fish stocks (Ciulli *et al.*, 2007; Panzarin *et al.*, 2012; Vendramin *et al.*, 2013a). In this study, we report, for the first time, the occurrence of a severe VNN outbreak affecting a large number of *Epinephelus* specimens in different northern Tunisian regions. This mortality episode seems to become recurrent phenomenon in accordance with the augmentation of water temperature. Indeed, the investigation conducted with professional fishermen and diving clubs in the region of Tabarka showed that the mortality episodes of groupers had existed during warm season (August to November) since 1984. The phenomenon sporadically reappeared in the following years, and became recurrent with more emphasis after 2003, affecting annually a larger number of fish species (*Balistes carolinensis*, *Corvina nigra*, *Diplodus sargus*) and extending to a wider geographic area (Bizerte, Cap Serrat, Cap Negro, Kef Abed and Kelibia) (Fig. 1), although the involvement of betanodavirus in this mortality has never been officially investigated. It is generally assumed that the water temperature is a major factor for the development of clinical disease. In northern Tunisia, the average of sea surface temperatures has

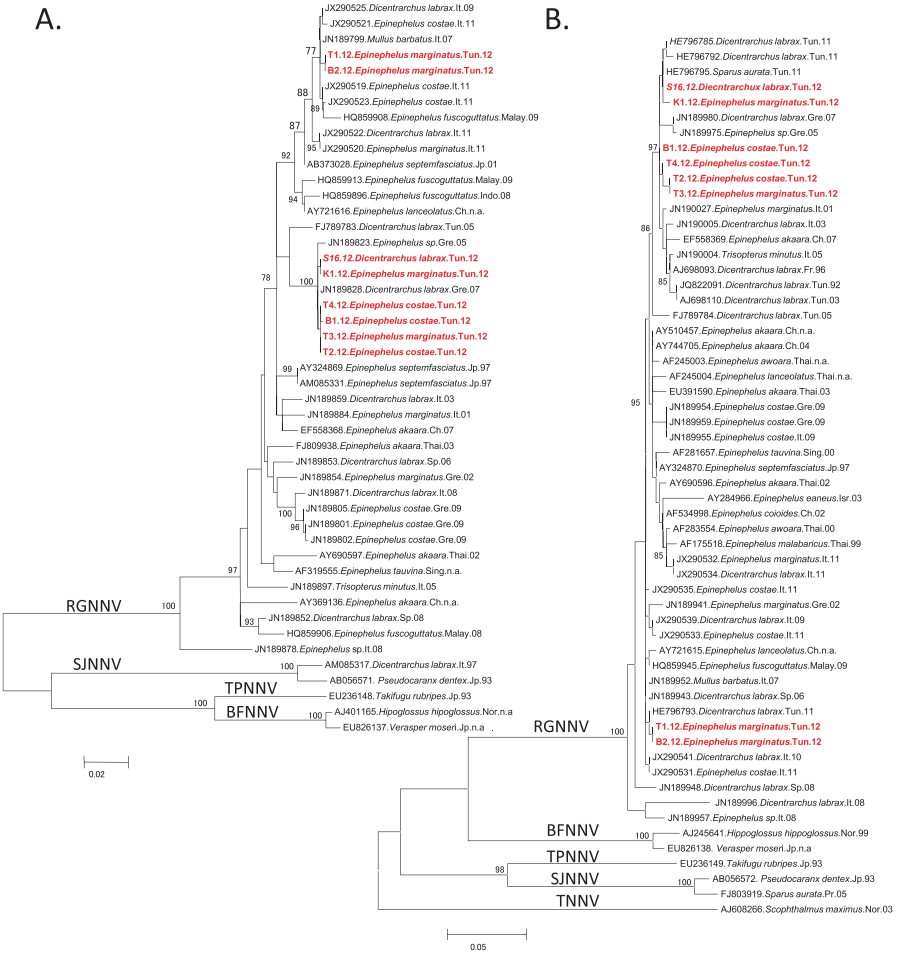


**Fig. 2.** Comparative phylogenetic analysis of the investigated viruses based on the partial RNA1 and RNA2 sequences: (A) Partial RNA1 sequence based tree; (B) Partial RNA2 sequence based tree. The phylogenetic tree was inferred through the Maximum Likelihood method with 1000 bootstrap replicates (only values ≥ 75 are reported at branch points). The genotype subdivision is shown at the main branches. The scale bar represent the number of substitutions per site. Tunisian viruses obtained from wild golden grouper, dusky grouper and European sea bass, in 2012, are labeled in red. n.a., not available.

increased by 0.9°C during the last 50 years, according to the Simple Ocean Data Assimilation database, and the largest rises occurred between 2000–2009, the period from which this disease has spread (data not shown). This significant increase of temperature could have disastrous consequences on ecosystems and fish species such as groupers. It can be also considered as a stimulating factor of virulence of some pathogens including betanodaviruses. Many cases of betanodavirus outbreaks have been reported at 24–30°C water temperature (Fukuda *et al.*, 1996; Chi *et al.*, 1999; Athanassopoulou *et al.*, 2003) as well as the increase of betanodavirus pathogenicity under experimental conditions (Nishizawa *et al.*, 2012; Vendramin *et al.*, 2013b). However, it is difficult to assess whether the onset of clinical disease is a consequence of the optimal temperature for virus multiplication, or it is caused by the physical stress of fish induced by elevated temperatures. Both factors may probably have a role in the apparition of clinical disease.

Our phylogenic data suggest the occurrence of viral exchange between farmed and wild fish populations as previously hypothesized by Vendramin *et al.* (2013a). Notably, in this case, Tunisian aquaculture facilities are mostly located in the eastern coasts, far from Tabarka and Bizerte regions (more than 100 km) and it is known that *Epinephelus* species are territorial, generally standing in rock caves. Thus, we speculate that the transmission of viruses might occur via vectors such as other wild fish. The contamination via food chain should also be considered since groupers are carnivore, especially piscivore. However, it is difficult to assess the origin of the infection: *Epinephelus* species can constitute a reservoir acting as a potential source of virus and at the same time farmed fish might shed high levels of virus in the environment during outbreaks, causing contamination of different wild species. More investigations are needed to evaluate the role of *Epinephelus* species in the dynamics of betanodavirus infections.

In the concern of general decline of endangered fish groupers, the control of the disease by combination of good management practices and vaccination of adults and/or fries could preserve wild fish populations by limiting viral exchanges, sustaining virus rejection in the environment and in the same time could reduce severe economic losses in aquaculture facilities.

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