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Original Research Article

THE FIRST OUTBREAK OF VIRAL ENCEPHALOPATHY AND RETINOPATHY IN FARMED SEA BASS (*Dicentrarchus labrax)* IN SLOVENIA

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Abstract: Viral encephalopathy and retinopathy (VER) is considered a serious disease of several marine fish species, caused by RNA virus belonging to the family *Nodaviridae*, genus *Betanodavirus*. The disease is spread almost worldwide and causes significant losses among diseased fish. It is characterised by vacuolation of the central nervous system and the retina.
In July 2018, behavioural abnormalities i.e. altered swimming, swirling and vertical floating as well as lethargy and anorexia were observed in farmed sea bass (*Dicentrarchus labrax*) in the Gulf of Piran (Slovenia), associated with significant mortality. The disease initially occurred in juvenile sea bass, but later market-sized fish also became affected. Diseased fish displayed ocular opacity and multifocal skin ulceration on the head. Emaciation in some fish was also evident. Histopathology revealed characteristic vacuolation in the brain and retina. Performing a RT-PCR and RT-qPCR techniques, we have identified and confirmed the presence of betanodavirus nucleic acid in ocular and brain tissues. In addition, concentrations of the causative agent of VER in spleen and kidney did result in significantly higher viral yield than expected. Phylogenetic analysis showed that Slovenian isolate belongs to RGNNV species of betanodaviruses. Based on the clinical signs, gross and typical microscopic lesions and results of molecular analyses, we can conclude that farmed sea bass from the Gulf of Piran were affected with VER. To the best of our knowledge, this is the first report of VER in Slovenia.

Key words: viral encephalopathy and retinopathy; betanodavirus; sea bass; histopathology; RT-qPCR

Introduction

Viral encephalopathy and retinopathy (VER), also termed as viral nervous necrosis (VNN), is a serious neuropathological disease of more than 50 fish species almost worldwide (1). It occurs mostly in marine environment, but the outbreaks in freshwater fish have also been reported (2, 3, 4). In marine aquaculture, VER is considered one of the most devastating infectious diseases (5), and sea bass (*Dicentrarchus labrax*) seems to be one of the most commonly and severely affected species (6). The disease affects mostly the larval and juvenile stages (1, 7), however, in several fish species, such as sea bass (8, 9) and grouper (*Epinephelus septemfasciatus*) (10), mass moralities have been also reported in adult and market-sized fish (1). Additionally, clinical signs and mortalities associated with VER were reported in wild fish species (11).

The causative agent is small (approximately 25 nm in diameter) spherical non-enveloped RNA virus, belonging to the genus *Betanodavirus* within family *Nodaviridae* (1, 12).

Based on the phylogenetic analysis of the RNA sequence of the T4 variable region, betanodaviruses have been clustered into four

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species, named striped jack nervous necrosis virus (SJNNV), red-spotted grouper nervous necrosis virus (RGNNV), barfin flounder nervous necrosis virus (BFNNV) and tiger puffer nervous necrosis virus (TPNNV) (13). It is reported that RGNNV exhibits the widest host range of warm water species (14, 15), including sea bass. Furthermore, two reassortants RGNNV/SJNNV and SJNNV/RGNNV have been described and reported to infect different fish species in Mediterranean (5, 16, 17, 18). Betanodaviruses have been often detected in apparently healthy wild marine fish (19, 20, 21).

VER is characterized by typical changes in swimming pattern associated with affected nervous system (15), such as whirling, spiralling or looping, erratic swimming, lying down on the bottom, keeping vertical positions, lying on their sides or belly up and body curved (8, 9, 10, 22). In addition, lethargy, changes in skin pigmentation, skin erosion in the head region, ocular opacity and exophthalmia have been described (8, 11). Histopathological findings, most commonly characterized by vacuolation and necrosis of nerve cells of the brain, retina and spinal cord, are remarkably consistent among the various affected fish species (15, 23).

In this paper we describe the first occurrence of VER in Slovenia, including clinical signs, gross pathology, histopathological lesions and the results of molecular diagnostic procedures. Some epidemiological aspects are also discussed.

Case presentation

Case history and clinical signs

In July 2018, abnormal swimming behaviour associated with heavy mortalities occurred in sea bass reared in floating cages in the Gulf of Piran. Affected fish showed erratic swimming, impulsive movements, swirling, belly up or keeping vertical position with either head or caudal peduncle upside. Some were laying on their sides and body curved. Moreover, lethargy, anorexia, change in skin pigmentation, endo– or exophthalmia, ocular opacity and congestion of the head were observed. The disease initially occurred in older juveniles (125 g) and later market-sized fish became affected. There was no significant mortality observed in younger juveniles (50 g). Sea bream (*Sparus aurata*) also remained clinically unaffected (Table 1).

The marine fish farm concerned is the only one in Slovenia with the annual production of 100 tons and had no history of VER. No vaccination was carried out at that time; the juveniles introduced into the farm in 2017 had already been vaccinated against *Listonella anguillarum*, but not against other pathogens. In fact, the number of introduced juveniles was higher than in previous years, yet the density was only about 4.7 kg/m^3. Otherwise, husbandry practices and epizootiology in the area in the year of the outbreak generally did not differ from previous years.

In spring 2018, only sea bream juveniles were introduced into the farm, while the last introduction of sea bass juveniles took place in autumn 2017. At the time of the disease outbreak, the water temperature exceeded 26°C.

The outbreak characterized by high losses lasted until the beginning of October 2018. The mortality firstly decreased in the population of juvenile sea bass (125 g) in the middle of September at the water temperature range 23–25°C. About two weeks later at water temperature below 20°C, the disease mitigated in adult sea bass as well (Figure 1). Nevertheless, after the mortality rate in autumn had decreased, abnormal swimming behaviour was still present in some fish and was regularly observed for months.

Gross pathology

Samples of clinically affected sea bass were collected from various cages of older juveniles

Table 1: Cumulative mortality, from July 1st to December 31st, 2018, of different fish populations in the fish farm

Fish species and category	**Adult sea bass**	**Adult sea bass**	**Juvenile sea bass**	**Juvenile sea bass**	**Juvenile sea bream**	**Juvenile sea bream**
An average weight on July 1st, 2018 [g]	870	300	125	50	110	20
Cumulative mortality from July 1st to December 31st, 2018 [%]	51	48	45	8	2	4

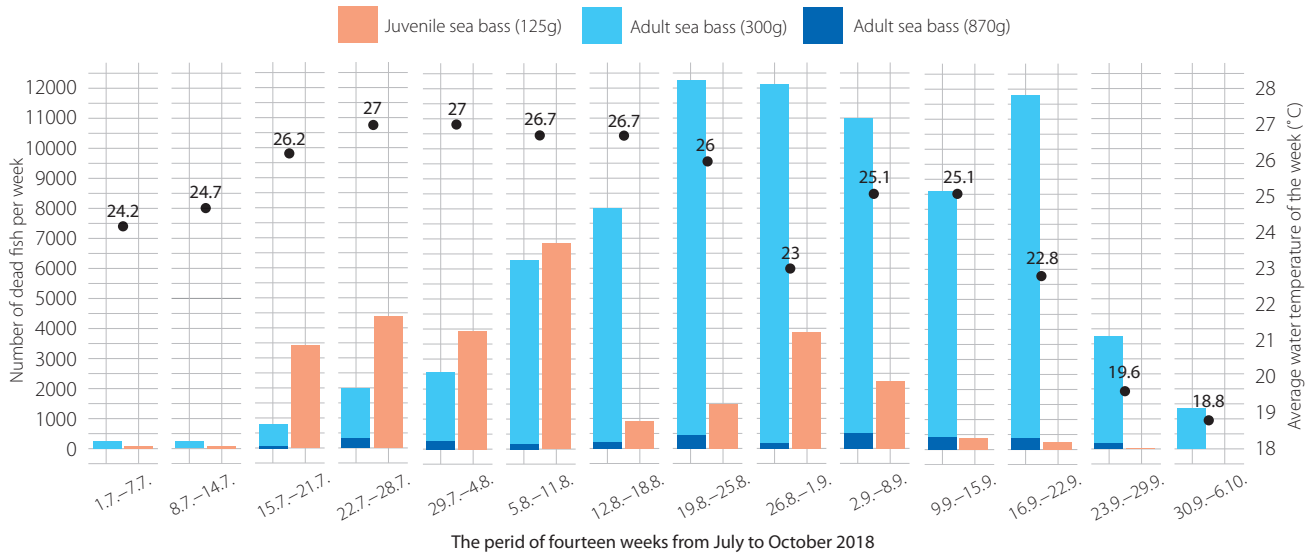

Figure 1: Disease pattern regarding the number of dead fish per week in most affected populations (juvenile sea bass (125 g) and adult sea bass (300 g and 870 g)) of sea bass at different sea temperature (an average weekly temperature)

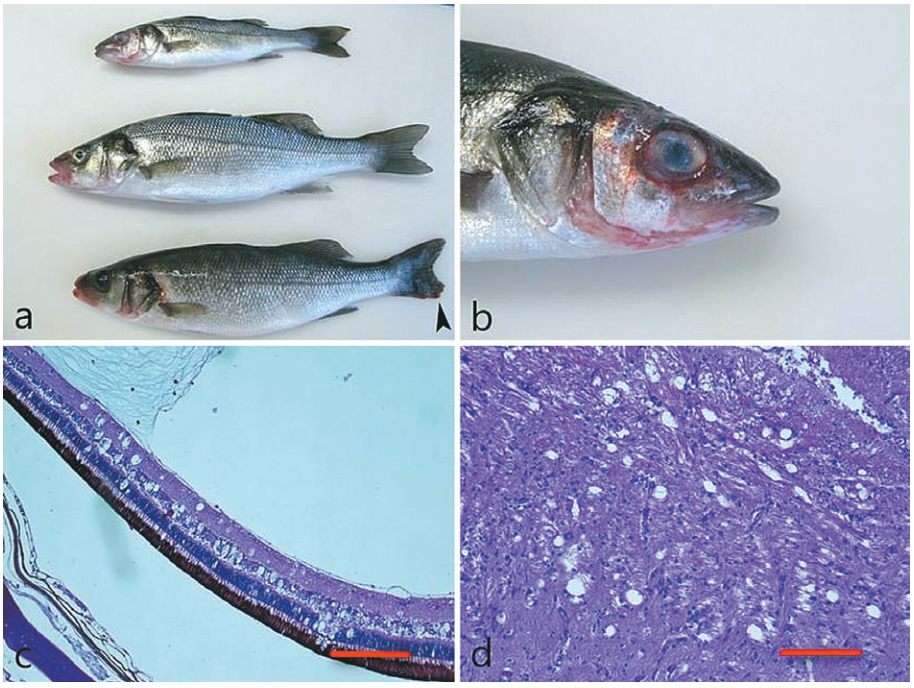

Figure 2: Viral encephalopathy and retinopathy. (a) Gross lesions included emaciation, ocular opacity and congestion of the head. In one fish, caudal fin erosion was noticed (arrowhead). (b) Close-up of the gross lesions showing ocular opacity and congestion on the head. (c) Characteristic vacuolation of the retina. HE. Scale bar: 100 µm; magnification: 100×. (d) Characteristic vacuolation in the neuropil of the brain. HE. Scale bar: 100 µm; magnification: 200×

(125 g) and adults (300 g). Seven fish (four older juveniles, three adults) were subjected for necropsy. External examination revealed lesions limited to the head, which consisted of multifocal skin ulceration and congestion, ocular opacity, exophthalmia or endophthalmia (Figures 2a, b). Additionally, one fish was emaciated, and caudal fin erosion was evident in another one (Figures 2a). No lesions were observed with examination of internal organs.

Histopathology

Eye and brain samples were fixed in 10% buffered formalin and routinely embedded in paraffin for histopathological examination. Four-µm thick tissue sections were first deparaffinised and then stained with haematoxylin and eosin (HE). Stained sections were examined with a light microscope. Microscopically, characteristic vacuolation in the retina and the brain was observed at different

degrees (Figures 2c, d). Brain vacuolation was mostly present in the neuropil and only single vacuoles were found in the neurons. Only in one fish, multifocal mild perivascular lymphocytic infiltrates and gliosis were found in the brain stem.

Virological analysis

The brain tissue and eyes as well as spleen and kidney were pooled separately and submitted for laboratory viral diagnostics. Fish organ homogenates were screened for betanodavirus by RT-PCR and RT-qPCR following the methods documented by Nishizawa et al. (24) and the World Organisation for Animal Health (OIE) in Manual of Diagnostic Tests for Aquatic Animals (2016) (1). All tissue samples were stored at –75°C for future analysis. Total RNA was extracted from supernatant of the organ homogenates (samples) using QIAamp Viral RNA (Qiagen, Germany). The extraction procedure was performed following the manufacturer's instructions. The RNA obtained was eluted in RNAse-free water. As a negative control, pool of negative fish tissue was processed alongside the virus isolate. Total RNA was added to a one-step RT-PCR for amplification of the certain genomic region within coat protein gene. A 427 nucleotide (nts) region targeting the T4 variable region of the RNA2 segment from diagnostic cases was amplified (Figure 3). RT-qPCR method was also introduced in the laboratory diagnostics of VER/VNN. A part of the RNA2 segment of viral genome was successfully detected using specific primers and MGB probe following OIE standard protocol (1).

Using molecular method RT-PCR we detected the presence of viral nucleic acid of betanodavirus successfully. With the RT-qPCR method, specific viral RNA in clinical samples can be detected quickly and specifically. Ct values obtained ranged from 15.88 (brain and bulbus) to 25.65 (spleen and kidney).

Confirmation of the positive results by both molecular methods, specific RT-PCR amplicon length for RNA2 genome region and the Ct values for RT-qPCR, corresponded 100%.

The RNA2 segment of Slovenian betanodavirus isolate was sequenced and compared with known isolates from betanodavirus coat protein sequences from different countries and hosts. A partial sequence of coat protein of 286 nts was aligned and used in phylogenetic analysis.

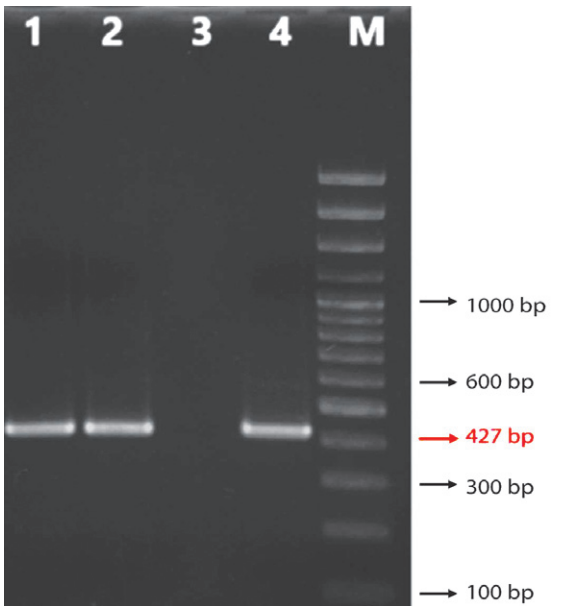

Figure 3: Agarose gel electrophoresis of RT-PCR positive samples of fish brain (1) and visceral organs (2) tested with F2/R3 primer pair. Reference negative (3) and positive (4) control. The red arrow indicates the expected size for the 427-bp amplicon. M = 100-bp size marker

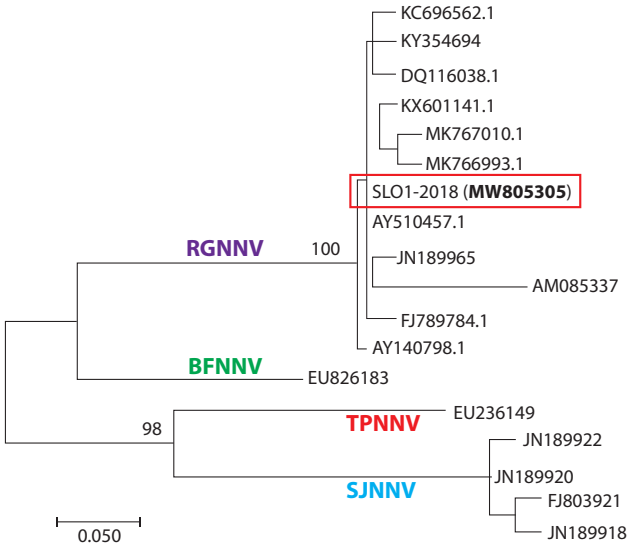

Figure 4: Maximum-likelihood (ML) phylogenetic tree based on partial RNA2 sequences depicting the phylogenetic relationships within genus *Betanodavirus*; subdivision of genus *Betanodavirus* is displayed by labeling the branches with different colors (violet: RGNNV; green: BFNNV; red: TPNNV; blue: SJNNV); ML bootstrap values >60% are reported next to the nodes; scale is shown at the left as substitutions per site.

Phylogenetic analysis was generated with the program MEGA version 7.0. and employed the Maximum-likelihood method using the Kimura two-parameter model (25) (Figure 4). The significance of the branching order was assessed

Table 2: Data related to the 18 betanodavirus isolates investigated in the phylogenetic analysis; abbreviations: unpubl., unpublished; n.d., data not available; t.s., this study

Isolate	Year of isolation	Country of outbreak	Reference	GenBank accession no.	Betanodavirus species
GMNNV-Korea	unknown	Korea	Cha et al., unpubl.(26)	DQ116038.1	RGNNV
9Gr.A.2012	2012	Greece	Bitchava et al., 2019(27)	MK767010.1	RGNNV
SpPm-IAusc1586.10	2010	Spain	Olveira et al., 2013(28)	KC696562.1	RGNNV
G9508KS	1995	Taiwan	Chi et al., 2003(29)	AY140798.1	RGNNV
28Gr.A.2013	2013	Greece	Bitchava et al., 2019(27)	MK766993.1	RGNNV
SFRG08/2013BSMu3	2013	Korea	Kim et al., unpubl.(30)	KX601141.1	RGNNV
570.16.2008c	2008	Italy	Panzarin et al., 2012(17)	JN189965	RGNNV
"1"	2009	Tunisia	Chérif et al., 2010(31)	FJ789784.1	RGNNV
"Redspotted grouper nervous necrosis virus"	unknown	China	Lin et al., unpubl.(32)	AY510457.1	RGNNV
Sa-I-97c	1997	Italy	Toffolo et al., 2007(33)	AM085337	RGNNV
VNNV/S. aurata/I/425-10/Sep2008	2008	Italy	Toffan et al., 2017(5)	KY354694	RGNNV
SpSa-IAusc156.03c 2003 Larvae	2003	Spain	Olveira et al., 2009(16)	FJ803921	SJNNV
37.2.2005c	2005	Portugal	Panzarin et al., 2012(17)	JN189918	SJNNV
250.1.2009c	2009	Cyprus	Panzarin et al., 2012(17)	JN189920	SJNNV
292.1.2.2009c	2009	Greece	Panzarin et al., 2012(17)	JN189922	SJNNV
BF93Hok	unknown	Japan	Nerland et al., unpubl.(34)	EU826138	BFNNV
TPKag93	unknown	Japan	Okinaka, unpubl.(35)	EU236149	TPNNV
SLO1-2018	2018	Slovenia	t.s.	MW805305	RGNNV

by bootstrap resampling of 1000 replicates. Accession numbers of nucleotide sequences for VER/VNN worldwide isolates available at GenBank were cited and listed in Table 2.

Based on clinical signs, typical histopathological lesions followed by identification of the causative agent by molecular analysis, VER was diagnosed.

Discussion

Since late 80', mass mortalities in farmed sea bass showing abnormal swimming behaviour have been reported from Mediterranean region by several authors (8, 9, 36). In summer 1995, heavy losses associated with nodavirus infection occurred in juvenile and adult sea bass in several marine fish farms in Italy (9) and VER is currently considered to be endemic in Mediterranean basin (5). However, in Slovenia altered swimming behaviour associated with mass mortalities in marine aquaculture fish species had not been observed until recently. Clinical signs as well as gross and histopathological findings in our case were similar to those described by other authors (8, 9, 22, 37, 38).

The temperature range at the time of the outbreak in July 2018 was in accordance with an optimal *in-vitro* growth temperature for betanodavirus species RGNNV at 25–30°C (39), and mortality significantly decreased at water temperature below 20°C.

The source of infection in our case is difficult to define, considering that only sea bream was introduced into the farm in 2018. Transmission of the disease occurs mainly horizontally through contaminated water (1), but vertical transmission has also been demonstrated in several fish species (7).

An additional possibility of transmission of the betanodavirus is represented from infected, asymptomatic specimens (21, 40). Results of infection trials reported by Castric et al. (41) showed that experimentally infected sea bream with no clinical signs of the disease was able to infect the juvenile sea bass by cohabitation. Furthermore, betanodavirus was detected in several marine invertebrate species, including Mediterranean mussel (*Mytilus galloprovincialis*) (42), which is the main cultured mollusc species in Slovenia. Moreover, the mollusc farming area Seča is in the immediate vicinity to the relevant fish farm. Kim et al. (43) confirmed infectivity of either BFNNVs or RGNNVs from shellfish, which may represent a potential risk for transmission of nodaviruses to cultured and wild host species. The ability of the sea bass nodavirus to survive at least one month at 25°C and at least one year at 15°C indicates that once released into the marine environment, it could remain widely spread during either cold or warm seasons (44). Thus, control measures possibly effective in hatcheries by implementation of proper disinfection procedures followed by introducing of betanodavirus-free broodstock, have limited results in preventing betanodavirus infections of farmed fish exposed to the marine environment in on-growing sites (15).

The nucleotide diversity of RGNNV isolates worldwide has been shown to vary depending on host species and environmental conditions (18, 45). Isolates from Italy, Spain, Portugal, Cyprus, Greece and Tunisia represent Mediterranean Basin.

Viral isolates collected from certain geographic area are generally similar to each other. In the present study, the most revealing spatial trend was the clear separation of isolates from the same geographical location. According to the genotype and geographical origin, SJNNV isolates within Mediterranean region and Japanese isolate from TPNNV species form two independent clusters.

Maximum-likelihood phylogenetic tree based on partial RNA2 sequences determined that the selected isolates within RGNNV species showed spatial correlation. In this study, geographic clustering of the virus isolates from Greece and Italy was observed. It is also important to note that within betanodavirus species RGNNV, closer phylogenetic relatedness of Korean RGNNV isolates with those from Greece and Italy was detected.

The determined partial T4 nucleotide sequence of Slovenian isolate showed 88.93 to 100% identity at the nucleotide level to the sequences among selected betanodavirus isolates analysed, highly related to strain RGNNV. The evolutionary analysis showed the phylogenetic relationships of newly characterized Slovenian isolate with the RGNNV species. Interestingly, it also exhibited 100% identity to the virus isolate from China.

In this study, the determined partial RNA2 segment sequences representing four major betanodavirus species showed 65.04 to 100% identity at the nucleotide level to the selected sequences of 18 worldwide betanodavirus isolates.

In our case high viral load was detected also in spleen and kidney. Retina and central nervous system including the brain and spinal cord are key organs of the infection in which the virus actively replicates. Kidney and spleen are not considered the target organs and therefore not suitable for VER diagnosis, but nevertheless causative agent of the disease can be detected in many organs according to published data (1). Our results revealed that besides bulbus and brain tissue kidney and spleen could also be suitable tissues for analysis.

Considering the severity of the disease and based on available data suggesting the immunogenic characteristics of the NNV in sea bass, great effort has been made in vaccine development (46), including attenuated, inactivated, recombinant and DNA vaccines with promising results (47).

Recently, an inactivated injectable vaccine against VER caused by RGNNV species for sea bass has been authorised for use in the appointed Mediterranean countries: Spain, Italy, Croatia and Greece (Pharmaq) (48). It is to be administered to fish of a minimum weight of 12 g, and the expected reduce of mortality caused by nodavirus (RGNNV species) in sea bass is up to 12 months post vaccination.

In these aspects, we believe that introduction of already vaccinated juveniles into Slovenian marine aquaculture facilities would be strongly recommended.

Conclusion

VER is one of the most devastating diseases of marine fish species with great impact on marine aquaculture. It is endemic in Mediterranean Basin, but the first outbreak in Slovenia occurred only in 2018. The disease caused increased mortality in juvenile and adult sea bass, which led to final loss of about 50% of affected populations. Phylogenetic analysis of Slovenian RGNNV isolate indicates its

close relation to other isolates from Mediterranean Basin. Subsequently, an authorised vaccine for selected Mediterranean countries could reduce the mortality rate and economic losses caused by VER also in sea bass in Slovenia.

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PRVI IZBRUH VIRUSNE ENCEFALOPATIJE IN RETINOPATIJE PRI GOJENIH BRANCINIH (*Dicentrarchus labrax)* V SLOVENIJI

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Izvleček: Virusna encefalopatija in retinopatija (VER) je nevarna bolezen številnih vrst morskih rib, ki jo povzroča nevrotropni RNA virus iz družine *Nodaviridae*, rod *Betanodavirus*. Bolezen je razširjena skoraj po vsem svetu in povzroča visok pogin okuženih rib. Zanjo so značilne vakuole v centralnem živčnem sistemu in retini. Konec julija 2018 so v ribogojnici v Piranskem zalivu pri brancinih opazili nepravilno plavanje, vrtenje in postavljanje v vertikalno smer ter letargijo in neješčnost, brancini so množično poginjali. Bolezen se je najprej pojavila pri mladicah, nato tudi pri konzumnih kategorijah brancinov. Obolele ribe so imele sivomotna očesna zrkla ter multifokalne kožne razjede na glavi, posamezne so bile shujšane. S histopatološko preiskavo smo ugotovili značilne vakuole v možganih in retini. Z molekularnima metodama RT-PCR in RT-qPCR smo potrdili prisotnost nukleinske kisline betanodavirusa v očesnem zrklu in možganih. Koncentracije virusa, ki so bile signifikantno višje od pričakovanih, smo ugotovili tudi v vranici in ledvicah. Na podlagi kliničnih znakov, makroskopskih in tipičnih histopatoloških sprememb ter rezultatov molekularnih preiskav lahko zaključimo, da so gojeni brancini v ribogojnici v Piranskem zalivu zboleli za VER. Opisani izbruh je prvi potrjeni primer te bolezni v Sloveniji.

Ključne besede: virusna encefalopatija in retinopatija; betanodavirus; brancin; histopatologija; RT-qPCR