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J Vet Res 64, 247-251, 2020
DOI:10.2478/jvetres-2020-0037

Detection of koi herpesvirus (KHV) and carp oedema virus (CEV) in invasive round goby, *Neogobius melanostomus* Pallas, 1814, from Poland and Germany

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Received: September 25, 2019 *Accepted: May, 14 2020*

Abstract

Introduction: The aim of the study was to determine the transmission potential of carp edema virus (CEV) and koi herpesvirus (KHV) introduced to Europe by the invasive round goby (*Neogobius melanostomus*). **Material and Methods:** A total of 70 round goby specimens were collected from the Szczecin Lagoon, Poland, and locations in Germany in the third and fourth quarters of 2018. The fish were analysed to detect KHV and CEV by PCR. **Results:** Six fish specimens were positive for the presence of KHV, while none of the gobies examined showed the presence of CEV. **Conclusion:** The CEV genome was detected in the goby specimens from Germany and from Poland. Considering the high pace of the spread of the round goby and its effectiveness in acquisition of new ecological niches, it should be kept out during refilling of carp ponds. Further studies should focus on experimental cohabitation of CEV-infected round gobies and specific-pathogen-free (SPF) carp to investigate the potential for active virus transfer.

Keywords: round goby, virus transmission, Poland, Germany.

Introduction

The round goby (*Neogobius melanostomus*) occurs naturally in the Ponto-Caspian region where it inhabits the catchment areas of the Sea of Azov and the Black and Caspian Seas (17). As suggested by Polačik (24), the spread of this species took place *via* ballast water of cargo ships and was propagated by intensive sea transport in recent decades. The tolerance of the species to a wide range of salinity (5–25 ppm) (14), high temperature (critical thermal maximum 33.4°C) (6), and low level of oxygen in water (critical lethal threshold ranging from 0.4 to 1.3 mg/L) (4) facilitates and accelerates its adaptation to new reservoirs in Europe.

In European waters, beyond the area of its natural occurrence, the species was first found in 1990 in Gdańsk Bay (Poland), a confluence of the Baltic Sea and Martwa Wisła river (26). In the subsequent years, its presence was also noticed in nearby reservoirs (25), and probably since 1996, it has been present in the western Baltic Sea and the Oder River estuary (8). In the German region of the Baltic Sea, the round goby was first found in 1998 in fish catches in the vicinity of the Zicker Peninsula (Rügen Island) (31), while in 2002, it was found further to the west, near the Darß Peninsula (Darßer Ort) (5, 31). In the following years, the species colonised the Rhine Delta and the Lek River near the village of Schoonhoven (2004), the Elbe River (2008), the Scheldt and Albert Canals in Belgium (2010), and the Weser River (2012) (3, 13, 27, 28). At the beginning of the 21st century, the species was also seen in the northern and eastern parts of the Baltic Sea (21).

It is believed that the second route of colonisation of European waters by the round goby was the catchment area of the Danube River, as evidenced by the successive occurrence of this fish at sites in the middle and upper courses of the river (22, 24). For example, in the Austrian section of the Danube near Vienna, the

species was found in 2000 (30), while in the German section of the river between Passau and Straubing, it was noted in 2004 (23).

Such a rapid spread of the round goby is a potential threat, not only due to disturbance of the ecosystems it colonises, but also from the point of view of epidemiology, as it can be a source of pathogens introduced into the new environment. This aspect is particularly relevant as the species can penetrate into fish ponds during filling and become an intermediate host in the chain of transmission of parasitic diseases. With regard to viral diseases, there is a possibility of the round goby transmitting viral diseases to species of great economic importance, as it has been found in the stomachs of such species as cod, perch, pikeperch, and brill (29).

Material and Methods

The study materials were 35 individuals of round goby (*Neogobius melanostomus*) derived from the Szczecin Lagoon (at Trzebież) and the same number in total from the following regions in Germany: the Palmer Ort (Greifswalder Bodden) (6 individuals), Ostklune (Stettiner Haff) (5 individuals), Lassan (Peenestrom/Achterwasser) (6 individuals), Gummlin (Stettiner Haff) (6 individuals), Spandowerhagen (Greifswalder Bodden) (11 individuals), and Mukran (Rügen – Ost) (1 individual). The materials were collected in the third and fourth quarters of 2018. After transporting the caught fish to the laboratory, tissue samples from each of them were taken and pooled in order for koi herpesvirus (KHV) and carp oedema virus (CEV) to be detected. None of the investigated fish presented clinical signs of ongoing or past viral infection.

DNA was isolated using a DNA Mini Kit (Syngen Biotech, Wrocław, Poland). Qualitative and quantitative assessment of the extracted DNA was conducted by measurement of absorbance using the NanoDrop 2000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and electrophoretic separation on a 1.5% agarose gel (Prona, Tychy, Poland).

All samples were analysed molecularly, which allowed the presence of sequence fragments of KHV (Cyprinid herpesvirus 3, CyHV-3), and frequently co-occurring CEV to be confirmed. Detection was conducted using PCR and nested PCR, using four pairs of specific primers for KHV and two pairs of primers for CEV.

PCR products obtained by using the KHV-F/KHV-R primer pair were used as a template for the second nested PCR employing the KHV-1Fn/KHV-1Rn primer pair (2, 9). The second set comprised the KHV-TK-F and KHV-TK-R primers used in the first round, and the KHV-TK-Fn/KHV-TK-Rn used in the nested PCR step (1, 16). The PCR products of the CEVforB/CEVrevJ primer pair were used as a template for the second nested PCR with CEVforBint/CEVrevJint. The PCR assays were performed according to previously published methods. The primers and methods used in this study are presented in Table 1.

The products of each PCR were assessed by separation on a 1.5% agarose gel followed by bi-directional Sanger sequencing (Genomed, Warsaw, Poland). The results of sequencing were aligned and analysed using BLAST-N and GENEIOUS PRIME software (https://www.geneious.com).

Results

The qualitative and quantitative analysis of the DNA obtained from the samples demonstrated that the method employing spin columns yielded a high degree of purity of the obtained DNA samples (A260/A280 = 1.8–2.0). As a result of electrophoretic separation of the nested PCR products obtained using the KHV-CT-Fn/KHV-CT-Rn primer pair, the presence of KHV in six samples from round gobies was determined (Fig. 1). The obtained results confirmed the effectiveness of this method for detection of parts of the KHV genome.

Positive results were obtained from samples of one individual caught in the Spandowerhagen facility in the Greifswalder Bodden (Fig. 1, sample 52RG) and in five individuals from the Szczecin Lagoon on the Polish side (Fig. 1, samples 2Z, 23P, 14ZAL, 1ZAL, 4ZAL). For the remaining 64 samples, negative results of detection of the KHV genome fragment were obtained, or the number of copies of the virus was below the threshold of detection of the method.

Table 1. Primer pairs used in the study to detect the KHV and CEV genomes

Primer	Primer sequence (5′–3′)	Product size	References
KHV-F	GACGACGCCGGAGACCTTGTG	486 bp	Gilad *et al.* (9)
KHV-R	CACAAGTTCAGTCTGTTCCTCAAC	486 bp	Gilad *et al.* (9)
KHV-1Fn	CTCGCCGAGCAGAGGAAGCGC	414 bp	Bergmann *et al.* (2)
KHV-1Rn	TCATGCTCTCCGAGGCCAGCGG	414 bp	Bergmann *et al.* (2)
KHV-TK-F	GGGTTACCTGTACGAG	409 bp	Bercovier *et al.* (1)
KHV-TK-R	CACCCAGTAGATTATGC	409 bp	Bercovier *et al.* (1)
KHV-TK-Fn	CGTCGTGAGGAATACGACG	348 bp	Unpublished: Way in Kempter *et al.* (15)
KHV-TK-Rn	ACCGTACAGCTCGTACTGG	348 bp	Unpublished: Way in Kempter *et al.* (15)
CEVforB	ATGGAGTATCCAAAGTACTTAG	528 bp	Matras *et al.* (18)
CEVrevJ	CTCTTCACTATTGTGACTT-TG	528 bp	Matras *et al.* (18)
CEVforBint	GTTATCAATGAAATTTGTGTATTG	478 bp	Matras *et al.* (18)
CEVrevJint	TAGCAAAGTACTACCTCATCC	478 bp	Matras *et al.* (18)

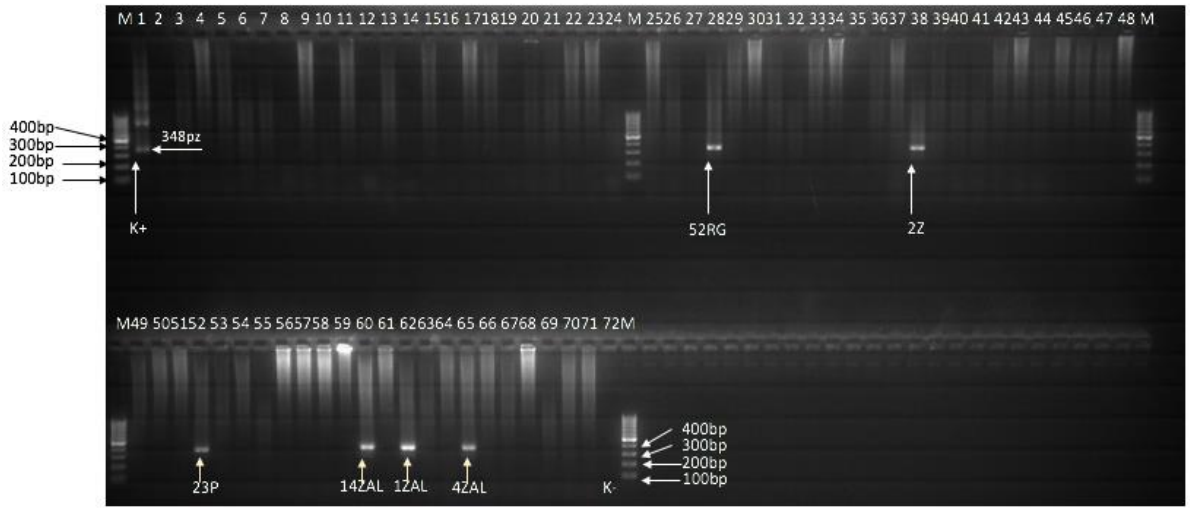

Fig. 1. Electrophoretic separation of nested PCR products using KHV-Fn/KHV-Rn primers

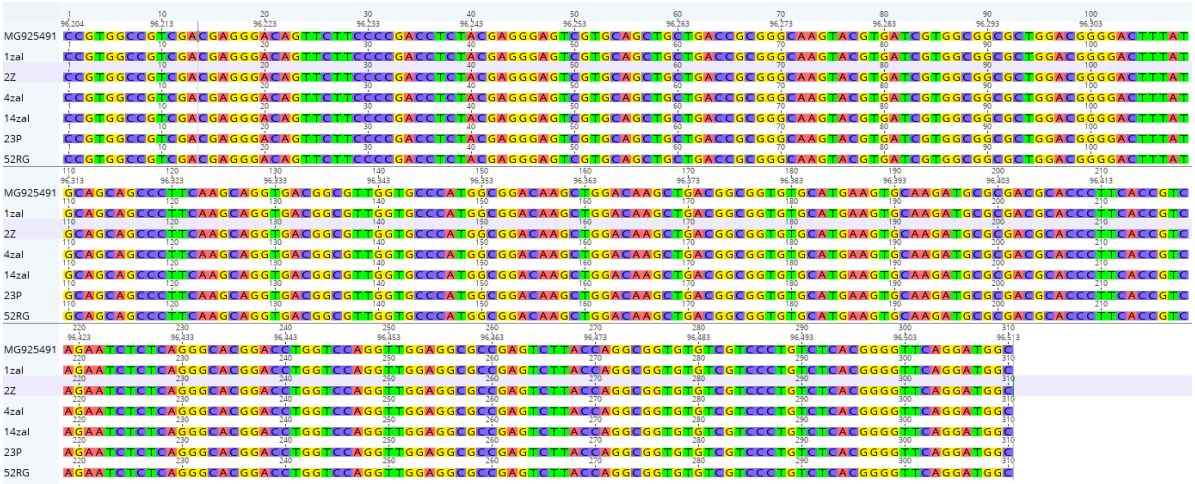

Fig. 2. Alignment of the obtained fragments of the KHV genome with the sequence submitted to GenBank under accession no. MG925491

The results of the assay aiming to detect CEV were negative for all 70 investigated round gobies. Bi-directional Sanger sequencing and alignment of sequences in the GENEIOUS and BLAST-N software confirmed that the obtained 310 bp fragments showed 100% similarity to the reference KHV sequences published in the National Center for Biotechnology Information (NCBI) GenBank database (Fig. 2).

Discussion

Viral infections are one of the causes of many mass mortalities among the indigenous ichthyofauna. The disease caused by KHV is a herpesviral infection (12) capable of inducing contagious and acute viraemia in common carp (*Cyprinus carpio*) and its varieties, such as koi and ghost carp (11). CEV disease, also known as koi sleepy disease, is caused by a poxvirus associated with outbreaks of clinical disease in koi and common carp. Water, as the environment of fish, is an important abiotic vector of viruses. However, a particular threat in the aquatic environment is posed by vector species, which are an important link in the chain of direct or indirect transmission, even though they do not allow virus replication. Animal vectors include other fish species, parasitic invertebrates, piscivorous birds, and aquatic mammals. The emergence of new invasive species in ecosystems, such as the round goby, constitutes a particular threat. Studies have shown that the round goby is one of the most invasive fish species not only in Poland but also globally (7). The appearance of an additional species, whose spread is so unpredictable, introduces an additional route of transmission of the virus into an ecosystem. Another factor facilitating a rapid spread of the pathogens of the round goby is the fact that the species inhabits both fresh and marine waters. Its presence in lakes (Z. Chełkowski, oral report, 1992) and the waters of the Puck Bay, the Vistula Lagoon and the Szczecin Lagoon, confirms that it can also introduce KHV into environments previously free of the virus. Due to the small body size of the round goby, with adult individuals reaching 20 cm in length, the species can be accidentally introduced into fish

ponds during filling. In natural waters, the species takes over the natural habitats of indigenous ichthyofauna and depletes its food resources. Therefore, it has been considered particularly harmful to the biodiversity of European waters. The round goby has been confirmed to be a carrier of the viral haemorrhagic septicaemia (VHS) virus in the Great Lakes on the border of Canada and the United States and in Lake Ontario (10). It has been found that VHS can be transmitted to other predatory species *via* the gut. A study by Więcaszek *et al.* (29) revealed the presence of round goby in the stomachs of predatory fish. This means that, potentially also in this case, transmission of koi herpesvirus can occur via the digestive tract when round goby are eaten by predators. A study by Nguyen *et al.* (20), revealing the presence of anguillid herpesvirus 1 causing the deaths of European eels (*Anguilla anguilla*), showed that the round goby was one of the five species in which the presence of the genome of this virus was confirmed. Under favourable conditions, round goby can reproduce all year round. In spring, it dwells near the shores where water temperature can vary from 9–26°C and where it spawns. Such environmental preferences facilitate the transmission of KHV, as the optimal temperature range for the replication of this virus is 16–23°C, it sometimes being possible in temperatures reaching even 28°C (32). However, there have been cases of gill necrosis and mortality induced by KHV even at 4°C (S.M. Bergmann, unpublished data, 2020). The overlap between the water thermal conditions optimal for the spawning of round goby and those conducive to the replication cycle of KHV, transmitted by this fish species, creates a very dangerous phenomenon. Matras *et al.* (19) confirmed the presence of the carp oedema virus in tench, crucian carp, Prussian carp, roach, bleak, and European perch. KHV was detected in 18 species besides the carp (15).

In conclusion, the results of our study suggest that as one of the most invasive species in European waters, the round goby not only jeopardises the biodiversity of the indigenous ichthyofauna but also poses an epidemiological threat in regards to KHV and possibly CEV. Its presence as a potential vector or carrier of KHV increases the risk and provides an additional opportunity for the transmission of the disease to new environments that this fish species colonises in the course of its expansion.

Conflict of Interests Statements: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This study was funded by the West Pomeranian University of Technology in Szczecin.

Animal Rights Statement: The experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Acknowledgements: The authors thank Dr Malte Dorow of the Landesforschungsanstalt für Landwirtschaft und Fischerei Mecklenburg-Vorpommern for help in obtaining material from sites in Germany, and Associate Professor Przemysław Czerniejewski of the West Pomeranian University of Technology in Szczecin for the information on the colonisation of European waters by invasive species.

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