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Potential for spread of carp edema virus (CEV) through vector fish species

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Summary

Introduction: Carp edema virus (CEV) is poorly understood in terms of its potential for horizontal and/or vertical transmission in aquatic environments. Because of the need to identify species that can latently transmit CEV, we undertook a study to determine the degree of infection of species other than common carp (*Cyprinus carpio*) in natural waters and farm facilities. The aim of the study was to identify vector species and determine the routes of virus transmission in northwestern Poland.

Materials and methods: 449 fish of 15 species were sampled for the study. The fish were collected in 2017-2018 from Lake Dąbie, Szczecin Lagoon, and three fish farms. Molecular analyses, which detected a fragment of the CEV gene encoding the P4a viral core protein, consisted of isolation of viral DNA, qualitative assessment of the purity of isolates obtained , and amplification of CEV genetic material by PCR reaction. The PCR reaction products were verified by real-time PCR.

Results: The study revealed the presence of species that could potentially be involved in virus transmission between natural waters and carp culture centers in northwestern Poland. These species included roach, bleak, crucian carp, and tench. The results obtained suggest the need for mandatory virological testing for CEV in fish intended for stocking, which should limit the uncontrolled transmission of the virus in natural waters and its introduction into carp farms during the flooding of breeding ponds.

Keywords: epidemiology, CEV transmission, latent species

Carp edema virus (CEV) has been detected in many European countries since 2004, both in wild and farmed koi. In Poland, it was first detected by Matras et al. (8) in archived samples from 2015-2017. As reported by the National Veterinary Research Institute in Puławy, Poland, the presence of CEV in carp was repeatedly confirmed only in breeding farms between 2019 and 2022 (10). In order to determine the transmission pathways, studies were also conducted to identify vector species under controlled conditions. These studies involved six species: bleak (*Alburnus alburnus*), crucian carp (*Carassius carassius*), European perch (*Perca fluviatilis*), Prussian carp (*Carassius gibelio*), roach (*Rutilus rutilus*), and tench (*Tinca tinca*) (9). Experimental cohabitation at 16°C showed that all of them can play a role as vector species in CEV infection. The authors also showed that even a short period of contact with infected fish can potentially be one of the routes of its spread, with a long period of elimination of this virus from vector species.

According to the Environmental Protection Agency, CEV is very difficult to diagnose, especially in asymptomatic fish. Infection associated with anatomopathological changes typical of CEV has been described only in common carp and its ornamental varieties. No fish carrying CEV have been diagnosed in fisheries or open waters. Given the limited and very unsystematic testing of wild fish for CEV carriage in Poland, it can be assumed that it is more prevalent precisely because of its presence in carp farms. As reported by Zrnčić et al. (15), an epidemiological survey confirmed the introduction of infected carp into one of the lakes in Croatia. The authors point out that the prevention of such incidents is of utmost importance and can be achieved by a regular testing of fish for CEV before moving them between farms.

In order to assess the prevalence of CEV in carp reared in ponds fed with water from natural watercourses, it is important to test as many species as possible in addition to common carp so as to define transmission pathways. Obtaining such data will provide a basis for determining the actual threat to wild fish in terms of the likelihood of introducing CEV into farms during pond flooding. The aim of this monitoring study was to determine the level of CEV infection in common carp *Cyprinus carpio* and selected fish species from the Odra river catchment area and selected carp farms. Identification of the vector species among the ichthyofauna of northwestern Poland will greatly expand the knowledge of the mechanism of CEV spread and will be useful for veterinary services and breeders in their efforts to minimize the risk of CEV transmission between farms and natural waters.

Material and methods

The material for virological studies consisted of fish collected from natural waters and carp culture facilities during the spring and fall seasons of 2017 and 2018. In 2017, a total of 355 specimens of 13 species were obtained from five locations. Those species were roach (*Rutilus rutilus*) (n = 101), zander (*Sander lucioperca*) (n = 60), European perch (*Perca fluviatilis*) (n = 76), round goby (*Neogobius melanostomus*) (n = 8), crucian carp (*Carassius carassius*) (n = 35), vimba bream (*Vimba vimba*) (n = 12), zope (*Ballerus ballerus*) (n = 9), European flounder (*Platichthys flesus*) (n = 14), bighead carp (*Hypophthalmichthys nobilis*) (n = 6), tench (*Tinca tinca*) (n = 5), European catfish (*Silurus glanis*) (n = 6), grass carp (*Ctenopharyngodon idella*) (n = 21), and pike (*Esox lucius*) (n = 2).

Samples from Lake Dąbie (n = 136) and the Szczecin Lagoon (n = 198) came from commercial fishing by local fishermen. The others were obtained from three fish farms: Fish Farm 1 (GR1 – RSD Lower Odra river) (n = 68), Fish Farm 2 (GR2 – Zagłówek) (n = 6), and Fish Farm 3 (GP3 – Ciasna) (n = 38) (Fig. 1).

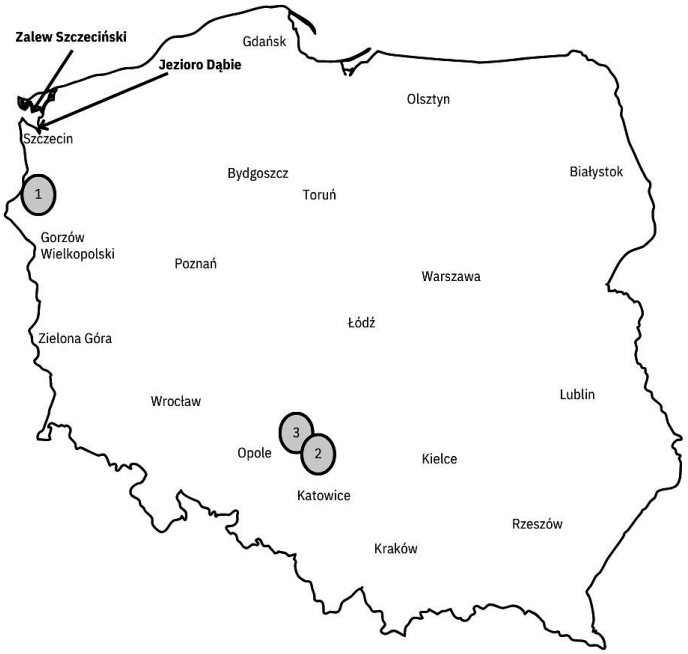

Fig. 1. Sampling locations where CEV was detected (1) and those where CEV was not detected (2, 3)

In 2018, a total of 94 fish of 6 species were obtained from two locations. Those species were roach (n = 10), tench (n = 10), crucian carp (n = 10), bleak (n = 10), European perch (*Perca fluviatilis*) (n = 10), and koi (*Cyprinus carpio*) (n = 44). The samples from the Szczecin Lagoon (n = 50) came from commercial harvests by local fishermen. The others originated from Fish Farm 1 (GR1 – RSD Lower Odra river) (n = 44).

After the fish were transported to the laboratory, tissue sections (gills, skin, kidney) were taken from each fish for CEV detection. Biological material was placed in disposable 1.5 ml Eppendorf tubes and cooled to 4°C for short-term storage until further analysis. For long-term storage, biological material was preserved by placing tissue fragments in isopropyl alcohol $(CH_3)_2CHOH$. Material preserved in this manner was stored at room temperature until further analyses.

DNA isolation. DNA extraction was performed with a ThermoMixer F1.5 (Eppendorf) and the Genomic Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions provided with the kit. Organ fragments weighing 10-15 mg were minced and placed in a 1.5 µl reaction tube. 100 µl TRIS buffer (Biotechnology, Poland), 50 µl LT lysis solution (Biotechnology, Poland), and 20 µl proteinase K (Biotechnology, Poland) were added. They were then incubated at 50°C until the tissue was completely digested (approximately 3 hours). The samples were mixed by vortexing several times during the digestion process. Once the cell homogenate was obtained, 150 µl of LT lysis solution was added and incubated at 70°C for 5 minutes. The samples were then vortexed intensively for 20 seconds and centrifuged at 10,000 rpm for 3 minutes. The resulting supernatants were transferred to silica columns. In the next step, the DNA material bound to the column was purified by washing the columns twice with 500 µl A1 wash buffer (Biotechnology, Poland) and 400 µl A2 wash buffer (Biotechnology, Poland). After adding each reagent, the sample was centrifuged at 10,000 rpm for 1 and 2 minutes, respectively. The dried minicolumns were transferred to new 1.5 ml Eppendorf tubes. The elution process was performed with TRIS buffer heated to 70°C in a volume of 200 µl. Samples were incubated for 2 minutes at room temperature and then centrifuged at 10,000 rpm.

Qualitative and quantitative evaluation of the DNA obtained was performed by agarose electrophoresis of the DNA isolates. A precise test determining the amount and purity of the DNA was also performed using a Nanodrop ND spectrophometer (Thermo Fisher Scientific Inc.) by measuring light absorbance at wavelengths of 260 and 289 nm. An agarose concentration of 1.5% was used. A solution containing a measured amount of BASICA GQT agarose (Prona) and TBE buffer (Fermentas) was heated until a clear and homogeneous liquid was obtained, then cooled, and ethidium bromide (EtBr, MP Biomedicals) was added. The liquid was placed in a forming rack, and after the gel solidified, 8 µl of the isolate was added to each well. Electrophoresis was performed in a Wide Mini-Sub Cell GT chamber (Bio-Rad) filled with TBE buffer (Fermentas) at 80 V for 45 min. The gel was then placed in a chamber (Gel Doc XR,

Bio-Rad) emitting UV light to absorb light. Gels were analyzed using the Quantity One Manual software. Absorbance measurements were then performed using a NanoDrop 2000 UV spectrophotometer (ThermoScientific).

PCR. Amplification of the material by real-time PCR (qPCR) was carried out at the Friedrich-Loeffler-Institut (FLI, Germany) using reagents from Roche (Germany). The analysis used primers described by Matras (8). The primers and molecular probes (Tab. 1) were synthesized by Genomed. PCR was performed in a total volume of 25 µl using GoTaq G2 MasterMix Colorless (Promega) (Tab. 4). At the 5' end, the probe was labeled with the FAM fluorescent dye, and at the 3' end, a fluorescence quencher BHQ-1 was placed. The reactions were conducted in the Mx 30005 P QPCR system (Stratagene). Similar to procedures for conventional and nested PCR, additional control samples, known as positive and negative controls, were included in each analysis. Both protocols – conventional PCR and real-time PCR – were developed in the CEFAS laboratory in Weymouth (UK).

The product of the PCR reaction with CEV-forB/CEVrevJ primers was used as a matrix for the second nested PCR reaction with CEV-forBint/CEVrevJint primers. Primers were synthesized by Genomed (Warsaw, Poland). PCR was performed in a total volume of 25 µl using GoTaq G2 Green MasterMix (Promega) (Tab. 2). Reactions were performed in a Mastercycler personal thermocycler (Eppendorf). Two additional samples were added to each reaction. The first was a so-called positive control, which was a CEV viral DNA sample obtained from the reference laboratory for KHV (State Institute for Viral Diseases in Riems, Germany). The second was the so-called negative control, which was a sample in which the DNA matrix was replaced with demineralized water. At the end of each reaction, the products were separated electrophoretically using parameters identical to those used to assess the quality of the isolates.

The results of each PCR reaction were evaluated by separating the PCR products on a 1.5% agarose gel followed by bidirectional Sanger sequencing of each PCR product.

Real-Time PCR. Amplification of the material by real-time PCR, or quantitative real-time polymerase chain reaction (qPCR), was performed at the Federal Institute for Animal Health at Insel Riems, Germany, using reagents from Roche (Germany). qPCR differs from other PCR methods in that one can analyze the increase in PCR reaction products after each reaction cycle, i.e. in real time. Primers described by Matras (8) were used for the analysis. The primers (Tab. 3) were synthesized by Genomed. The corresponding probes were designed and supplied by the same company. The PCR was performed in a total volume of 25 µl using GoTaq G2 MasterMix Colorless (Promega) (Tab. 6). The probe was labeled from the 5' end with FAM fluorescent dye. A BHQ-1 fluorescence quencher was added to the 3' end. Reactions were performed in a thermocycler of the Mx 30005 P QPCR system (Stratagene). As with conventional and nested PCR, additional samples, called positive and negative controls, were included in each analysis.

Tab. 1. Primers used to detect P4a protein in conventional and nested PCR

Primer name	Primer sequence	Product size	Source
CEV qFor1	5'-ATGGAGTATCCAAAGTACTTAG-3'	528 bp	Matras et al. 2017
CEV qFor1			
CEV qFor1			
CEV for B			
CEV rev J	5'-CTCTTCACTATTGTGACTTTG-3'	528 bp	
CEV for B - int	5'-GTTATCAATGAAATTTGTGTATTG-3'	478 bp	
CEV rev J - int	5'-TAGCAAAGTACTACCTCATCC-3'	478 bp	

Tab. 2. PCR mixture composition

Demineralized water (PCR grade)	GoTaq G2 Green Master Mix	Forward primer – F	Reverse primer – R	DNA matrix
6.5 µl	12.5 µl	0.5 µl	0.5 µl	5 µl

Tab. 3. Primers used to detect P4a protein in real-time PCR

Primer name	Primer sequence	Source
CEVqFor1	5'-AGTTTTGTAKATTGTAGCATTTCC-3'	Matras et al. 2017
CEVqRev1	5'-GATTCCTCAAGGAGTTDCAGTAAA-3'	
CEVqProbe1	5'-AGT TTGTTTCTTGCC ATACAAACT-3'	

Tab. 4. Composition of the mixture for real-time PCR

Distilled water (PCR grade)	GoTaq G2 Green Master Mix	Forward primer – F	Reverse primer – R	TaqMan probe	DNA matrix
6.25 µl	12.5 µl	0.5 µl	0.5 µl	0.25 µl	5 µl

Results and discussion

Electrophoretic separation of nested PCR products showed the presence of P4a protein in 22 out of 449 samples from 14 species. Positive results were obtained for five tench, eight roach, three crucian carp, and six bleak from the Szczecin Lagoon (Tab. 5 and 6).

Tab. 5. Fish species with confirmed CEV carriage

Genre	Latin name	Total number of fish	Confirmed carriers
Tench	*Tinca tinca*	14	5 (1.1%)
Roach	*Rutilus rutilus*	111	8 (1.7%)
Crucian carp	*Carassius carassius*	45	3 (0.6%)
Bleak	*Alburnus alburnus*	10	6 (1.3%)

Tab. 6. Locations with confirmed CEV carriers

Location	Number of fish examined	Confirmed carriers
GR1 – RSD Lower Odra river	68	14 (20.5%)
GR2 – Zagłówek	6	0
GR3 – Ciasna	38	0
Lake Dąbie	136	1 (0.73%)
Szczecin Lagoon	198	22 (11.1%)

The tench, a constant element in the stocking of open waters in the Szczecin Lagoon and Lake Dąbie (13), serves, according to our results, as a link facilitating the transmission of the CEV virus. Hatcheries and facilities responsible for the rearing of stocking material do not have procedures mandating virological examinations before introducing material into natural waters. As reported by Wawrzyniak et al. (13), fisheries in Poland are allowed to manage lakes and ponds in exchange for obtaining spawning fish from these waters. This practice poses a significant risk in managing ichthyofauna resources because the CEV virus may not only be introduced into the hatchery area, but also infect the entire stocking material produced. This risk extends to common carp, with less than 1% of fish examined carrying the CEV virus. Given the widespread presence of this species in stocking reports (PZW Zielona Góra, 2020), the risk of CEV virus transmission to open waters is very high.

Special attention should be paid to monitoring CEV presence in roach. The virus was detected in 1.7% of the 111 individuals examined. Roach is a species commonly used for stocking reservoirs, such as quarry ponds or small lakes (e.g., Lake Chłopskie). From an epidemiological standpoint, it might seem that the risk of transmission is low, since there is no connection between these reservoirs and rivers. Unfortunately, both roach and bleak, in which the authors confirmed the presence of the CEV virus, are often used by anglers as live bait. This is a factor facilitating horizontal transmission between so-called closed reservoirs and flowing waters.

Also interesting are the results showing the degree of CEV infection in specific locations. As shown in Table 6, fish farms in southern Poland were CEV-negative. The situation was completely different in the Odra river watershed, where Fish Farm 1 had an over 20% infection rate. Along the river's course, there was a dilution effect of CEV-positive fish (Lake Dąbie was just under 1%), and accumulation was observed in the waters of the Szczecin Lagoon, where over 11% of fish were CEV-positive.

Stocking open waters with untested stocking material also leads to the transmission of other viral diseases. This applies especially to vector species, such as Russian sturgeon (*Acipenser gueldenstaedtii*), Atlantic sturgeon (*Acipenser oxyrinchus*), European eel (*Anguilla anguilla*), and common carp (*Cyprinus carpio*).

Stocking with European eel has become an almost flagship program for institutions officially managing open waters, potentially contributing to the spread of anguillid herpesvirus (AngHV1) in Polish waters. As demonstrated by Nguyen et al. (11), this virus has been diagnosed both in glass eels imported into Poland from Denmark and among other species, such as silver carp (*Carassius gibelio*), perch (*Perca fluviatilis*), zander (*Stizostedion lucioperca*), sterlet (*Acipenser ruthenus*), and round goby (*Neogobius melanostoma*).

A similar situation occurs in the implementation of programs for the restitution of Atlantic sturgeon in Polish waters. Under this program, material that did not undergo full virological diagnostics was introduced into both the Odra and the Vistula rivers. As demonstrated by Hoffsoe-Oppermann's research (4), the presence of the white sturgeon herpesvirus (WSIV) genome was detected in wild individuals of both Russian sturgeon and Atlantic sturgeon.

In the case of carp, the presence of koi herpesvirus (KHV) is a concern not only for fish farms. Its presence in open waters is a result of the prevalence of this species in the lists of stocking programs carried out by individual users of fisheries. As shown by Kempter et al. (6), horizontal transmission of the KHV virus between vector species is possible, even in the absence of any clinical signs in fish.

Understanding how viruses are transmitted in the aquatic environment is one of the most important elements in controlling and limiting their spread. The rate of viral mutation is related to the constant search for new ways to replicate in host cells. For this reason, viruses that infect fish make constant attempts to alter the transcript of genetic information through mutations in genes, which in turn cause changes in the proteins that coat their genetic material. The modification of viral proteins makes it impossible for the cells of the immune system of fish to envelop and inactivate viral particles. Due to the well-known phenomenon of virus tropism to individual cells and tissues, they are attributed to transmission through host species, i.e. those in which intensive replication occurs, and vector species, in which the virus does not replicate, but is ready to infect other fish.

As demonstrated by Tolo et al. (12), even cormorant feces are a vector for the transmission of active CEV virus particles in aquatic environments. The authors pay particular attention to the occurrence of species other than common carp in lakes studied in the state of Minnesota. Our own research (5) shows that the koi herpesvirus (KHV) occurring in coinfections with CEV can be transmitted to taxonomically distant species without apparent anatomopathological changes. This means that the viruses that infect carp are constantly mutating, probably because of changes in water temperature in Europe.

The carp edema virus belongs to the *Poxviridae* family and infects fish living in inland waters. The World Organization for Animal Health (WOAH, former OIE) (2023) describes its mode of spread as a form of direct horizontal transmission in which the gills of infected carp play a crucial role (1). Our own research (3), however, has shown that CEV virus replication also occurs

in the skin and kidneys of fish. The tropism of the CEV virus for these cells has also been demonstrated by in situ hybridization. This is important in determining the direction and rate of mutation of the CEV virus, which in turn affects its ability to integrate into cells of species previously considered safe. This threat is recognized by the Environmental Protection Agency, whose report on CEV points out that the virus is difficult to detect, especially in its latent phase, which facilitates its uncontrolled spread in the environment. A study by Matras et al. (9) to indentify the vector species in Poland showed that in addition to carp, species such as bleak, Prussian carp (*Carassius gibelio*), crucian carp, roach, European perch, and tench were also infected after experimental cohabitation. However, there are no studies to determine the degree of CEV infection in natural waters and the presence of the virus genome in companion species in carp farms.

The present study revealed the presence of the CEV genome in only few individuals of tench, roach, crucian carp, and bleak from the natural waters of the Szczecin Lagoon. Considering the large amount of material examined, consisting of 449 fish, it is puzzling that the spread of the CEV virus in the environment was so limited. Of particular importance is the failure to find vector species among fish from carp farms where CEV has been found in crucian carp (2). One example is GR1 in waters near Gryfino, also infected with the koi herpesvirus (KHV) in previous years, according to a study by Kempter et al. (5). At that time, the virus caused massive mortality in carp. A study by Kempter and colleagues (5) on the presence of vector species in the cooling water of the Lower Odra river power plant, i.e. in the waters where soot was found with KHV-infected carp, showed the presence of up to 18 vector species. These included tench, roach, and crucian carp, which means that the aforementioned species have a high affinity in the genome. This makes it possible to transmit viral genomes, including those of both koi herpes viruses and carp edema viruses, in the aquatic environment.

Due to the widespread presence of tench, roach, and crucian carp in the waters of northwestern Poland, special care should be taken when flooding carp ponds to ensure that they do not enter newly developed carp breeding facilities. Tench is often a companion species in carp ponds because of its similar environmental requirements. Given its positive tropism for KHV and CEV, it should not be reared with crucian carp. It poses a direct threat of transmitting these viruses between hatcheries, carp farms, and natural waters. The common bleak, which at the same time proved to be a potential vector in the transmission of CEV in natural waters, did not show any tropism for koi herpesvirus.

These results allow us to conclude that co-infection with both viruses, i.e. the simultaneous occurrence of KHV and CEV, is not a consequence of the presence of specific vector species, but of a reduced immunity of sick and symptomatic fish, which release residual organic matter with viral particles into the water. This material, in turn, is absorbed by the digestive system and gill epithelium of other species present in the culture ponds, and it is most likely that the virus enters natural waters via vector species only after the ponds are drained. Information on the possibility of such transmission is provided by the results of an experiment of Matras et al. (9), which showed that among companion species infected at 16°C the CEV virus was present seven days after infection in only tench and crucian carp, whereas the other species used in the experiment became infected after a longer period of time. This means that tench and crucian carp are two indicator species for monitoring the presence of CEV in both carp farms and natural waters.

As shown in a study by Adamek et al. (1), direct horizontal transmission is an important pathway for CEV transmission in the environment, and vertical transmission is unlikely. Due to the lack of scientific information on the biophysical characteristics of the virus, it is difficult to determine the importance and potential for indirect transmission. This mode of transmission had not been defined for KHV until six years after its first detection in Poland. Research by Kiełpiński et al. (7) confirmed that *Unionidae* mussels may play an active role in KHV infection of carp. Therefore, it is reasonable to monitor invertebrate fauna present in carp ponds for involvement in the transmission of KHV.

As suggested by Way et al. (14), it is possible to improve the management and disease control in aquatic habitats, and the biosecurity principles applied to koi herpes viruses can be applied to carp edema virus disease (CEVD). However, Way et al. believe that there is a need to fill the gap in knowledge on disease pathogenesis and epidemiology that currently prevents an accurate assessment of the potential impact of CEVD on koi carp farms, as well as crucian carp and wild carp stocks.

In conclusion, the state of knowledge on the possible routes of environmental transmission of CEV is inadequate. Not only is there a lack of data on the possible species involved in CEV transmission in certain geographical regions, but also not enough is known about the presence of the CEV genome in the invertebrate fauna. Therefore there is a need for research to increase the knowledge of the biology and epidemiology of the carp edema virus both within farms and in the catchment areas of rivers that feed the ponds. This knowledge will be useful to veterinary services and farmers and will contribute to the development of procedures to minimize the risk of spreading CEV to wild populations, which in turn will improve the control of carp edema virus disease (CEVD).

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