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J Vet Res 68, 63-72, 2024
DOI:10.2478/jvetres-2024-0016

Assessing tropism and genetic traits of carp oedema virus isolates to enhance detection strategies

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Received: September 25, 2023 *Accepted: March 11, 2024*

Abstract

Introduction: Carp oedema virus (CEV) is a relatively understudied poxvirus. It exhibits an affinity for gill and skin epithelial cells. Investigations were conducted into selected aspects of CEV biology, with a focus on determining cell and tissue tropism of CEV, acquiring gene sequences and updating CEV tests in fish tissues. **Material and Methods:** A total of 238 common carp tissue samples from nine aquaculture farms were analysed. The study evaluated the efficacy of intermediate detection of CEV by real-time PCR and *in situ* hybridisation. The genes encoding protein P4a were sequenced, analysed and aligned in a phylogenetic tree using a molecular evolution model. **Results:** *In situ* hybridisation revealed the necessity to validate the Centre for Environment, Fisheries and Aquaculture Science protocols for sampling for CEV detection and to use the tissues for which the virus has the highest tropism, namely the skin and kidneys, rather than solely the gills. The level of genetic variability was determined, and it was shown that CEV mutates systematically. The creation of two distinct phylogenetic clades confirms certain strains' description as Polish isolates. **Conclusion:** Determining the localisation of CEV genetic material in organs and tissues is pivotal for shaping the World Organisation for Animal Health guidelines. The utility of molecular diagnostics has been demonstrated in the skin and kidney of carp, in addition to the gills, impelling their inclusion in diagnostic protocols. The clusters identified in the phylogenetic tree offer valuable insights for developing the current PCR primers. The prevalence of CEV infection in aquaculture, juxtaposed with its notably lower detection in wild fish, underscores the significance of mandatory molecular diagnostic testing for CEV in carp farming.

Keywords: carp oedema virus, virus tropism, fish, detection methods.

Introduction

Carp oedema virus (CEV) was initially detected in koi (*Cyprinus carpio*) in the Japanese prefectures of Hiroshima and Niigata in 1974, and since then its prevalence has expanded (16). In the 1990s, CEV was considered the aetiological agent of carp oedema disease, and in the year 2000, it was confirmed to also cause koi sleepy disease (KSD) (13). In Europe, it first appeared in the UK in 2009 (22), and in 2012, the virus was first detected in common carp, also in the UK (13). In Poland, its presence was first confirmed by Matras *et al.* (12), who further defined three distinct Polish isolates. According to data from the Community Reference Laboratory for Fish Diseases, CEV was detected in 2020 in the UK, Austria, Belgium, the Czech Republic, Italy, Hungary, Croatia, Denmark, France, the Netherlands, Ireland, Germany, Serbia and Poland (21).

Carp oedema virus belongs to the poxvirus group and exhibits an affinity to the epithelial cells of gills and skin (13). It poses no threat to humans but induces pathological changes in all age groups of cultured carp. Mortality rates are high, ranging from 80% to 100%, especially among young individuals exposed to stress, such as occurs during transport to other aquaculture facilities (10). Miyazaki *et al.* (14) noted that KSD outbreaks occurred 4–5 days after infection, with mortality reaching 76%. Fish deaths due to CEV in Japan were observed at water temperatures ranging from 13–25°C, while in the UK they occurred between 6°C and 9°C (13).

Visible symptoms in infected fish include pronounced lethargy. In such cases, fish tend to gather near the water's surface, exhibiting signs of respiratory distress, or they lie on the bottom of the pond. This abnormal behaviour results from the slowing of vital processes in fish due to

oxygen deprivation caused by pathological changes in the gills. Infected fish also display oedema and necrosis of the gills, hyperplasia, sunken eyes, haemorrhages in the fins, pathological skin changes, often around the oral and anal regions, and additional swelling of the entire body in young individuals (4, 10).

The virus does not replicate in immortalised cell lines. Therefore, molecular techniques are employed for diagnosis of CEV infections (12). In Poland, research by Matras *et al.* (13) providing data up to 2022 detected CEV in carp and koi on numerous fish farms between 2015 and 2017. In 2018, it was found on 13 out of 55 farms; in 2019, on 5 out of 48; in 2020, on 1 out of 50; and in 2021, the virus was not detected in any of the 50 locations. In 2022, however, the virus was detected on 2 out of 14 farms.

As indicated by the report of a workshop meeting of relevant National Reference Laboratories, molecular methods for the detection of CEV are based on biological material collected solely from the gills (21). The emergence of new CEV variants observed in recent years makes it necessary to verify its affinity for carp cells and tissues. There is also a need to update the knowledge of the genetic status of newly emerging CEVs through the sequence analysis of genes responsible for the synthesis of the core protein P4a and the comparison of these isolates with others published in the National Center for Biotechnology Information (NCBI) GenBank database.

Material and Methods

The samples for the study consisted of common carp (*Cyprinus carpio*) obtained from nine fish farms: 15 specimens from the first, 61 from the second, 15 from the third, 17 from the fourth, 70 from the fifth and 15 each from the sixth, seventh, eighth and ninth. In total, 238 carp from these nine fish farms were collected for molecular analysis. The locations of the sample collection sites are shown in Fig. 1.

After the fish were transported to the laboratory, tissue fragments (kidney, spleen, gills and skin) were collected from each one for CEV detection. Biological material was placed in disposable Eppendorf-type tubes of 1.5 mL and cooled to 4°C for short-term storage or preserved by being placed in isopropyl alcohol ($(CH_3)2CHOH$) for storage exceeding two months.

Sample preparation. DNA extraction was carried out using the Genomic Mini Kit (A&A Biotechnology, Gdańsk, Poland), following the manufacturer's instructions and employing an F1.5 ThermoMixer (Eppendorf, Hamburg, Germany). A gill fragment weighing 10–15 mg was homogenised and placed in a 1.5 mL reaction tube. Subsequently, 100 µL of tris buffer (A&A Biotechnology), 50 µL of LT lysis solution (A&A Biotechnology), and 20 µL of proteinase K (A&A Biotechnology) were added. The mixture was then incubated at 50°C and periodically vortexed until complete tissue digestion had taken place (approximately 3 h). After obtaining a cellular homogenate, 150 µL of LT lysis solution was added, and the solution was incubated for 5 min at 70°C. The samples were vigorously vortexed for 20 s and then centrifuged for 3 min at 10,000 rpm. The resulting supernatants were transferred to columns with silicon filler. The next step involved purifying the DNA material bound to the column by washing the columns twice with 500 µL of A1 and 400 µL of A2 washing buffers (A&A Biotechnology, Poland). After each addition of the reagent, the sample was centrifuged respectively for 1 and 2 min at 10,000 rpm. The dried columns were transferred to new 1.5 mL Eppendorf-type tubes. Elution was performed with 200 µL of tris buffer heated to 70°C. The samples were incubated for 2 min at room temperature and then centrifuged again at 10,000 rpm.

Fig. 1. Locations of fish sampling sites

Qualitative and quantitative assessment of the obtained DNA was conducted *via* agarose gel electrophoresis of the extracts. A 1.5% agarose concentration was used. A solution with a measured amount of agarose (Basica GQT, Prona, Burgos, Spain) and tris/borate/ethylenediaminetetraacetic acid (EDTA) (TBE) buffer (Fermentas, Vilnius, Lithuania) was heated until clear and homogeneous, then cooled, and ethidium bromide (EtBr, MP Biomedicals, Solon, OH, USA) was added. The liquid was placed in a gel-forming stand, and after the gel solidified, 8 µL of the isolate was applied to each well. Electrophoresis was carried out in a Wide Mini-Sub Cell GT chamber (Bio-Rad, Hercules, CA, USA) filled with the TBE buffer at 80 V for 45 min. Subsequently, the gel was transferred to a Gel Doc XR chamber (Bio-Rad) emitting UV light, enabling gel light absorption. Gels were analysed using the Quantity One program (Bio-Rad). Absorbance measurements were performed using a NanoDrop 2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Table 1. Primers used for the detection of the carp oedema virus P4a protein in conventional and nested PCRs

Primer name	Primer sequence	Product size (base pairs)	Reference
CEV qFor1 CEV for B	5′-ATGGAGTATCCAAAGTACTTAG-3′	528	12
CEV rev J	5′-CTCTTCACTATTGTGACTTTG-3′	528	
CEV for B - int	5′-GTTATCAATGAAATTTGTGTATTG-3′	478	
CEV rev J - int	5′-TAGCAAAGTACTACCTCATCC-3′	478	

CEV – carp oedema virus; q – quantitative PCR; For/for – forward; rev – reverse; int – internal

Table 2. Composition of the PCR mixture used for detection of the carp oedema virus P4a protein in a second nested PCR

Deionised water (PCR grade)	GoTaq G2 Green Master Mix	Forward primer	Reverse primer	Template DNA
6.5 mL	12.5 mL	0.5 mL	0.5 mL	5 mL

Table 3. Primers used for the detection of the carp oedema virus P4a protein in a real-time PCR

Primer name	Primer sequence	Reference
CEV qFor1	5′-AGTTTTGTAKATTGTAGCATTTCC-3′	12
CEV qRev1	5′-GATTCCTCAAGGAGTTDCAGTAAA-3′	
CEV qProbe1	5′-AGAGT TTGTTTCTTGCC ATACAAACT-3′	

Table 4. Composition of the real-time PCR mixture for detection of carp oedema virus P4a protein

Distilled water (PCR grade)	GoTaq G2 Green Master Mix	Forward primer	Reverse primer	TaqMan probe	Template DNA
6.25 mL	12.5 mL	0.5 mL	0.5 mL	0.25 mL	5 mL

Table 5. Summary of carp tissue samples (kidney, spleen, gills and skin) subjected to *in situ* hybridisation

Sample collection site	Study material	Species	Symbol
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC1
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC1
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC2
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC2
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC4
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC4
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC7
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC7
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC8
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC8
Farm 2	kidney, spleen	Carp (*Cyprinus carpio*)	DC9
Farm 2	gills, skin	Carp (*Cyprinus carpio*)	DC9
Farm 9	kidney, spleen	Carp (*Cyprinus carpio*)	9SK
Farm 9	gills, skin	Carp (*Cyprinus carpio*)	9SK

Amplification of the CEV genetic material was conducted following the thermal profile published by Matras *et al.* (12). The initial denaturation occurred at 95°C for 5 min and was followed by denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min, the polymerisation reaction at 72°C for 1 min, a final extension for 10 min, and cooling of the samples to 4°C. The DNA sequence fragment encoding the P4a protein was detected using a nested PCR with the primers listed in Table 1.

The product of the PCR reaction using the CEV for B/CEV rev J forward and reverse primers was used as a template for the second nested PCR reaction using the CEV for B int/CEV rev J - int internal primers. The primers were synthesised by Genomed (Warsaw, Poland). The polymerase chain reaction was performed in a total volume of 25 µL using GoTaq G2 Green MasterMix (Promega, Madison, WI, USA) (Table 2), and the reactions were carried out in a Mastercycler personal thermocycler (Eppendorf). Each reaction

included two control samples: a positive control consisting of CEV virus DNA (Friedrich-Loeffler-Institut (FLI), Riems, Germany) and a negative control, in which the template DNA was replaced by deionised water. After each reaction, the products were separated by electrophoresis under the same conditions used for assessing the quality of the specimens.

The results of each PCR reaction were evaluated by separating the PCR products on a 1.5% agarose gel and bidirectionally Sanger sequencing them.

Real-time PCR. Amplification of the material by real-time PCR was carried out at the FLI using reagents from Roche (Penzberg, Germany). For analysis primers described by Matras *et al.* (12) were used, which together with the molecular probes (Table 3) were synthesised by Genomed. The reaction was performed in a total volume of 25 µL using GoTaq G2 MasterMix Colorless (Promega) (Table 4). At the 5′ end, the probe was labelled with the fluorescein amidite fluorescent dye, and at the 3′ end, the Black Hole Quencher 1 (BHQ-1) was placed to reduce the fluorescent signal. The reactions were conducted in the Mx 30005 P QPCR system (Stratagene, La Jolla, CA, USA). Similarly to the procedures for the conventional and nested PCRs, additional samples as positive and negative controls were included in each analysis. Both protocols – the one for the conventional PCR and the one for its real-time PCR counterpart – were developed in the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) laboratory (Weymouth, UK).

Sequence analysis. Bidirectional Sanger sequencing was performed on behalf of the FLI by Genomed using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany).

***In situ* hybridisation.** Virus detection was performed using a reagent kit (Roche) in accordance with an accredited methodology developed at the FLI laboratory. *In situ* hybridisation was conducted using a molecular probe to locate the specific CEV virus sequence in histological preparations. Four selected fish tissue samples from farm 2 (n = 6) and farm 9 (n = 1), previously diagnosed as infected and preserved in isopropanol were used after re-fixation in 10% formalin as materials for the study. The tissues utilised for hybridisation included kidney, spleen, gills and skin from seven individuals (Table 5). Tissue fixation was carried out using the Davidson method. Dehydration, impregnation and embedding were performed automatically using a Tissue-Tek VIP machine (Sakura Finetek, Torrance, CA, USA). The preserved preparations were sectioned on a microtome and transferred to basic Silane-Prep slides (Sigma-Aldrich Chemie, Taufkirchen, Germany). The reaction area on the slide was outlined with a peroxidase–anti-peroxidase (PAP) Pen marker (Merck, Darmstadt, Germany) to prevent the leaking of reagents from the examined tissue. Subsequently, the marked area was treated with proteinase K (Appligene, now Thermo Fisher Scientific, Illkirch, France) (100 mg/mL) suspended in TE buffer (50 mM tris and 10 mM EDTA), 200 mL of this being added to each preparation, and the slide was incubated for 20 min at 37°C. The samples were then rinsed with 99.8% ethanol and left to dry for 1 min. After drying, the preparations were outlined again with the PAP Pen marker.

The examined tissue sections were covered with a slip and placed on a preheated Mastercycler Gradient thermal cycler plate at 95°C (Eppendorf) for 5 min to induce denaturation. After denaturation, the preparations were rapidly cooled on ice for 2 min. *In situ* hybridisation was conducted through the addition of 5 µL of homogenates from soft tissues isolated from koi and farmed carp. The slides were placed in a moist chamber at 42°C for 12 h. The hybridisation solution (200 mL per preparation) consisted of Denhardt's solution, saline–sodium citrate (SSC, pH 7.0), bovine serum albumin and molecular probes. The preparations were washed with 0.4× SSC (2 × 5 min) heated to 42°C to remove non-specifically bound probes. The preparations were then incubated at 42°C for an additional 10 min. Solutions of digoxigenin (DIG) 1 (0.1 M maleic acid (11.61 g) and 0.15 M NaCl (8.766 g); Sigma-Aldrich Chemie) and DIG 2 (1g of reagent from the DIG Nucleic Acid Detection Kit in 100 mL of DIG 1 buffer; Boehringer Mannheim, Mannheim, Germany) were used for antibody staining. Volumes of 200 mL of each solution were added to the examined tissues and the tissue and solution mixture was incubated for 30 min under foil at room temperature. The next step involved equilibrating the examined sessions with DIG 3 solution (0.10 M tris (12.11 g), 0.10 M NaCl (5.844 g) and 0.05 M $MgCl_2$ (10.165 g); Sigma-Aldrich Chemie) for 10 min at room temperature. The bleaching process was then stopped using DIG 4 solution (100 mM Tris-HCl and 0.001 M EDTA; Sigma-Aldrich Chemie). To differentiate the level of infection in cells, contrast staining was carried out using Bismarck Brown Y counterstaining (Sigma-Aldrich Chemie).

Bioinformatics analysis. Sequencing products were subjected to bioinformatics analysis using Geneious Prime 8.0 (Biomatters, Auckland, New Zealand) and BLAST-N available in the NCBI database to determine the level of similarity between amplicons. In Geneious Prime 8.0, all obtained CEV virus sequences were aligned. The analysis of *in situ* hybridisation results was conducted using an Eclipse E-600 microscope (Nikon, Tokyo, Japan). Data were processed and calculated using Excel spreadsheet software (Microsoft, Redmond, WA, USA).

Results

Gel electrophoresis. Electrophoretic separation of nested PCR products revealed the presence of CEV P4a gene DNA in 16 out of 238 samples obtained for the study. Positive results for CEV virus genome carriage were obtained on three out of nine tested fish farms

(farm 2, where prevalence was 11%; farm 5, where it was 1%; and farm 9 with 7%). A map of the epizootic area is presented in Fig 2.

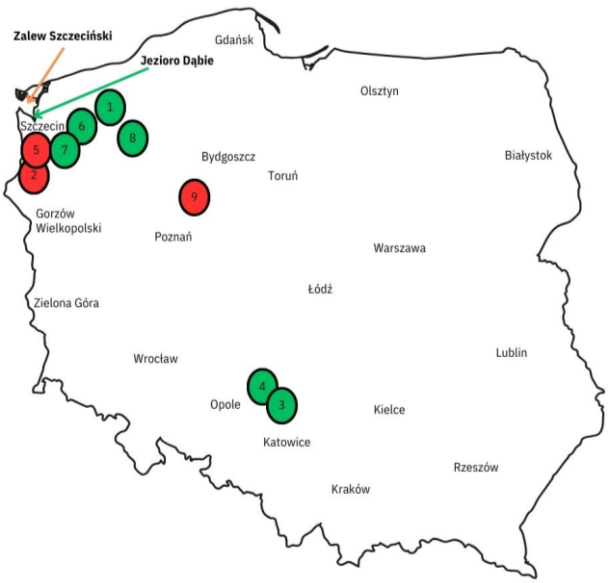

Fig 2. Sampling sites in the study. Locations with positive test results (in red) and locations free from carp oedema virus (in green). Outline of the map obtained from the website fabrykapuzli.pl and graphically processed by the author using canva.com

Table 6. Carp oedema virus–positive samples in common carp (*Cyprinus carpio*) detected using real-time PCR

No.	Sample code	Sample collection site	Threshold cycle
1	DC 4	Farm 2	36.67
2	DC 7	Farm 2	28.26
3	DC 8	Farm 2	24.44
4	DC 9	Farm 2	23.47
5	DC 10	Farm 2	25.08
6	DC 11	Farm 2	24.55
7	DC12	Farm 2	24.82
8	DC 5	Farm 5	28.2

Table 7. List of positive samples in carp obtained from kidney, spleen, gills and skin using *in situ* hybridisation

Sample collection site	Code	Kidney	Spleen	Gills	Skin
GR 2	DC1	−	−	+	+
GR 2	DC2	+	−	+	+
GR 2	DC4	−	−	+	−
GR 2	DC7	+	−	+	−
GR 2	DC8	+	−	+	−
GR 2	DC9	+	−	+	−
GR 9	9SK	+	−	+	+

Sequencing. Positive samples were sequenced. The sequences obtained from 16 carp specimens were aligned and registered in the NCBI GenBank database under accession numbers from OQ469756 to OQ469771.

Real-time PCR. The threshold cycle values for the isolates ranged from 23.47 to 39.35. Any value below 37 was considered a negative result, being below the method's detection threshold. Eight positive results were obtained (Table 6).

***In situ* hybridisation.** The presence of CEV virus genetic material was observed in all individuals selected for testing using this methodology (Figs 3–10).

The virus showed affinity for cells and tissues in the following descending order: gills, kidney and skin. It was found to have a negative tropism for the spleen related to its replication ability (Table 7).

Molecular sequence analysis. The sequences of positive samples were compared to the CEV virus sequence logged by Matras *et al.* (12) in the GenBank database under accession number KX253999 (Fig. 11). To obtain data on the evolutionary relationship between the sequences obtained during the research conducted in this study and sequences of a similar genome fragment responsible for the synthesis of protein P4a deposited in GenBank, a molecular evolution model was used. A phylogenetic tree was constructed based on the Tamura–Nei (TN93) model (19) (Fig. 11). Sequence comparison revealed that those obtained in the current study formed two distinct clades. The obtained sequences were registered in GenBank under accession numbers from OQ469756 to OQ469771.

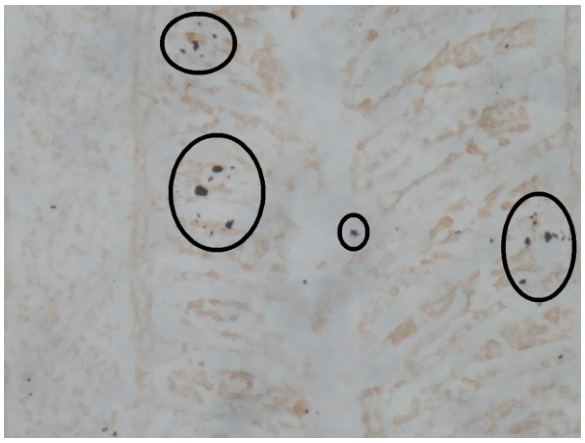

Fig. 3. Confirmation of CEV genetic material in carp gills

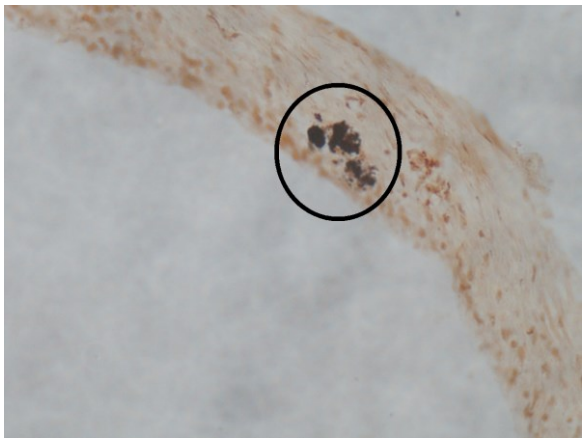

Fig. 4. Confirmation of CEV genetic material in skin

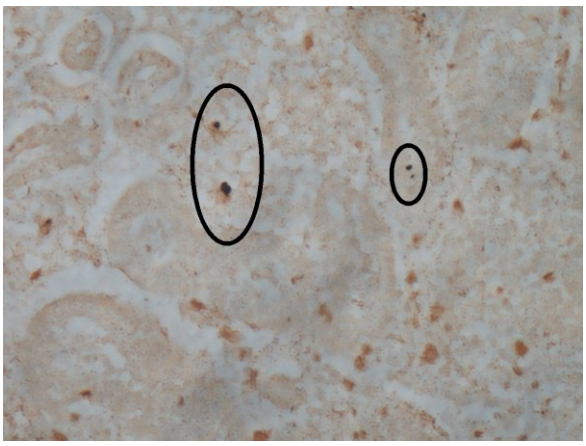

Fig. 5. Confirmation of CEV genetic material in kidney

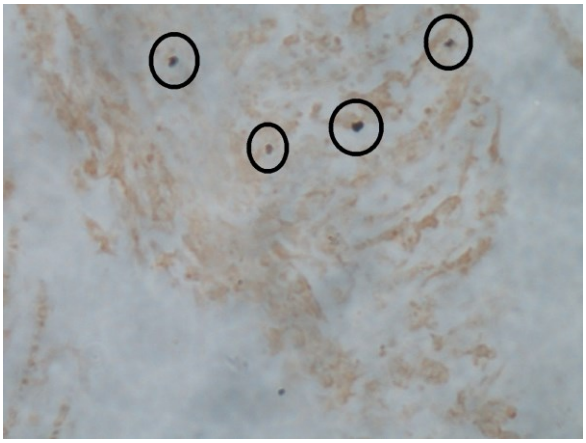

Fig. 6. Confirmation of CEV genetic material in gills

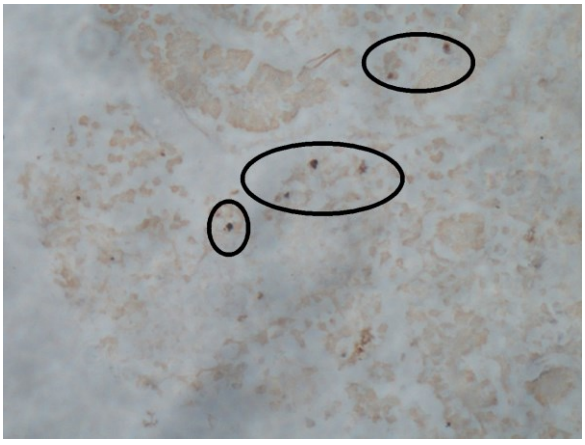

Fig. 7. Confirmation of CEV genetic material in kidney

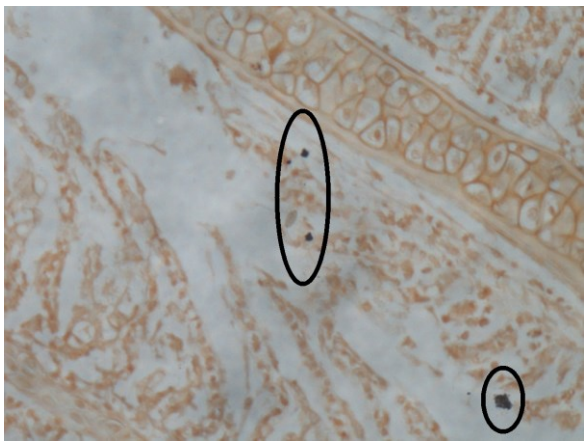

Fig. 8. Confirmation of CEV genetic material in gills

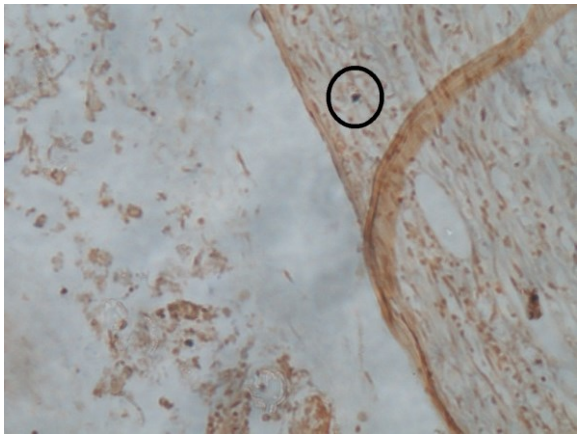

Fig. 9. Confirmation of CEV genetic material in skin

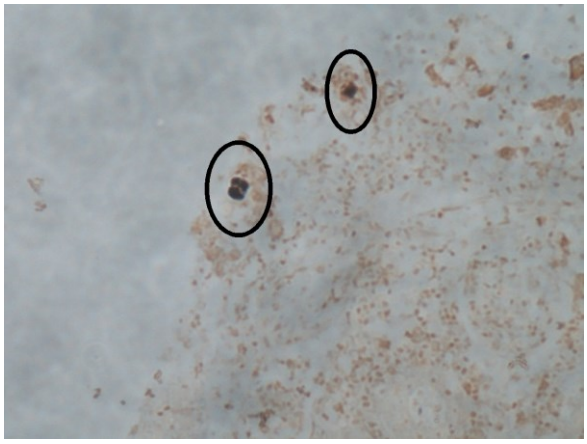

Fig. 10. Confirmation of CEV genetic material in kidney

Discussion

Viral diseases in fish pose a significant problem because of the practical impossibility of eliminating them from breeding facilities. A viral disease diagnosis often leads to restrictions on the sale of live fish and implementation of measures such as mandatory pond drying, disinfection through liming, and exclusion of ponds from use for at least one season. For the aquaculturist, this disrupts the production cycle and generates financial losses.

After the severe harm done to many carp farms by koi herpesvirus infection, the failure to make a rapid diagnosis has been seen to doom the aquaculturist to incur near-certain massive mortality of all age groups, even up to 100% of the population (23). A similar situation may occur with carp oedema virus, which has spread worldwide in a very short time. Its first detection took place in the 1970s in Japan (14), where it was observed in young koi, causing clinical symptoms of body swelling and swimming at the water's surface or lying on the pond bottom.

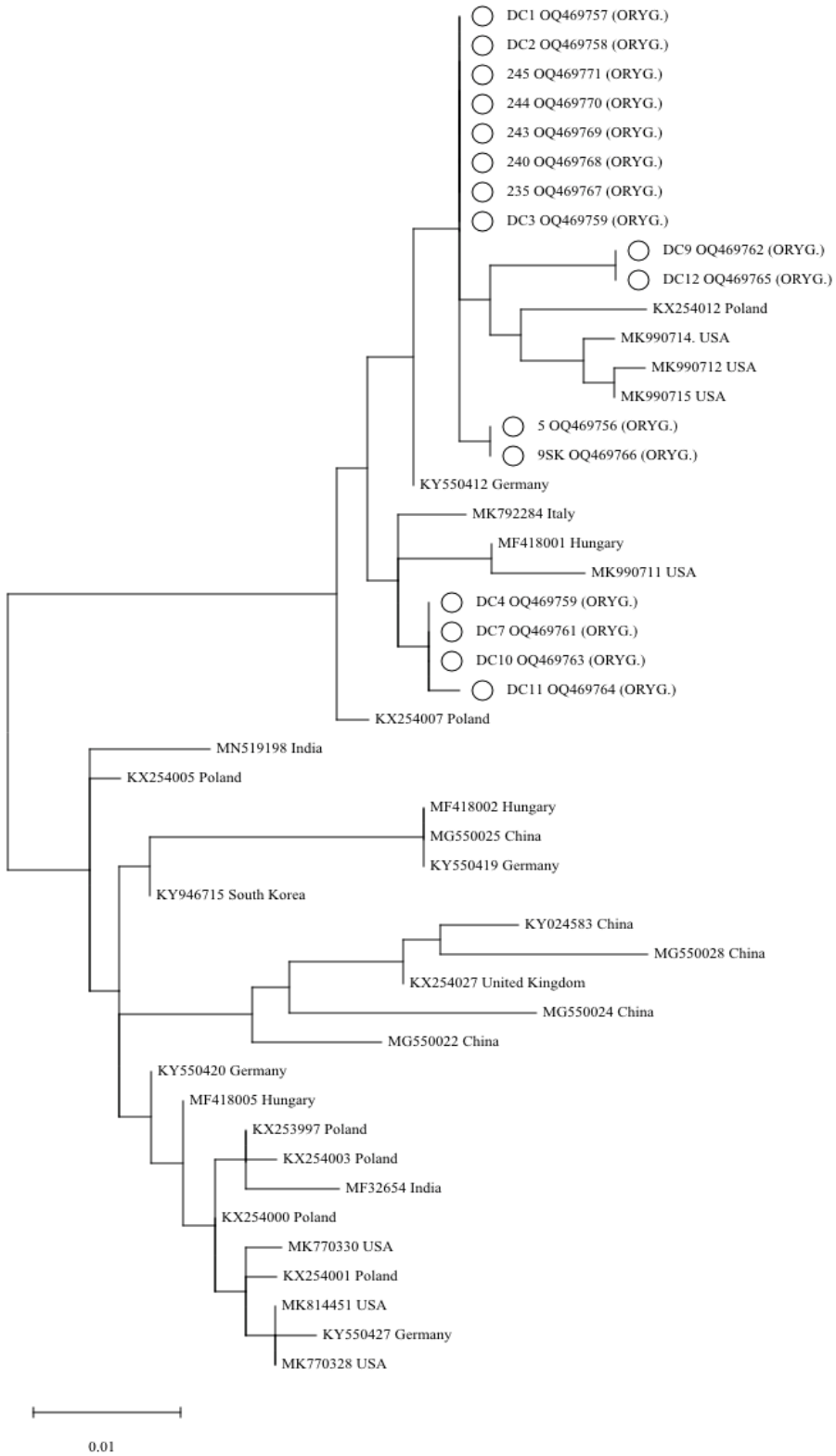

Fig. 11. Maximum-likelihood tree constructed using the Tamura–Nei (TN93) model for the gene P4a sequences of carp oedema virus obtained from GenBank and the authors' sequences (designated by "ORYG.") with accession numbers OQ469756–OQ469771. Scale – substitution frequency

As literature data indicate, CEV was not observed and diagnosed in Europe until around 40 years after its initial detection in Japan. The first detection in Europe occurred in the United Kingdom in 2009 (23), and subsequent detections were in France and the Netherlands (7), Austria and Germany (8, 9), Hungary (1), North America (10), the Czech Republic and Slovakia (11), India (19), Thailand (17), and Croatia (25).

In Poland, the first diagnosis of CEV was the result of a reanalysis of samples collected as part of the koi herpesvirus (KHV) surveillance programme from 36 fish farms (12). Despite the archival origin of the

samples subjected to molecular analysis (their collection having taken place between 2013 and 2015), CEV genetic material was confirmed in 47% of them. Therefore, the question arises whether the absence of CEV in Poland for nearly four decades from its confirmation in Japan was actually due to the lack of clinical symptoms or mortality or was the result of a lack of appropriate diagnostic tools.

It is highly likely that the clinical symptoms observed in carp farms in Poland in 2015, of fish lethargy, pale, necrotic changes in the gills and mortality reaching 90% of the population led farmers to attribute these changes to KHV infection. Unfortunately, the clinical picture does not allow distinguishing between infections caused by KHV and CEV without specialised molecular diagnostics. The confirmation of the presence of CEV genetic material in three additional locations indicates a high probability that the virus has been transmitted so far only between farms.

Carp oedema virus belongs to the *Poxviridae* family, which are DNA viruses with the largest size, complex structure, and a present capsid, and their replication occurs exclusively in the cytoplasm of cells (5). It can be assumed that the gills of fish, formed from gill filaments and lamellae covered with a thin epithelium, create an ideal cellular space for virus replication. As Evans *et al.* (6) demonstrated, the function of fish gills is not limited to breathing, but also includes maintenance of ion balance and removal of metabolic products. As indicated by the *in situ* hybridisation method, CEV virus particles accumulate mainly at the base of the gill lamellae and on the gill lamellae themselves. As virus replication progresses and individual gill fragments lose functionality, virus particles detach from the gill arches and enter the water. This mechanism is important for determining the role of water as a medium with suspended organic matter content, mainly including fragments of gills in the necrotic stage and skin and scales of the fish living in it. Water with these constituents is a medium facilitating the transmission of CEV between farms and between ponds and the natural environment.

The studied fish farms where CEV was detected did not use closed systems. Material was collected as far as possible from spawning sites, but a larger part came from hatcheries. Information from farmers also indicated that all hatcheries released water from ponds when they are dried (or from pools during cleaning), which directly promotes the penetration of CEV into the wild ichthyofauna.

From the current study defining the role of individual organs in carp with CEV genome component presence, it can be concluded that this virus does not only show tropism for carp gills, as indicated by the WOAH (24) – carp skin and kidney should also be mentioned as places where virus replication is possible. The applied *in situ* hybridisation method indicated the presence of a part of the CEV genome, which points to skin and kidney cells as having a use in screening diagnostics. These results mean that the likely scenario for the expansion of new CEV genogroups, as defined by Adamek *et al.* (2), involves other organs besides the gills. This has significant clinical implications because the gills are the first to be affected by CEV exposure (exhibiting swelling and necrosis), and the possibility of replication in other organs increases the number of infected cells.

The explanation and definition of CEV pathogenesis encompass the steps of the virus' entering the fish's body, replicating in susceptible cells, bypassing the local host defence, spreading to other organs, and ultimately transferring from the infected organism to a new host. The identification of the skin and kidneys as new organs of CEV tropism indicates that through mutations and genome evolution, the CEV virus has found new types of cells where it can replicate.

The concentration of CEV virions in koi tissues was also analysed by Adamek *et al.* (3), who used a quantitative PCR (qPCR) specific for virus specimens known to infect koi carp. The newly developed qPCR could be a useful research tool for CEV pathobiology. However, there are no studies that have introduced a different methodology from determining the copy numbers of the virus in tissues, although a novel method could improve CEV diagnostics. This is also reflected in the inclusion of this methodology in the list of those used in Laboklin accredited veterinary diagnostic laboratories. Laboklin offers its services in 38 countries worldwide, and everywhere its CEV diagnostic methodology is based solely on real-time PCR, with gill tissues as the starting material (https://laboklin.com/en/products/infectious-diseases-pathogens-and-antibody-detection/viruses/carp-edema-virus-cev/carp-edema-virus-cev-pcr/).

Comparisons of PCR-based methods for detecting genetic variants of CEV were made by Adamek *et al.* (1) in 2017. They analysed 39 samples from different geographical regions, subjecting them to molecular analysis according to five available protocols. The authors showed that PCR tests developed at CEFAS exhibited the highest diagnostic sensitivity. As indicated by our current study, the use of PCRs or nested PCRs is not the only available methodology to minimise the acquisition of false-negative results.

The development and optimisation of *in situ* hybridisation conditions presented in this work allow for the expansion of CEV detection to new organs and tissues. This also defines the direction of CEV genome evolution, which is likely to lead to the emergence of new "Polish" CEVs in the future, analogous to what happened with koi herpesvirus. This is evident from the constructed phylogenetic tree, which showed that the sequences obtained in this study formed two distinct clusters. Previous sequences from Poland, which served as the basis for defining the CEV genogroups isolated in 2016, are located between sequences from Hungary, India, the USA and Germany.

Rehman *et al.* (18) noted that current genomic data on CEV are limited to a fragment of the DNA sequence

encoding the core protein P4a. Sequences submitted to the NCBI database up to 2020 showed significant variability in this protein; therefore, detection of CEV using only PCR methods can lead to inaccuracies because of the high genetic variability of the P4a protein.

The phylogenetic tree analysis revealed that sequences from the UK clustered among sequences detected in China and the clade with sequences from Poland isolated in 2016 (all of them), along with those from the USA, Germany and Hungary. The trade in koi originating from China and the trade in stocking material largely from Hungary may represent one of the potential epidemiological pathways of CEV transmission in Poland.

It is very likely that the genetic distinctiveness of sequences from South Korea results from the limited scale of importation of fish from this country. The genetic distinctiveness of CEV sequences isolated between 2009 and 2014 and those isolated after 2019 support the assumption that a process of evolutionary emergence of local isolates is occurring, analogous to what happened with koi herpesvirus. Unfortunately, this provides the potential for the number of false-negative results to rise. This means that the primers used by accredited specialised veterinary diagnostic laboratories offering services worldwide (*e.g*. CEFAS and Laboklin) should change, and updated real-time PCR reaction protocols should be designed. These forthcoming protocols are to be considered obligatory for CEV, taking into account the variability between isolates obtained from different geographical regions. This would have a significant impact on minimising the unintended transmission of CEV between countries that trade in live carp, primarily koi.

Various exhibitions and koi fairs, where fish offered by exhibitors and hobbyists are not subject to mandatory testing for the presence of CEV genetic material, also represent a major source of its spread worldwide. As pointed out by Way *et al.* (22), improving water environment management and disease control is possible, and the principles of biological safety applied to koi herpesvirus can equally be applied to CEV diseases. However, it is necessary to fill the knowledge gap regarding the pathogenesis and epidemiology of these diseases, which currently prevents a precise assessment of the potential impact of CEV on koi, common carp and wild carp populations.

Conclusion

It can be noted that global aquaculture is becoming increasingly important because of the rising demand for food for the world's growing population. The carp species is crucial because of its resistance to oxygen deficiency and its relatively rapid growth rate. Viral diseases have a significant impact on carp production, as evidenced by the decimation of aquaculture farms in the Czech Republic, Poland and Hungary between 2003 and 2006. The most serious viral diseases include koi herpesvirus disease, spring viraemia of carp, CEV disease and KSD. As data from the National Veterinary Research Institute in Puławy (unpublished data, 2023) show, there are no comprehensive compilations of losses in aquaculture caused by viral diseases. Therefore, every effort should be made to limit their transmission both between aquaculture farms and between farms and the natural environment. This can only be achieved by identifying vector species and updating detection protocols. All new data on virus biology and transmission routes will provide a basis for modern and rational water environment management.

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

Financial Disclosure Statement: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Animal Rights Statement: The authors declare the experiments on animals were conducted in a manner in accordance with subsequently introduced Ethical Committee laws and regulations as regards care and use of laboratory animals.

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