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Characterisation of a newly isolated SVCV strain in Ukraine

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Spring Viraemia of Carp Virus (SVCV) has been well documented since the 1960s. This virus infection generally results in an acute haemorrhagic syndrome with septicaemia and associated high mortality. During a fish health inspection in carp farms in the Eastern region of Ukraine, SVCV was isolated from yearlings of the common carp *Cyprinus carpio*. For virus isolation and characterisation, methods of cell culture, EM, PCR, and sequencing were used. Preliminary examination of infected fish revealed a range of lesions, particularly in spleen and kidney tissues. The virus grew in fish cell lines of FHM and EPC with infectious titre of $10^{6.2–6.5}$ and $10^{6.9–7.4}$ $TCID_{50}$/ml, respectively. Investigation by electron microscopy demonstrated that ultrastructurally the isolated virus was similar to rhabdoviruses. Virions were non-enveloped with a typical bullet profile, approximately 80 to 180 nm in length and 60 to 90 nm in diameter. In addition, the nucleotide sequence of the glycoprotein gene G fragment in size of 579 base pairs was analysed. The nucleotide sequence was registered at GenBank under the accession number MH043331. The phylogenetic analysis revealed a close relationship of East Ukrainian isolates of SVCV with sequences that represent the strains from genogroup Ib.

Keywords: SVCV, common carp, molecular identification

INTRODUCTION

Spring viraemia of carp (SVC) is a viral disease caused by Spring viraemia of carp virus (SVCV) or *Carp sprivivirus*, which is classified as a member of the family *Rhabdoviridae* belonging to the genus *Sprivivirus* (ICTV, 2018 Virus Taxonomy: 2018 Release). The genome of SVCV is a linear single-stranded negative RNA molecule that encodes five structural proteins: nucleoprotein

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(N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and viral RNA-dependent RNA polymerase (L) in the order: 39-N-P-M-G-L-59 (Ahne et al., 2002).

The World Organization for Animal Health classified SVC as an especially dangerous disease of cultured fish (OIE, 2000). SVCV is distributed worldwide (Ashraf et al., 2016; Woo, Bruno, 2011). SVCV is an economically important fish pathogen since cyprinids are the main aquacultured species in Ukraine. The annual carp yield of stocked species amounts to 20,000 tons. In Ukraine, carp mortality caused by SVCV (mainly that of fingerlings) usually amounts to 30–40%, but sometimes can reach up to 70% (Matvienko et al., 2014).

Today, molecular epidemiology is instrumental for the investigation and control of the disease due to its ability to reveal the possible sources of infection. The molecular characterization of a viral pathogen allows to trace phylogenetic relationships of the virus and to determine its evolutionary history. Therefore, the aims of our study were to investigate SVCV from carp species reared in Eastern Ukraine using molecular approaches. In this study, we present the results of PCR and nucleotide sequence analysis as well as cell culture detection and electron microscopy studies of the East-Ukrainian isolates of SVCV sampled from carps from March to June in 2017 and 2018.

MATERIALS AND METHODS

Sample collection. During spring periods of 2017 and 2018, a total of 40 fish samples were continuously collected from alive and moribund carp fingerlings in fish farms ((1) "Chervona Dolyna" and (2) "SlavaTES", Slavyansk district; (3) "Oleksandrivka" and (4) "Mayachka", Oleksandrivka district; (5) "Nitrius" and (6) "Krasnolymanske", Lymansk district) of PC "Donrybkombinat" in Donetsk region. The capacity of PC "Donrybkombinat" is 5000 ha with of up to 2800 tons of annual fish production. The samples that displayed key lesions or signs of disease were considered for laboratory testing. At least five fish with symptoms of disease from each affected pond or cage were collected and placed into a separate submission form for each site. The samples of internal organs (kidney and spleen) were removed from individual fish and placed into a 1.5 ml microcentrifuge tube. Samples were transported to the laboratory on ice and processed immediately.

Cell lines. All collected samples were tested using cell culture assay. FHM and EPC cell lines were maintained in MEM medium (PAA) supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% foetal bovine serum (FBS Gold, PAA). The samples of internal organs were homogenized with MEM and filtered through the 0.45 μm membrane (Sarstedt). Then the virus suspension was inoculated onto 24-hours cell monolayer growing in 25 cm^2 flasks. After absorption for 60 min at 20°C, MEM medium supplemented with 2% of FBS was added to cells. When a complete viral cytopathic effect (CPE) was evident, the tissue culture supernatant was harvested and centrifuged at 2500 × *g* for 10 min at 4°C to remove cell debris. The 50% tissue culture infective dose ($TCID_{50}$ ml^{-1}) of the resulting supernatant was determined (Dougherty, 1964).

Virus purification. The virus was purified from tissue culture supernatant by the method of ultracentrifugation. Briefly, after cell debris was separated by centrifugation at 2500 × *g* for 10 min at 4°C, the pellet was discarded and the supernatant was centrifuged in Beckman L5-50B in a rotor of SW-40 for 60 min at 70500 × *g* at 4°C. The virus pellet was suspended in TNE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.5) and centrifuged at 2500 × *g* for 5 min at 4°C. Then the virus suspension was used for electron-microscopy investigation and viral RNA extraction. For electron-microscopy investigation, the viral suspension was stained with 2% uranyl acetate (Chen et al., 2009).

RNA extraction and cDNA synthesis. Genomic viral RNA was extracted using GeneJET™ RNA Purification Kit (ThermoScientific) as described in manufacturer's protocol. The cDNA synthesis was conducted using

RevertAid™ Premium First Strand cDNA Synthesis Kit (ThermoScientific).

RT-PCR. In order to compare different approaches for virus identification, the reverse transcription PCR (RT-PCR) was used to analyse all collected samples. Amplification of a 714 bp fragment of SVCV cDNA was performed using primers derived from sequences of the region coding for the glycoprotein gene: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR-RTC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH-ACN-CAY-3' SVCV R2). If the CPE in cell culture was not extensive, the second round of amplification was used. We used the semi-nested assay to amplify 606 bp fragment using primers: SVCV F1 and SVC R4 with the following sequence 5'-CTG-GGG-TTT-CCN-CCT-CAA-AGY-TGY-3' (Stone et al., 2003). The PCR mixture consisted of 12,5 μl of DreamTaq Green PCR MasterMix (ThermoScientific), 20 pmol of each primer, 1 μl of cDNA and the nuclease-free water up to a total volume of 25 μl. The amplification was conducted with 35 temperature cycles of: 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C followed by a final extension step of 10 minutes at 72°C. The PCR products were analyzed by 2.0% agarose gel electrophoresis.

Sequence analysis. The PCR products were purified with the Silica Bead DNA Gel Extraction Kit (ThermoScientific) and subjected to nucleotide sequence analysis using a 3130 Genetic Analyzer (Applied Biosystems). The sequences were aligned with available SVCV sequences in the GenBank database (NCBI) according to the CLUSTAL W by the software of Molecular Evolutionary Genetics Analysis (MEGA) v. 6.0. The nucleotide sequence was registered in GenBank under accession number of MH043331.

RESULTS

Clinical signs. All moribund and dead carps were on the surface of fish tanks while the samples were being screened. External signs of moribund fish included uncoordinated spiral swimming and violent flexing of the body. The diseased fish were notably darker in colour and appeared weak and lethargic. Acute infection was systemic, and the haemorrhages in different organs could be recognized. Liver, kidney, and digestive tract lesions were noted. Clinical symptoms of the disease included muscle lesions, gill oedema, deformation of internal organs, and tissue necrosis.

The level of mortality on the fish farms of PC "Donrybkombinat" was reported in a number of different ways. Two different figures for each farm reflected the monthly and the cumulative mortality (Table 1). Monthly mortality was calculated as the percentage of fish lost on a farm within March–June. It was measured for assessing the health of the fish on the each farm. The cumulative mortality over a full production cycle was the percentage of fish lost on a whole facility of each farm of PC "Donrybkombinat" during the entire period, given as a percentage of the total number of fish that were initially stocked on the farm. It was reported once the entire farm has been fully harvested after a epizooty. The cumulative mortality in inspected fish farms was 45–55% on the average (Table 1).

Virus reproduction in cell culture. The reproduction of the isolated virus in fish continuous cell cultures of FHM and EPC was investigated. These cell lines were sensitive to virus. The virus caused morphological changes and cells rounding. Subsequently cells scaled from the surface and CPE of virus on cells was visible. For FHM the complete destruction of monolayer was noted at 4-5 days after infection (d.a.i.). For EPC cells the CPE and complete destruction of cell monolayer were marked at 3–4 d.a.i. Infectious titres of SVCV isolates for cell lines FHM and EPC were $10^{6.2-6.5}$ and $10^{6.9-7.4}$ $TCID_{50}$/ml respectively. The highest infectious titre was observed for EPC cells, which is appropriate as this cell line was derived from the common carp – a natural SVCV host. That is why the EPC is the most appropriate cell lines for SVCV diagnostic.

TEM. The results of our electronic-microscopy investigations of purified viral particles

Table 1. **The monthly and cumulative mortality (%) of carp *Cyprinus carpio* caused by SVCV in fish farms of PC "Donrybkombinat" during March-June 2017 and 2018**

Fish farm*	2017					2018				
	March	April	May	June	CM**	March	April	May	June	CM
1	5.0	19.0	21.0	4.0	50.0	6.0	23.0	25.0	8.0	62.0
2	6.5	20.2	23.0	5.0	54.7	7.0	23.0	24.0	6.0	60.0
3	3.0	16.0	23.0	8.0	50.0	4.5	16.5	23.0	7.0	51.0
4	5.5	18.0	22.5	7.5	53.0	6.0	18.4	22.0	8.0	54.4
5	8.0	13.0	14.0	3.0	38.0	7.0	14.0	14.0	5.0	40.0
6	8.0	15.5	13.0	4.0	40.5	19.0	20.5	23.0	4.0	66.5
	Total per period				**47.70**	Total per period				**55.65**

* Fish farms: (1) "Chervona Dolyna" and (2) "SlavaTES", Slavyansk district; (3) "Oleksandrivka" and (4) "Mayachka", Oleksandrivka district; (5) "Nitrius" and (6) "Krasnolymanske", Lymansk district;

** CM – cumulative mortality;

*** The average water temperature during the period is +12°C (+2°C in March and +22°C in June);

**** Fish density was 100,000 fingerlings/ha (each fish is 50–100 g in size).

revealed basic characteristics for rhabdoviruses morphology and ultrastructure. The virions were non-enveloped with a typical bullet profile, approximately 80 to 180 nm in length and 60 to 90 nm in diameter.

Phylogenetic analysis. The size of RT-PCR products after the first round of amplification was 714 bp. The total number of samples was 40 and only five (5) of them were positive after the first round for SVCV using semi-nested RT-PCR. Mainly, more positive SVCV samples were detected by means of 606 bp fragments. The total amount of SVCV positive probes proved by semi-nested PCR was 27. It should be noted that after the first round of PCR, not all products were visible on agarose gels. To avoid this, we used the semi-nested assay using the second round and increased sensitivity of PCR. Only six RT-PCR positives samples were sequenced with reason to cover one sample for each facility. All samples shared the same nucleotide sequences. The results of sequencing showed that amplified PCR products were identical to fragments of SVCV glycoprotein gene G deposited in GenBank by other researchers. The nucleotide sequence was registered in GenBank under the accession number of MH043331. East-Ukrainian 2Ukr2017 isolate showed a high level of nucleotide sequence diversity (82.7% to 99% identity) among other SVCV isolates. The highest identity of 99.0% was noted for the sequence of glycoprotein G in SVCV isolate 2/90 (accession number AJ538060) isolated in Moldova (Stone et al., 2003). Both isolates represent the Ib genogroup. Interestingly, amino acid sequences of 2Ukr2017 and 2/90 isolates were 100% identical.

The identity between Ukrainian 2Ukr2017 and 1Ukr2014 (accession number MH043330) SVCV isolates was 92.6% and 94.3% in nucleotide and amino acid sequences, respectively. The phylogenetic analysis revealed a close relationship 1Ukr2014 with SVCV isolates that represent the Fijan strain and genogroup Id, which is traditionally associated with SVC in Europe (Figure). However, the 2Ukr2017 isolate was more similar to the strains previously isolated in Ukraine, Moldova, and Russia in the late eighties; also, it has less homology with the virus isolate 1Ukr2014. Therefore, it could be assumed that the origin of the East-Ukrainian isolate differs from the one described for 1Ukr2014 (Table 2).

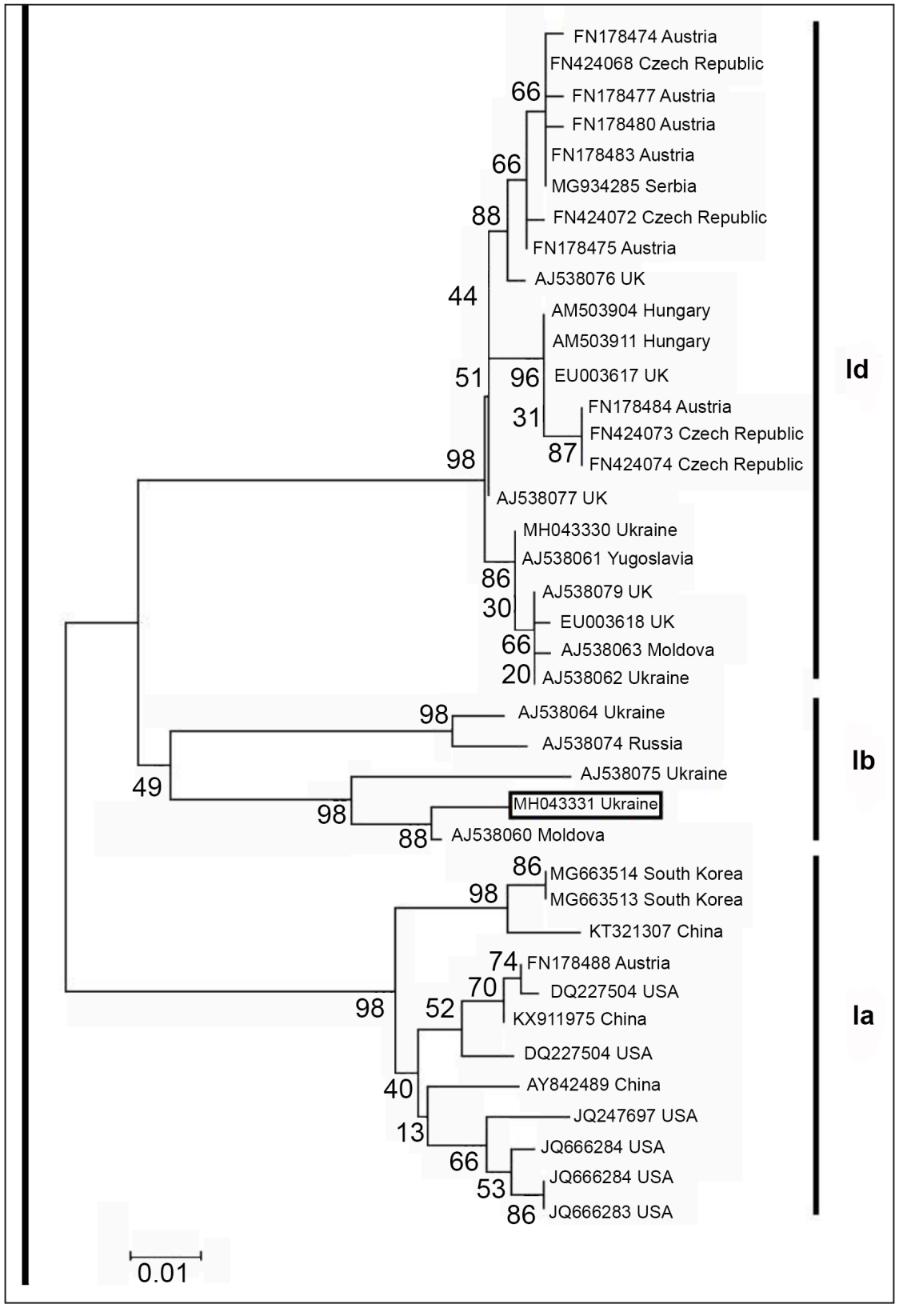

Figure. Phylogenetic analysis of G gene fragment of the new Ukrainian isolate of SVCV (accession number MH043331). The tree was generated by means of a neighbour-joining algorithm in MEGA software v. 6.0 (bootstrap 1000)

Table 2. **Isolates of SVCV and G gene sequence data analysed**

Isolate name	Host name	Year of isolation	Country of isolation	Genogroup	Accession number
N3-14	Bighead carp	1986	Ukraine	Id	AJ538062
N1-5	Grass carp	1986	Ukraine	Ib	AJ538064
RHV	Rainbow trout	1989	Ukraine	Ib	AJ538075
1_Ukr	Common carp	2014	Ukraine	Id	MH043330
2_Ukr	Common carp	2017/2018	Ukraine	Ib	MH043331
P4	Common carp	1983	Russia	Ib	AJ538074
M2-76	Common carp	2007	Moldova		EF593136
M2-78	Hypophthamichthys molitrix	1983	Moldova	Id	AJ538063
2/90	Common carp	1990	Moldova	Ib	AJ538060
17312/5	Common carp	1981	Hungary	Id	AM503904
17417/3	Sheatfish	1981	Hungary	Id	AM503911
S30	Common carp	1971	Yugoslavia	Id	AJ538061
DE/1999	Common carp	1999	Serbia	Id	MG934285
SO/2001	Common carp	2001	Serbia		MG934280
PI05	Pike	2009	Czech Republic	Id	FN424068
V-636	Carp koi	2009	Czech Republic	Id	FN424073
V-637	Siberian sturgeon	2009	Czech Republic	Id	FN424074
V-632	Common carp	2009	Czech Republic	Id	FN424072
66-94	Common bream	1994	Austria	Id	FN178474
92-94	Carp koi	1994	Austria	Ia	FN178475
39-97	Common carp	1997	Austria	Id	FN178477
122-02	Goldfish	2000	Austria	Id	FN178480
37-06	Carp koi	2006	Austria	Id	FN178483
38-06	Common carp	2006	Austria	Id	FN178484
3911-07	Common carp	2007	Austria	Ia	FN178488
970395	Common carp	1997	UK	Id	AJ538076
940626	Tench	1994	UK	Id	EU003617
880062	Common carp	1988	UK	Id	AJ538079
770346	Common carp	1977	UK	Id	AJ538077
880163	Common carp	1988	UK	Id	EU003618
212364	Carp koi	2002	USA	Ia	DQ227501
322383	Carp koi i	2004	USA	Ia	DQ227504
500549	Common carp	2007	USA	Ia	JQ666282
566087-1	Largemouth bass	2008	USA	Ia	JQ666283
566087-2	Bluegill	2008	USA	Ia	JQ666284
MN/27923/11	Common carp	2011	USA	Ia	JQ247697
992	Common carp	2004	China	Ia	AY842489
SH150514	Goldfish	2015	China	Ia	KT321307
Shlj3	Common carp	2015/2016	China	Ia	KX911975
SH150518	Carp koi	2015	China	Ia	KT321308
ADC-SVC2016-1	Common carp	2016	South Korea	Ia	MG663514
ADC-SVC2016-3	Largemouth bass	2016	South Korea	Ia	MG663513

DISCUSSION

In this report we described the isolation of SVCV from carp *C. carpio* fingerlings in fish farms of PC "Donrybkombinat" that is located in Donetsk region. Several SVCV isolates were described in Ukraine during 1986, 2006 and the period of 2013–2014 (Stone et al., 2003; Miller et al., 2007; Matvienko et al., 2014). In almost all cases they were isolated in the southern, western, and central parts of Ukraine. Based on the comparison of previous Ukrainian and current European SVCV isolates we suggest that East-Ukrainian SVCV 2Ukr2017 relates to Fijan strain but is quite distinct from isolate 1Ukr2014 (Stone et al., 2013; Padhi & Verghese, 2012). SVCV isolate 2Ukr2017 belongs to the Ib genogroup of viruses isolated in Ukraine, Moldova, and Russia, while 1Ukr2014 isolate is closer to European strains of the Id genogroup.

Ukrainian isolates were assigned to Genogroup I and were primarily isolated from the common carp; however, viruses assigned to this group were also isolated from another fish species such as the silver carp, the bighead carp, and the grass carp. It should be noted that in comparison of two Ukrainian isolates, the case of branch separating and evidence that SVCV has probably evolved independently in different areas. On the other hand, the 2Ukr2017 isolate could represent a virus strain from the former post-Soviet countries that existed or were brought to Donetsk region across the wide area.

An interesting point would be a thorough phylogenetic analysis of the isolates. According to recent publications, SVCV isolates are classified into four genogroups, Ia, Ib, Ic, and Id. Genogroup Ia contains isolates from Asia (Zhang et al., 2009; Kim et al., 2018) and the Americas, while isolates from Eastern Europe mainly belong to genogroups Ib and Id (Basic et al., 2009; Padhi, Verghese, 2012). Most likely, the 2Ukr2017 isolate was brought from Russia or even Moldova, because south and east regions are on the border and the cooperation between fish farms is very significant. This could be a novel finding and should therefore be confirmed by analysis of all East Ukrainian strains. Another important fact is that there veterinary control over the transboundary fish movement in Ukraine is insufficient.

During SVC epizootics, virus concentrations in the water may reach levels which are more than sufficient to infect fish. Up to 10^5 TCID/ml^{-1} were detected in troughs with carp fingerlings during an epizootic. A number of papers describe the stability of the virus in the environment (Hoffmann et al., 2002; Bandin, Dopazo, 2011). Therefore, rapid and accurate diagnosis of SVCV infection is critical to the control of the virus because carp surviving infections as juveniles may become life-long carriers and shed live virus.

The screening of samples using RT-PCR would potentially be more sensitive than tissue culture; however, suitable cell lines also can be used for surveillance of cyprinids for SVCV. Additionally, molecular techniques are required in the identification of the serotype and genotype of the isolated SVCV-strains. Since the virus is horizontally transmitted, the detection of the virus in broodstock, even in the absence of the disease, often means the destruction of fish population. There will be a need to screen not only all farmed populations of carp in the eastern regions of Ukraine, but also to confirm the presence of SVCV in wild populations of common carp *Cyprinus carpio* or another cyprinids species.

The general geographical distribution of SCVC strains is known. The Fijan strain was mainly identified in Europe (Fijan et al., 1971), but another SVCV strains occur in most of major cyprinid-farming countries in Asia, South Africa, and Americas (Dikkeboom et al., 2004). The virus is shed mostly with the faeces and urine of clinically infected fish and by carriers. Waterborne transmission is believed to be the primary route of infection, but bloodsucking parasites like leeches and the carp louse may serve as mechanical vectors of SVCV. Ukrainian carp farms closely collaborate with each other and fish-farms of the neighbouring countries; consequently, it might be one of the putative ways of spreading the SVCV to/from Ukraine.

Therefore, we suggest that the movement of fish to different countries should be stopped, since this movement increases the risk of spreading the virus and causes new disease outbreaks in new geographical areas.

In this research, the SVCV was identified in Donetsk region indicating a wide spread of the virus in Eastern Ukraine. The RT-PCR assay and nucleotide sequence analysis confirmed the prevalence of isolated virus to the Fijan strain of SVCV. In our opinion, a semi-nested PCR assay should be used for rapid virus identification, because in the case of a low concentration of target RNA only this molecular approach was capable to identify the virus. The complete monitoring of SVCV and its diagnostic in cyprinids cultivated in fish farms or native ponds of Ukraine will result in total data of virus distribution and identification of other strains widespread in Europe.

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NAUJAI IŠSKIRTI KARPIŲ PAVASARINĖS VIREMIJOS VIRUSO TIPAI UKRAINOJE

Santrauka

Karpių pavasarinės viremijos viruso diagnozė pradėta fiksuoti nuo XX a. 7 dešimtmečio. Šis virusas paprastai sukelia ūminį hemoraginį sindromą ir septicemiją, o tai lemia didelį mirštamumą. Patikrinus žuvų būklę rytinio Ukrainos regiono karpių fermose, karpių pavasarinės viremijos virusas buvo išskirtas iš paprastųjų karpių (*Cyprinus carpio*). Viruso išskyrimui ir apibūdinimui buvo naudojamos ląstelių kultūros, EM, PGR ir sekvenavimas. Preliminariai ištyrus užkrėstas žuvis, nustatyta daugybė pažeidimų, ypač blužnies ir inkstų audiniuose. Virusas išaugo FHM ir EPC žuvų ląstelių linijose, jų infekcinis titras buvo atitinkamai $10^{6,2–6,5}$ ir $10^{6,9–7,4}$ $TCID_{50}$/ml. Elektroninės mikroskopijos tyrimas rodo, kad išskirtas virusas struktūriškai panašus į rabdovirusus. Virionai neturėjo apvalkalo su tipišku kulkos profiliu, jų ilgis apie 80–180 nm, skersmuo 60–90 nm. Išanalizuota 579 bazių porų ilgio glikoproteinų G geno fragmento nukleotidų seka. Nukleotidų seka buvo užregistruota „GenBank“ (registracijos numeris MH043331). Filogenetinė sekų analizė atskleidė glaudų Rytų Ukrainos karpių pavasarinės viremijos viruso izoliatų ryšį su Ib geno grupės tipo sekomis.

Raktažodžiai: karpių pavasarinės viremijos virusas, paprastasis karpis, molekulinė identifikacija