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INVESTIGATION OF STARRY STURGEON *ACIPENCER STELLATUS* IN THE BLACK SEA AND SEA OF AZOV FOR ACIV-E INFECTION

Rud Yu.P. – PhD, Senior Research Scientist
Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine
rudziknew@ukr.net

Bushuiev S.H. – PhD, Senior Research Scientist
Institute of Marine Biology of the National Academy of Sciences of Ukraine
bsg1956@gmail.com

Khabarov V. – engineer
Institute of Fisheries and Marine Ecology,
khabarovnauka@gmail.com

Hrytsyniak I.I. – D.Sc., Director
Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine
info.iforgua@gmail.com

Buchatsky L.P. – D.Sc., Leading Research Scientist
Institute of Fisheries of the National Academy of Agrarian Sciences of Ukraine
iridolpb@gmail.com

Specific sturgeon nucleocytoplasmic large DNA viruses (sNCLDV) infect several species of the *Acipenseridae* family. sNCLDV have previously been referred to as unclassified members of the family *Iridoviridae*. They have recently been moved from *Iridoviridae* to *Mimiviridae* family. One of these viruses, *Acipenser iridovirus-European* (AcIV-E), is present in farms across Europe where it has occasionally caused mild to severe losses in sturgeons. In this study, we provide data on AcIV-E search in a susceptible species such as starry sturgeon (*Acipencer stellatus)* from the Black sea and Sea of Azov. In 2020, starry sturgeons were sampled in Odessa region of the Black Sea and Zaporizhzhia region of the Sea of Azov. None of the sampled fish demonstrated pathological signs of disease and were healthy upon visual observation all individuals. Total DNA was extracted and submitted to a generic PCR targeting different fragments of major capsid protein (MCP) gene of AcIV-E. No virus-specific products were obtained in any starry sturgeon sample from both seas, while the expected products of MCP gene fragments as well as the fragments of beta-actin gene in control reactions were successfully amplified using fish and viral DNA of AcIV-E isolated from Siberian sturgeon in 2018–2019. Although AcIV-E is difficult to identify in asymptomatic wild fish, but it probably can play a role in starry sturgeon pathology across the Black Sea basin. A high risk of virus spread to wild populations is possible since this virus was identified in sturgeon farms upstream the Dnipro river. Therefore, AcIV-E, even in the presence of other pathogens, should be studied in sturgeon hatcheries as well as in possible vectors of disease, but the huge area of inspection could be a challenge.

Key words: iridovirus, starry sturgeon, Black Sea.

Viruses are the most abundant among all marine organisms and have been recognized as causative agents of diseases in bacteria, protists, mollusks, crustaceans, fish and mammals. Studies of marine viruses have become an important and independent field in marine biology, driven by a growing awareness of the significant and diverse role of viruses in the marine ecosystem (Munn 2006).

Viruses can restrict aquaculture production dramatically. For example, viruses cause significant economic losses in sturgeons, in both aquaculture and wild populations. Sturgeon nucleocytoplasmic large DNA viruses (sNCLDV) have been found to infect several species of the *Acipenseridae* family. These viruses are present in hatchery-reared and wild sturgeon populations across the North America and Europe and pose a potential disease risk (Mugetti et al. 2020a). sNCLDV have previously been referred to as unclassified members of the family *Iridoviridae*. The first iridovirus to

infect sturgeon was identified in the 1980s in North America, where the disease of white sturgeon (*A. transmontanus*) (WSIV) was observed (Hedrick et al. 1990). Other iridoviruses have since been found in the North America in different sturgeon species: MRSIV (Kurobe et al. 2010), NV (Clouthier et al. 2013), SNSV (Lapatra et al. 2014), BCWSV (Clouthier et al. 2015). AcIV-E was identified in Europe in six sturgeon species: Russian (*A. gueldenstaedtii*), Siberian (*A. baerii*), Adriatic (*A. naccarii*), Starry (*A. stellatus*), Sterlet (A. *ruthenus*) and Beluga (*Huso huso*) (Bigarre et al.,2017; Mugetti et al. 2020b). The overall epidemiological situation is poor and remains unclear, since lack of disease control and active fish trade allowed the virus to spread to different regions of Europe. To date, AcIV-E has been identified in France (Bigarre et al. 2017), Italy (Ciulli et al. 2016), Sweden (Axen et al. 2018), Poland (Stachnik et al. 2019), and Ukraine (Rud et al. 2020).

Aquaculture is becoming an increasingly important sector of agriculture and production of sturgeons is an essential part of it. In this study, we describe the data regarding AcIV-E search in starry sturgeon (*A. stellatus*) in the Black Sea and Sea of Azov in Ukraine with a purpose to identify the virus and to analyze its possible distribution.

Materials and Methods

Sample collection. In 2020, a total of 15 fish samples were collected from alive starry sturgeons near Odessa and Berdyansk, Zaporizhzhia regions during trawl catch of sprat (*Sprattus sp*.) that was characterized by high selectivity of production of the main object and insignificant by-catches of other non-target species. By-catches of Red Book species, such as starry sturgeon, have been recorded. Actions in this situation are determined by the Rules of Fisheries in the Black Sea Basin and the annual fishing regime. By-catches of sturgeon and other species were considered incidental, caught fish released into the water live. Before the release of sturgeons, smears were taken from their gills, mouth, barbels and skin of head using sterile inoculating loop which was rinsed in 1.5 collection tube with DNA/RNA-Shield solution. Smears were sampled from the skin, gills and mouth in purpose to obtain the upper layers of epithelium. Sampling did not affect fish viability as well as no physical damage was inflicted on the fish. Fish parameters were recorded and alive starry sturgeons were released back into the Sea. The samples were collected from individual fish and transported on ice to the laboratory and processed immediately.

DNA extraction. The collected samples from individual fish were used for total DNA extraction. DNA was purified using Quick-DNA Miniprep Kit (ZymoResearch) as described in the manufacturer's protocol.

PCR. Conventional PCR (cPCR) was performed using primer sets specific to different fragments of AcIV-E major capsid protein (MCP) gene: setA (oPVP339-0PVP340), set B (oPVP341-oPVP344), and set C (oPVP341-oPVP345) (Bigarre et al. 2017). Additionally, each DNA sample was tested with primers specific to reference sturgeon beta-actin gene (Burcea et al. 2018). All samples showed positive results for beta-actin gene suggesting sufficient DNA quality. In control reactions, the DNA sample of AcIV-E from infected Siberian sturgeon was used. cPCR reactions were performed in 25 µl with 250 ng of total DNA, 0.2 µM of each primer of a pair and DreamTaqGreen PCR master mix (ThermoFisher Scientific).The following cycles were applied: one step of 8 min at 95°C, followed by 35 cycles at 94°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 10 min (Bigarre et al. 2017). A volume of 20 µl was run on a precasted 2% agarose gel (Sigma-Aldrich) for 30 min before observation under UV.

Results

In 2020, 15 samples of starry sturgeon were collected in the framework of the scientific study for the fish stock assessment in the Black Sea. None of the fish caught demonstrated pathological signs of any disease and all individuals were healthy upon visual observation. The average size of starry sturgeons from the Black Sea were 42.3/52.5 cm (standard/total length) (Table). The same procedure was carried out in the Sea of Azov, where starry sturgeons of average length of 57.6/63.0 cm were caught during a monitoring in 2020. All the fish were apparently healthy without obvious external signs of any disease (Table 1).

None of the sampled starry sturgeon from both the Black and Azov seas were positive for the AcIV-E. Using different sets of primers specific to different portions of MCP gene, we failed to amplify the corresponding fragments of viral DNA (Fig. 1a). It should be noted that the sensitivity of the primer sets used was sufficiently high, and all primers in a dilution experiment of viral DNA obtained from infected Siberian sturgeon were able to amplify the expected fragments with low DNA concentration. As a result, 535 bp for the set C were successfully amplified in control samples (Fig. 1a). The reference beta-actin gene was amplified in all samples, indicating proper DNA quality (Fig. 1b).

Discussion

In this study, we attempted to identify AcIV-E in wild populations of the starry sturgeon from the Black Sea and the Sea of Azov. According to recent publications, AcIV-E is usually identified in fish with signs of the disease and in the vast majority of cases it occurs at sturgeon farms rather than in wild stocks. In addition, fish age also plays a role in virus detection. Virus mainly affects fingerlings, but no signs of the disease appear in fish of older age groups.

Table 1

Samples of sturgeons tested for AcIV-E using cPCR: BS – the Black Sea; SA – Sea of Azov; Nd, not determined; - negative result; + positive result

Sample No.	Host species	Origin	Size, cm	AcIV-E	Beta-actin	Genbank
1	Starry sturgeon	BS	41/51	-	+	Nd
2	Starry sturgeon	BS	44/53	-	+	Nd
3	Starry sturgeon	BS	44/55	-	+	Nd
4	Starry sturgeon	BS	39/49	-	+	Nd
5	Starry sturgeon	BS	40/52	-	+	Nd
6	Starry sturgeon	BS	46/55	-	+	Nd
7	Starry sturgeon	SA	70/76	-	+	Nd
8	Starry sturgeon	SA	75/82	-	+	Nd
9	Starry sturgeon	SA	72/79	-	+	Nd
10	Starry sturgeon	SA	51/56	-	+	Nd
11	Starry sturgeon	SA	41/45	-	+	Nd
12	Starry sturgeon	SA	43/47	-	+	Nd
13	Starry sturgeon	SA	54/59	-	+	Nd
14	Starry sturgeon	SA	48/53	-	+	Nd
15	Starry sturgeon	SA	64/70	-	+	Nd
16	Siberian sturgeon	Dnipro	12/15	+	+	MT645223

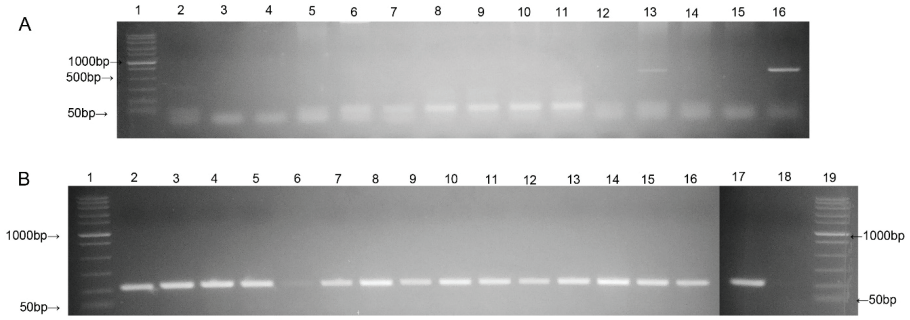

Fig. 1. Detection of AcIV-E in starry sturgeon (*A. stellatus*) by generic PCR (1A): 1 – Fast DNA ladder N3238S (New England BioLabs), 2 – control reaction (all components without DNA), 3–8 – samples from the Black Sea, 9–15 – samples from the Sea of Azov, 16 – AcIV-E, isolate of *A. baerii* UA 1/2018 (fragment of MCP gene - 535 bp); Amplification of the reference beta-actin gene (161 bp) on all samples (1B): 1, 19 – Fast DNA ladder N3238S (New England BioLabs), 2–7 – samples from the Black Sea, 8–16 – samples from the Sea of Azov, 17 – DNA sample of AcIV-E infected Siberian sturgeon, 18 – control reaction (all components without DNA)

In our study, all studied fish apparently of 2–5 years old appeared to be healthy. Therefore, the chances to identify the AcIV-E in these fish were not high. Another important factor is that the samples were collected from alive fish, and therefore it was not possible to select internal organs such as the heart, kidney and spleen, or even gills, which are also target organs for virus.

All samples were collected and stored in accordance with the recommendations for virological testing and delivered to the laboratory in time. Starry sturgeons were carefully tested using different sets of sensitive primers specific for the MCP gene of AcIV-E (Bigarre et al. 2017). We were unable to identify the viral DNA using high-sensitivity PCR tests, but instead, the control DNA was amplified successfully. Just like in 2018, when the AcIV-E was firstly detected in a Siberian sturgeon in Ukrainian farms, only specific PCR was used (Rud et al. 2020). But in that case, the most important factor of positive results in AcIV-E diagnosis was the stocking density of fish at the farms. Aside from PCR, there is no other method for virus diagnosis. AcIV-E does not propagate on cell cultures, therefore, developing new methods for its diagnosis, perhaps even more sensitive, is now a challenge (Pallandre et al. 2019).

The densities of starry sturgeon populations in the sea and at fish farms are dramatically different. Accordingly, when an infection occurs at a fish farm, the virus spreads rapidly and results in high morbidity and mortality. The likelihood of effective transmission of AcIV-E between susceptible species in the sea is extremely low. However, despite this, the threat still exists. Firstly, there are several farms for starry sturgeon reproduction for restocking in the Black Sea region. Secondly, the virus was identified in Ukraine at farms that have connections with rivers of the Dnipro basin and, consequently, the Black Sea. Thirdly, there is currently no data available on the spread of AcIV-E in the Black Sea countries where sturgeon aquaculture is actively developing. The main goal of sturgeon aquaculture is to increase the production of black caviar and to achieve this goal, several sturgeon species are usually cultivated at the same farm. Therefore, virus-susceptible species are kept together. Taking into account the national programs for the restoration of endangered sturgeon species in the Black Sea basin, there is a real threat for wild sturgeon populations to be infected with an emerging AcIV-E. The infection may not manifest itself in acute form, but may adversely affect vital processes, including reproduction, and transmit the virus to offspring. Therefore, in our opinion, it would be advisable to carry out surveillance procedures of farms participating in sturgeon stocking programs in order to minimize the risk of disease spread.

As it was shown previously, one European isolate of AcIV-E is closely related to NV detected in Canada (95% identity) (Bigarre et al. 2017). It demonstrates the presence of two well-formed strains of sturgeon iridoviruses in Europe: the newest AcIV-E strain and the Namao Virus (NV), which most likely was introduced to Europe by importing white sturgeon (*A. transmontanus*) or other species. The AcIV-E strain probably poses different virulence, although the data are insufficient. There are two AcIV-E variants identified in Europe using SNPs technique (Bigarre et al. 2017). Both var1 and var2 carry unique sequences in the MCP gene. Analysis of the RFC gene within a single viral population also indicates the presence of two AcIV-E variants (Pallandre et al. 2018). The PCR-HRM was used to monitor the virus in France. The most commonly encountered was the var2, one or occasionally mixed with the var1. Interestingly, the var1 was never found alone (Pallandre et al. 2019). Therefore, other regions of Europe, including Danube countries, should be monitored.

The lack of sufficient viral DNA sequences, as well as morphological characteristics that differ from classical iridoviruses complicate current classification of sNCLDV (Hedrick et al. 1990; Clouthier et al. 2013; Kurobe et al. 2011). In addition, according to recent data, there is a close genetic link between the MCP genes of NV, AcIV-E and *Cafeteria roenbergensis virus* (CroV), a giant virus from the *Mimiviridae* family (Clouthier et al. 2013). The striking relationship of sNCLDV to *Mimiviridae* was confirmed by genomic analysis (Clouthier et al. 2018). AcIV-E can thus be renamed as ESMV (*European sturgeon mimivirus*). The complete sequences of sturgeon iridoviruses could be important for determining the relationships between sNCLDV and *Mimiviridae* family.

The total trade of caviar and sturgeon products worldwide increased to 180.000 tonnes in 2016 (FAO 2018). One of the key factors of sturgeon aquaculture development is availability of national and international programs for the conservation of these endangered species with a purpose to restore their populations (Bronzi, Rosenthal 2014). It is very important to constantly monitor viral diseases at fish farms, which grow fish for restocking of natural waters, as there is a high risk of transmission of dangerous diseases from aquaculture species to natural ones, which can significantly worsen the overall epizootic situation. No less important is the diagnosis of infectious agents in natural populations of valuable and endangered species, including sturgeons. In this research, we tried to identify one of the most dangerous viruses for sturgeon – AcIV-E in the starry sturgeon population in the Black Sea and the Sea of Azov, which can initiate monitoring studies on the ecology of this pathogen and could help to assess the biological risks of sNCLDV for sturgeon wild populations and aquaculture in the future.

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ДОСЛІДЖЕННЯ ВІРУСУ AcIV-E У СЕВРЮГИ *ACIPENCER STELLATUS* У ЧОРНОМУ ТА АЗОВСЬКОМУ МОРЯХ

Рудь Ю.П., к.б.н., с.н.с.
Інститут рибного господарства НААН
rudziknew@ukr.net

Бушуєв С.Г., к.б.н., с.н.с
ДУ «Інститут морської біології Національної академії наук України»
bsg1956@gmail.com

Хабаров В., інженер
Інститут рибного господарства та екології моря
khabarovnauka@gmail.com

Грициняк І.І., д.с.-г.н., професор, директор
Інститут рибного господарства НААН
info.iforgua@gmail.com
Бучацький Л.П., д.б.н., професор, провід.н.с.
Інститут рибного господарства НААН
iridolpb@gmail.com

Специфічні осетрові нуклеоцитоплазматичні великі ДНК-вмісні віруси (sNCLDV) інфікують декілька видів з родини *Acipenseridae*. sNCLDV раніше відносили до некласифікованих представників родини *Iridoviridae*. Нещодавно їх перенесли до родини вірусів *Mimiviridae*. Один із цих вірусів, *Acipenser iridovirus-European* (AcIV-E), присутній у господарствах по всій Європі, де іноді спричиняє помірні або навіть чималі втрати осетрових. У цьому дослідженні ми наводимо дані ідентифікації вірусу AcIV-E у чутливого до виду – севрюги (*Acipencer stellatus*) з Чорного та Азовського морів. У 2020 році було відібрано зразки від севрюги в Одеській та Запорізькій областях в акваторіях Чорного та Азовського морів відповідно. Жодна з відібраних риб не демонструвала патологічних ознак захворювання, і у разі візуального спостереження всі особини були здорові. Зі зразків виділяли загальну ДНК та проводили ПЛР з використанням праймерів, націлених на різні фрагменти гена основного капсидного білка (MCP) вірусу AcIV-E. У жодному з досліджуваних зразків севрюги з обох морів не виявлено вірусоспецифічний генетичний матеріал, тоді як у контрольних реакціях очікувані продукти фрагментів гена MCP, а також фрагменти гена бета-актину були успішно ампліфіковані зі зразків ДНК риби та вірусу AcIV-E, виділеного від сибірського осетра в 2018–2019 роках. Хоча AcIV-E важко ідентифікувати у безсимптомних риб з природних популяцій, імовірно, вірус може відігравати свою роль у патології севрюги у басейні Чорного моря. Високий ризик поширення вірусу на природні популяції можливий, оскільки вірус був виявлений у осетрових господарствах, що базуються на річці Дніпро. Тому AcIV-E навіть за присутності інших патогенних мікроорганізмів слід досліджувати в риборозплідниках осетрових риб, а також у можливих векторах хвороби, але величезна площа моніторингу може бути проблемою.

Ключові слова: іридовірус, севрюга, Чорне море.