

Detection of white sturgeon iridovirus (WSIV) in wild sturgeons (*Actinopterygii: Acipenseriformes: Acipenseridae*) in Poland

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Abstract

Introduction: White sturgeon iridovirus (WSIV) disease is caused by a virus of the eponymous family and is mostly triggered by stressful environmental conditions, *i.e.* high rearing density, excessive handling, or temporary loss of water. The aim of this study was to develop the most effective diagnostic method for quick and efficient confirmation or exclusion of the presence of WSIV. **Material and Methods:** A total of 42 samples (spleen, gills, intestine, skin, kidney, and brain) were collected from eight sturgeon (*Acipenser gueldenstaedtii* and *A. oxyrinchus*) aged $\leq 5+$ farmed or caught between 2010 and 2014 in open waters (Dąbie Lake and Szczecin Lagoon). They were tested for WSIV presence using conventional PCR, qPCR, and *in situ* hybridisation (ISH). **Results:** In gross examination, all fish appeared to be healthy. Neither species showed clinical signs typical of WSIV infection. In the majority of cases, fragments of iridoviral DNA were found using molecular methods in the kidneys, and also in the liver, gills, and skin. The detection rate using ISH was 47.37% and most commonly the brain and kidney tissues were positive. The most efficient of the methods used was real-time PCR, with 100% effectiveness in detection of WSIV DNA. **Conclusion:** The study demonstrates the capabilities for WSIV diagnosis available to sturgeon farmers and water administrators, indicating useful methods of adequate sensitivity as well as organs to sample in order to achieve the highest probability of viral detection.

Keywords: *Acipenser gueldenstaedtii*, *Acipenser oxyrinchus*, disease control, freshwater aquaculture, vectors.

Introduction

Acipenseridae is one of the few living animal families which represent the remnants of prehistoric life on Earth as “living fossils”. As has been shown in genetic studies, a species which historically occurred in European waters was the Atlantic sturgeon (*Acipenser oxyrinchus*), whose current natural presence is limited to the St. John River in Canada. In the 19th century, sturgeons were subject to intensive economic exploitation, and as they occurred in high abundance, there was no reason to implement any protection measures. Unfortunately, excessive and uncontrolled catches along with overexploitation of the species as a source of valuable caviar caused a rapid decline in the population of Atlantic sturgeon in Poland. The last

individual caught in the Vistula River weighed 136 kg and measured 281 cm in length (Anonymous, 1965). Since 2004, work has been undertaken to restore the Baltic sturgeon species in Poland. The material used in restoration comprised fertilised eggs and hatchlings obtained from Atlantic sturgeon spawners from the St. John River and imported from Canada (13). Due to the increasing interest in rearing sturgeon of various species in Poland, the risk of penetration of species other than *A. oxyrinchus* into open waters is also increasing. The risks are not only those associated with competition for feed or breeding sites, but also that of the transmission of viral, bacterial, or parasitic diseases. According to Skóra (unpublished data), the occurrence of a foreign species in the environment carries a risk of transmission of parasites and pathogens, which may lead to a significant

decline in the abundance of the indigenous species. Cases such as the accidental transfer of approximately 50,000 individuals of the Siberian sturgeon (*A. baerii*) and Russian sturgeon (*A. gueldenstaedtii*) into the Wieprza River and Baltic Sea in August 2017 due to a water level rise in the Grabowa River which affected a farm in Jeżyczki (West Pomerania, Poland), also create a risk of occurrence of natural hybrids of *A. baerii* and *A. gueldenstaedtii* with *A. oxyrinchus*. This accident will nullify the restitution programme, following a long and laborious effort to restore the gene pool and a stable population of this species in the Polish waters, and will cause hybridisation. As part of the sturgeon health monitoring programme in natural waters and in fish stock farms, individuals representing *A. oxyrinchus* and *A. gueldenstaedtii* were subjected to a molecular analysis for the very dangerous and poorly detected viral infection caused by white sturgeon iridovirus (WSIV) in Poland. As a result of analyses carried out in the 1990s, WSIV was first isolated from white sturgeon (*A. transmontanus*) by Hedrick *et al.* (9) who had earlier conducted the molecular characterisation of the virus (8). With a size of 120–200 nm, the virus is replicated partly in the cytoplasm and its genetic material is double-stranded genomic DNA. Among the primary facilitators of the emergence of serious outbreaks of the disease are stressors such as high stock density, sudden changes in the environmental conditions (*e.g.* changing water temperature or oxygen content) or poorly selected feed (4). Unfortunately, horizontal transfer is not the only route that can lead to infection. There is a possibility of the virus being transmitted from adult individuals to offspring, *i.e.* by vertical transmission. To reduce or preferably eliminate this route of transmission, fish eggs are disinfected using iodine tincture (16). However, whether after such treatment fish viruses will be sufficiently degraded to prevent their further replication is a question current studies are still aiming to elucidate.

Stress in fish is one of the main factors with a significant influence on the rate of viral replication in the host cells, which is directly associated with the host's poor resistance (4). Considering cage cultures, stressogenic factors such as stock density in the cages or environmental conditions are also very important in determining the rate and degree of viral infection. These factors are of key importance in both vertical and horizontal transfer of WSIV. They also affect the degree of infection of healthy fish by vectors (the asymptomatic carriers) (5, 7). Therefore, good farming practices require that after a positive diagnosis of WSIV disease on the fish farm, the infected fish be immediately isolated, a thorough disinfection performed, and the stressor eliminated as much as possible (11, 16).

The first significant sign of a WSIV infection is cessation of feeding by the fish, which leads to the deterioration of their overall condition. It is hypothesised that it is due to the infection of the sensory epithelium in the olfactory organ (20). Another clinical

sign are oedematous and pale gills, which leads to problems with oxygen uptake. A characteristic but less common sign of infection is reddening along the ventral plates, which may subsequently turn into petechiae (15). For better understanding of the aetiology of WSIV infection, juvenile individuals of white sturgeon were experimentally infected by immersion in a suspension of viral particles to understand and assess the degree of infection of tissues. The results of the experiment confirmed that tissues in contact with water, such as the gills, skin, nostrils, barbels, and epithelium of the pectoral fins, are the most suitable for the detection of the virus (21). The presence of iridovirus, observed to date in white sturgeon, has also been confirmed in other species. In 1993, the virus reached northern Europe and decimated the local populations of *A. gueldenstaedtii* (1). To date, the occurrence of a virus similar to WSIV has been detected in shovelnose sturgeon (*Scaphirhynchus platorynchus*), pallid sturgeon (*Scaphirhynchus albus*), and sturgeons originating from the Missouri River (17).

The aim of this study was to develop the most effective diagnostic method that would allow a quick and efficient confirmation or exclusion of the presence of WSIV infection, not only in the internal organs for which the virus demonstrates tropism, but also in the skin or gills of fish (non-invasive methods). We tested and verified three diagnostic methods, assessing their sensitivity and effectiveness in the monitoring of WSIV infections.

Material and Methods

Samples. Sturgeon samples were obtained between 2010 and 2014 from open waters (Dąbie Lake and Szczecin Lagoon) and from fish farms located in Poland (Table 1).

Table 1. List of sturgeon species caught wild or obtained from a fish farm in Poland

Species	N	Open water	Intended for restocking (farmed)
		n	n
<i>Acipenser gueldenstaedtii</i>	2	1	1
<i>Acipenser oxyrinchus</i>	6	4	2

N = total number of individuals sampled; n = number of individuals sampled from the given origin

A total of 42 samples were collected from eight individuals aged between 0+ and 5+ (Table 2). Tissues including sections of the spleen, gills, intestine, skin, kidneys, and brain were extracted separately from each fish from the *A. gueldenstaedtii* and *A. oxyrinchus* species. The samples were kept at 4°C until arrival at the laboratory and then stored at –20°C or preserved in 70% isopropanol alcohol until analysis. Data concerning the

names and locations of the fish farms cannot be disclosed due to the confidentiality of such information.

Detection of WSIV using PCR. Total DNA from 42 collected fish samples was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. A polymerase chain reaction (PCR) assay was used for the detection of a DNA amplicon from the WSIV genome which encodes the putative major capsid protein (MCP) gene using the primers WS 229F/WS 245R and cycling conditions described by Kwak *et al.* (14). Based on thermal gradient PCR, a primer annealing temperature of 55°C was established. The 25 µL PCR mixtures contained REDTaq ReadyMix™ PCR Reaction Mix (Sigma-Aldrich, Taufkirchen, Germany), 0.2 mM of each primer pair and 1 µL of DNA template. In total, the PCR-based tests for the detection of WSIV were conducted for all samples. Subsequently, the obtained PCR products were resolved using gel electrophoresis and verified using comparison with a molecular weight standard (GeneRuler 100 bp Plus DNA ladder, Thermo Fisher Scientific, USWAQ, USA). Bidirectional sequencing of the obtained PCR products was ordered from Genomed (Warsaw, Poland). DNA sequence assembly and similarity analyses were completed using Geneious 8.0, BLAST, and MEGA5 software (2, 12, 19).

Detection of WSIV using real-time PCR. The primers and probe for the detection of 75 bp of the WSIV genome were designed based on the MCP consensus sequence obtained during the previous step. The real-time PCR was performed using 1 µL of DNA template, 5 µL of RT2xHS PCR MasterMix (A&A Biotechnology, Gdynia, Poland), 0.2 µL of 0.2 mM PHofFor (5'-CSTCAACCRCGTGCCRA-3'), 0.2 µL of 0.2 mM PHofRev (5'-TCCCYGATGCGGACAAGT-3'), 0.13 µL of 0.13 mM TaqMan probe (5'-6FA-CTTACTGGA YGCACCTATCTTCTCTBHQ-3'), and 3.47 µL of H₂O diethyl pyrocarbonate. The real-time PCR reaction was carried out on a Mastercycler ep realplex2 thermocycler (Eppendorf, Hamburg, Germany). The thermal profile consisted of polymerase activation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 61°C for 1 min. In each run, 42 samples were analysed including a positive and a negative control.

In situ hybridisation (ISH). During *in situ* hybridisation, a molecular probe and virus detection were conducted by PCR using a kit of reagents from Roche (Ritsch-Rotkreuz, Switzerland) and the Mastercycler thermal cycler in accordance with a certified methodology developed by the Institute of Infectology at the Friedrich-Loeffler-Institut in Riems (Germany). Pieces of the following tissues (total n=19) were formalin-fixed and paraffin-embedded according to standard protocols: spleen (n=4), gills (n=4), skin (n=4), intestine (n=3), brain (n=2), and kidney (n=2). Specimens of 5–7 µm were placed on Superfrost microscope slides (Microm International, Walldorf,

Germany) for 18 h at 62°C, dewaxed using 2 × Roti-Histol (Carl Roth, Karlsruhe, Germany) for 10 min and 2 × 100% ethanol for 10 min, and air-dried. The slides thus-prepared were framed with a PAP Pen (Merck, Darmstadt, Germany), treated with proteinase K (100 µg proteinase K mL⁻¹) in TE buffer (50 mM Tris, 10 mM EDTA) for 20 min at 37°C and fixed again with 95% ethanol followed by 100% ethanol, each for 1 min. After air-drying, the sections were framed with the PAP Pen again and, for equilibration, were covered by the hybridisation mixture (ISH-M) containing 4 × standard saline citrate (SSC), 50% formamide, 1 × Denhardt's reagent, 250 µg of yeast tRNA mL⁻¹ and 10% dextran sulphate, and incubated for 1 h at 42°C in a humid chamber. DIG-labelled probes prepared with the primer pair (14) were added to the sections and covered with a coverslip, placed on the *in-situ* plate of the Mastercycler, and heated to 95°C for 5 min for denaturation of DNA. Subsequently, the slides were cooled on ice for 2 min and incubated overnight at 42°C in a humid chamber for hybridisation. The following day, the coverslips were removed by washing in 2 × SSC twice for 10 min. For the removal of unspecifically bound probes, the slides were incubated in 0.4 × SSC at 42°C for 10 min. The sections were then counterstained with Bismarck -brown Y for sharpening and contrasting a possible positive signal, which becomes visible as violet-black foci in infected cells. Analysis of *in situ* hybridisation results was conducted using an Eclipse E-600 fluorescence microscope (Nikon, Tokyo, Japan) and finally arithmetic data were calculated using an MS Excel spreadsheet.

Results

In gross examination, all fish appeared to be healthy. Neither of the two species *A. gueldenstaedtii* or *A. oxyrinchus* showed clinical signs typical of a WSIV infection. Molecular analysis revealed a fragment of WSIV DNA in 42 organ samples (Table 2). In the majority of cases, fragments of WSIV DNA were found in the kidneys (six out of six samples), and liver, gills, and skin (six out of eight samples). Detailed information on the number of positive and negative results in different organs is given in Table 2. The most efficient method was real-time PCR, with 100% effectiveness in detection of WSIV DNA (Table 3). The detection rate using ISH was 47.37%. Most commonly, the virus was found in the brain (two out of two), kidney (two out of two), gill (two out of four), and skin (three out of four) tissues (Fig. 1). A clear cytopathic effect in the form of morphological changes was visible as “balloon-like” cells. Within these structures, hybridisation of the probe with WSIV DNA fragments occurred. The presence of the pathogen was also indicated by the specimen colour changing due to a reaction at the site of probe hybridisation with the defined fragment of the viral genome in the tissue.

Table 2. Summary of the results of WSIV DNA detection (positive isolates) using real-time PCR and conventional PCR in various sturgeon organs

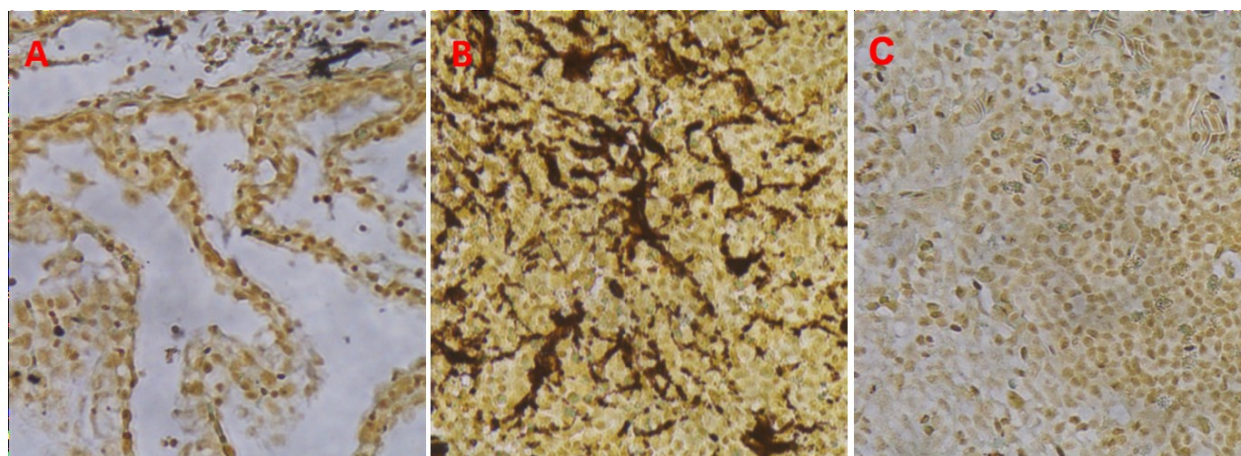
Species	N	Age (n)	Organ	Number of organs analysed	Method	
					Real-time PCR	PCR
Russian sturgeon, <i>Acipenser gueldenstaedtii</i>	2	0+ (1) 5+ (1)	Liver	2	2	2
			Gills	2	2	2
			Skin	2	2	2
			Kidneys	1	1	1
			Gut	2	2	2
			Brain	NA	NA	NA
Atlantic sturgeon, <i>Acipenser oxyrinchus</i>	6	0+ (2) 1+ (3) 2+ (1)	Liver	6	6	4
			Gills	6	6	4
			Skin	6	6	4
			Kidneys	5	5	5
			Gut	5	5	3
			Brain	5	5	3

N = number of individuals studied; n = number of individuals of a certain age; NA = not applicable

Table 3. Summary of the results of WSIV detection in fish tested using PCR and real-time PCR

Species	N	Positive isolates			
		PCR		Real-time PCR	
		n	(%)	n	(%)
<i>Acipenser gueldenstaedtii</i>	2	9	100	9	100
<i>Acipenser oxyrinchus</i>	6	25	75.75	33	100

N = total number of individuals studied; n = number of positive isolates

**Fig. 1.** Tissues of *Acipenser oxyrinchus* infected with WSIV. A – gills; B – kidney; C – negative control

Discussion

Molecular diagnosis offers the possibility of fast and effective intervention in the event of a positive result, but it also poses the risk that its negative results may be false when the testing method used has a low threshold of sensitivity. Therefore, the assessment of the applicability of common diagnostic methods, such as PCR, real-time PCR or *in situ* hybridisation, is an extremely important element of molecular analysis. All these methods are based on the principle of detection and characterisation of a fragment of viral genomic DNA and, in particular cases, sequencing of the obtained DNA

fragments. The last procedure can be used not only to determine the reference sequence of viral DNA, but also to track the evolution of each virus. Such advanced methods of molecular analysis allow diagnosing cases of viral infections, as well as maintaining surveillance of the rise of new virus isolates in the given environment which may differ in their degree of virulence. For this reason, intense research on the biology and evolution of pathogenic viruses in fish has been conducted, which includes testing of the available experimental methods. In a report on the diagnosis of an iridovirus infection in *A. transmontanus* in British Columbia, Stephen pointed to juvenile individuals as asymptomatic carriers and the

possibility of detection using microscopic analysis (18). A US team of researchers demonstrated the effectiveness of an invasive diagnostic method for the detection of WSIV (6). They compared methods of detection of WSIV in *A. transmontanus* based on DNA extracted from the pectoral fin (*in vivo*) and brain (post-mortem). The results showed no differences between the methods. A similar study was conducted in five *Acipenseridae* species originating from farms in Poland, Germany, and Italy (10). However, in this case, the authors established that detection of WSIV was the most effective in material derived from the kidneys (97.9%), gut (97.5%), skin (94.9%), and gills (93.2%). The most efficient method was real-time PCR followed by standard PCR and *in situ* hybridisation (49.2%).

Due to the potential risk of WSIV infections in Polish fish farms, in which sturgeon may not be the leading species but is often present as an accompanying one, it is indicated to conduct routine diagnosis of it. This is of particular importance in the trade of live sturgeons not only for consumption but in particular as spawners for reproduction and the subsequent stocking of open waters. The diagnostic methods mentioned in this article demonstrate in both in wild and farmed-for-stock sturgeons that these fish are potential vectors likely to contribute to the spread of WSIV in open waters. According to US researchers (7), there are potential differences in the resistance to the virus between individual populations of *A. transmontanus*. For this reason, the authors suggest exclusion from spawning of those individuals which have given offspring susceptible to WSIV infections. Following this suggestion in the management of sturgeon in hatcheries could potentially increase protection of the entire *A. transmontanus* population against WSIV-induced kills. Watson *et al.* (20, 21) paid special attention to the role of water temperature in their experiment involving a controlled infection of juvenile *A. transmontanus* individuals with WSIV. Chronic emaciation of the fish at 10°C was due to the more severe and extensive clinical changes in the mucosa of the olfactory organ of the fish used in the experiment. The lack of clinical signs in WSIV carriers was confirmed in a study by Watson *et al.* (20, 21). Four days after the experimental infection of white sturgeon with WSIV, the authors observed microscopic lesions in tissues, despite the absence of any systemic pathological changes. Arun *et al.* (3) considered viral diseases of fish as one of the main factors putting at risk the sustainable management of aquaculture globally. They indicate the potentially expensive but often only effective solution to be vaccination against viral diseases. These diseases are responsible not only for serious economic losses on fish farms, but also for the introduction of the virus into new environments *via* trade in live fish without virological monitoring. Our study proposes a ready-to-use methodology for veterinary administrative services responsible for fish health protection, and this methodology is predicated on the need clear from the considerations above. Moreover, the study demonstrates

the capabilities for WSIV diagnosis available to fish farmers and water administrators, indicating useful methods of adequate sensitivity, as well as the organs to sample in order to achieve the highest probability of detection of the virus in sturgeons.

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