Isolation and characterization of an atypical Siberian sturgeon herpesvirus (SbSHV) strain in Russia: novel North-American *Acipenserid herpesvirus 2* strain in Europe?

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Abstract

Siberian sturgeon herpesvirus (SbSHV) was isolated in Russia for the first time in 2006. Nine SbSHV isolates were recovered from different fish hatcheries producing the same CPE in cell cultures, the same clinical signs and mortality kinetics in virus-infected fish, the same virus neutralization pattern, and shared identical nucleotide sequences. In 2011 a new isolate was recovered from juvenile sturgeon, which caused completely different CPE. That isolate was not readily neutralized by Siberian sturgeon hyperimmune antisera and its DNA was not recognized by the routine PCR developed for SbSHV detection. Molecular study of the novel isolate revealed that it was more closely related to North-American Acipenserid herpesvirus 2 (AciHV-2) isolates from white sturgeon, while the genome sequences of the former SbSHV isolates showed high similarity to the AciHV-2 isolated from shortnose sturgeon. While clinical signs and mortality caused by the novel isolate in infected Siberian sturgeon were similar to those of the formerly described SbSHV isolates, the incubation period and mean time to death produced by the novel isolate were twice as long. The differences between the former isolates and the recent one suggest that a novel SbSHV strain emerged in Europe and the molecular findings imply its North-American origin.

1. Introduction

Siberian sturgeon herpesvirus (SbSHV) was discovered in 2006 in moribund fingerling Siberian sturgeon (*Acipenser baeri*) which experienced an acute outbreak of disease in a sturgeon hatchery located in the Central European part of Russia (Shchelkunov et al., 2009).

Since then, it has been found widespread in cultured sturgeon species in Russia. Two isolates were recovered from Kazakhstan and Finland (Shchelkunov & Shchelkunova, unpublished). SbSHV is the cause of an acute necro-haemorrhagic skin syndrome complicated by secondary opportunistic infections (fungal, myxobacterial, or protozoan) (Shchelkunov et al., 2009). Partial sequence analysis of the viral genome determined that the SbSHV was a potential member of the genus *Ictalurivirus* within the family *Alloherpesviridae* under the order *Herpesvirales* (Doszpoly and Shchelkunov, 2010). It was also hypothesized that the SbSHV is probably not a new virus species, but a novel genotype virus of the species *Acipenserid herpesvirus 2* (AciHV-2) (Doszpoly and Shchelkunov, 2010). Up to now, four AciHV-2 isolates from North-America have been described and partially sequenced (Kelley et al., 2005; Kurobe et al., 2008). The SbSHV is most closely related to the SSHV-99-CAN strain isolated from shortnose sturgeon (*Acipenser brevirostrum*) in Canada. Between 2006 and 2011, nine SbSHV isolates were recovered from different parts of Russia, and all of them closely resembled the first isolate SK1/0406 in their properties (Shchelkunov & Shchelkunova unpublished). In 2011 a novel isolate (SIz6/0311) was recovered from Siberian sturgeon fingerlings. That isolate caused CPE which was markedly different from that produced by the formerly described SbSHV isolates. In this study, we provide biological and molecular genetic analysis to differentiate between this newly revealed SbSHV isolate and the earlier isolates of the virus.

2. Material and methods

2.1. Virus isolation and DNA extraction. A viral isolate (SIz6/0311) was obtained from a disease outbreak in Siberian sturgeon fingerlings reared in a fish farm in Izhevsk (near the Ural mountains, Russia). The fish showed typical signs of the chronic

SbSHV disease. Virus isolation technique was described in detail elsewhere (Shchelkunov et al., 2009). For virus isolation, tissue from the siphon of the diseased fish was used. Virus was propagated in white sturgeon spleen (WSS-2) (Hedrick et al., 1991) and Siberian sturgeon pooled liver/spleen/kidney (SSO-2) (Shchelkunov et al., 1997) cell lines.

After successful propagation of the virus, viral DNA was extracted from 100 µl cell culture supernatant by using a commercial kit (Central Research Institute of Epidemiology, Moscow). The extracted DNA was placed on Whatman filter paper and thus transported to the molecular biology laboratory where it was eluted with 250 µl nuclease free water (after having been soaked at 4°C for 3 hours). Subsequently, the samples were concentrated to a volume of 50 µl in a vacuum centrifuge (Speed-Vac) and stored at -20°C until further use.

2.2. PCR and sequencing. For routine disease diagnostics and identification of SbSHV, a conventional PCR was used, based on a primer pair (Table 1) complementary to sequences of the DNA polymerase gene of SK1/0406 strain presented in the GenBank (Acc. No.: GU253908.1), with the following program: 95°C for 2 min – 1 cycle; 94°C for 20 s, 55°C for 30 s and 72°C for 40 s – 30 cycles. Since this PCR failed to detect the DNA from the recent virus isolate, another PCR was chosen. This PCR (primers in Table 1) was developed for detection of ictaluriviruses (Ictalurid herpesvirus 1 and 2, and Acipenserid herpesvirus 2) (Doszpoly and Shchelkunov, 2010). This PCR was successful, which suggested that the recent isolate (SIz6/0311) was an ictalurivirus, so more detailed molecular characterization was carried out. Different parts of the genome were selected for amplification and comparison with the reference SK1/0406 strain (hereafter type I SbSHV). A 7 kb long region was amplified between the DNA polymerase gene (ORF57) and the first exon of the ATPase subunit of terminase (ORF62) as described previously (Table 1) (Doszpoly and Shchelkunov,

2010). Sequence for this region was already available from the type I SbSHV (Doszpoly and Shchelkunov, 2010).

Additional genes were amplified and sequenced from both type I and type II (SIz6/0311) SbSHV for a more comprehensive comparison. These genes were the ORF39 (major capsid protein) and ORF46 (membrane glycoprotein). The primers (Table 1) were designed using the sequences of the AciHV-2 isolates (Doszpoly et al., 2011b). PCRs were carried out in 50 µl final reaction volume. The reaction mixture consisted of 34 µl distilled water, 10 µl of 5×HF buffer (Phusion), 0.5 µl thermo-stable DNA polymerase enzyme (Phusion), 1 µl (10 µM) of each (forward and reverse) primer, 1.5 µl of dNTP solution of 10 mM concentration (Phusion), and 2 µl target DNA. The following programs were used: initial denaturation at 98°C for 5 min, followed by 45 cycles of denaturation at 98°C for 10 s, annealing at 56°C for 30 s, and elongation at 72°C for 1 min/1000 bp. The final extension was performed at 72°C for 5 min. After electrophoresis, the PCR products were cut out from 1% agarose gels, and were purified with the QIAquick Gel Extraction Kit (Qiagen). The amplification products were cloned into plasmid using the CloneJet PCR Cloning Kit (Fermentas), according to the protocol of the manufacturer. The plasmid containing the amplified target was sequenced with pJETfo and pJETre primers (Fermentas), and then primer walking was applied. The sequencing reactions were performed with the use of the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem). Sequencing electrophoresis was carried out in an ABI 3100 Automated Capillary DNA Sequencer.

2.3. Production of hyperimmune antisera and serum neutralization test. Serological identification of the isolate SIz6/0311 was performed in serum neutralization test (SNT) with antisera raised in Siberian sturgeon juveniles hyperimmunized with the reference strain SK1/0406 as described before (Shchelkunov and Prokaeva, 2009). Briefly, eleven

individual 2 yr old Sberian sturgeon (approximate body weight of 350 – 400 g) were infected with SbSHV using the water bath method. For this, the fish were accommodated in a basin with 40L of aerated water at 15 – 17°C. The viral strain SK1/0406, isolated from integumental tissues of naturally diseased fingerling Siberian sturgeon, and grown in the SSO-2 cell line, was added to a final concentration of $10^{3.85}TCID_{50}$/ml water. After one hour exposure, the fish were transferred to a 250L basin with flow through water at 15 – 17°C and regularly fed with specialized commercial pelleted feed. In few weeks the fish showed clinical signs of herpesviral disease and cumulative mortality approached 36.4%.

At 2.5 months after the challenge, when surviving fish had recovered, the five strongest and healthiest individuals were selected for further hyperimmunization. The water temperature was gradually increased from 15°C to 19 – 20°C. At 113 days post challenge first sera were sampled from fish for virus neutralizing antibodies (NA). For this, each individual fish was marked and blood samples were taken from the caudal vein under MS-222 (85 – 100 mg/l) anesthesia. The blood was allowed to clot at 20°C for one hour and the clots retracted overnight at 4°C. The serum was separated by low speed centrifugation, diluted two-fold, aliquoted, thermoinactivated at 45°C for 30 min (Watson et al., 1995) and kept frozen at – 18°C before use. Preimmune sera were also sampled and processed in the same way.

At the day of blood sampling the first reimmunization was performed. For this purpose, the water temperature was lowered to 15°C and $6x10^{4.85}TCID_{50}$ of virus grown in the white sturgeon skin (WSSK-1) cell line (Hedrick et al., 1991) was injected i.p. to each anesthetized fish. In 3 weeks the water temperature was increased to 19 – 20°C and the fish were sampled for sera for the second time one month after virus injection followed by repeated sampling every 2 – 3 weeks. Second reimmunization was done in a similar way one year after the water bath infection, at which time the number of selected fish was reduced to two.

150 The serum neutralization test was performed according to established protocol with
151 constant virus working dose of 32 $TCID_{50}$ per 96-well microplate well (Wizigmann 1980).
152 Serial 2-fold antiserum dilutions were used to determine the antibody titre, or with constant
153 antiserum working dose at dilutions of 1:50 or 1:100, depending on the serum antibody titre,
154 to produce about 20 neutralizing units/well. At this case, serial 10-fold virus dilutions were
155 used to calculate the neutralization index (NI). The preimmune Siberian sturgeon sera at the
156 same dilution were used as a negative antibody control. Virus and serum dilutions were
157 incubated at 21.5^{o}C for 1 hr, then transferred to the 96-well microplates with WSS-2 cells and
158 incubated further at 15^{o}C for 10 – 15 days with daily checks for CPE. NI values were
159 calculated and interpreted as follows for each virus and sera used:
160 NI = virus titre with normal serum / virus titre with hyperimmune serum,
161 where $\leq$ 10 = negative NI, 11 – 49 = equivocal NI, $\geq$ 50 = positive NI.
162
163 **2.4.** ***In vivo*** **experiments.** To determine if virulence of the SbSHV types I and II
164 differed, and if there were fish host age dependent virulence patterns, virus challenges of fish
165 were performed. Healthy Siberian sturgeon of various ages were obtained from a virus free
166 sturgeon farm. Two- and 3-month-old fingerlings and 2-years-old sturgeon were infected with
167 the reference strain SK1/0406 (type I SbSHV) using one hour long bath (about 10^4 $TCID_{50}$/ml
168 water, 14 – 15^{o}C). Four-month old sturgeon fingerlings were infected in a similar way using
169 the isolate SIz6/0311 (type II SbSHV). After exposure, the fish were transferred to 120L
170 aquaria with flow-through aerated water of 14 to 17°C, in which they were held and regularly
171 fed with commercial pelleted feed. Clinical signs and mortality was monitored daily until
172 100% mortality occurred or complete recovery of remaining fish was achieved. The number
173 and mean body weight of infected fish in the tests were as follows: 2-month-old: 48, 12.5 g;

3-month-old: 17, 15.0 g; 2-year-old: 11, 350 g; 4-month-old: 42, 17.5 g. In total 118 fish were infected. Each test was replicated only once.

The same numbers of negative control fish of the same age were similarly mock-infected with virus-free cell culture liquid. After a test was completed, daily mortality data was plotted as cumulative mortality curves, and survival analysis was performed using the Kaplan-Meier (KM) method. Signficant differences among the probability of survival of pertinent pairs of experiments were conducted using both log rank and Wilcoxon tests. The mean time to death (MTD) was calculated for each group of virus infected fish.

3. Results

3.1. CPE-based discrimination between the two viruses. The WSS-2 cell line used for virus isolation was found to be the most susceptible to both SbSHV types (Shchelkunov & Shchelkunova, unpublished). After the cells were inoculated with the reference strain SK1/0406 or other closely related virus isolates, large syncytia could be easily recognized, harboring dozens or hundreds of nuclei. In contrast, the same cell line inoculated with the isolate SIz6/0311 showed completely different CPE, which consisted of granulation and vacuolization of cell cytoplasm, and diffuse rounding of cells. This pattern of CPE was readily reproduced in serial virus passages in WSS-2 cells. No CPE was observed in uninfected cell culture (Figure 1).

3.2. PCR and sequencing. The diagnostic PCR specific for the type I SbSHV produced the predicted 635 bp long amplicon in all type I isolates, however, no product was found with SIz6/0311 DNA sample. The consensus PCR for the ictaluriviruses (Doszpoly and Shchelkunov, 2010) gave positive result (277 bp long fragment, data not shown) using the

199 SIz6/0311 DNA sample as a target. By sequencing this fragment it was found that the latter
200 was definitely an alloherpesvirus (ictalurivirus) showing high similarity (93%) to the North-
201 American AciHV-2 SRWSHV (Idaho) isolate from Snake River white sturgeon.
202 Subsequently, longer DNA sections of the viral genome were amplified and sequenced. The
203 region between the DNA polymerase and terminase genes is 7048 bp long in the genome of
204 type I SbSHV (Doszpoly and Shchelkunov, 2010). However, it was found to be 7058 bp long
205 in the type II SbSHV isolate. The amplified genome region containing the major capsid
206 protein (ORF39) was 3726 and 3864 bp long in the SbSHV type I and type II, respectively,
207 while a 472 bp long intergenic fragment between ORF 44 and ORF 83 was deleted from type
208 I SbSHV genome. The G+C content of the sequenced genome segments proved to be 38.8%
209 and 38.5% in type I and type II virus, respectively. The sequences were deposited to GenBank
210 (Acc. No.: KT183703-KT183707). Results of the overall nucleotide comparison (ORF38-40;
211 ORF44-47 and ORF57-62 regions with 15kb long sequences) of the two types of SbSHV and
212 the SRWSHV isolates are as follows: type I shows 86% similarity to both type II and the
213 SRWSHV, while the type II shows 92% similarity to the North-American SRWSHV strain.
214 There is a 44 aa long deletion in the major capsid protein (from 547 aa to 596) of the type I
215 SbSHV compared to type II SbSHV or the SRWSHV isolate (Figure 2). At the same time, the
216 ORF83 is longer by 90 bp in type I SbSHV compared to the other two viruses.
217
218 **3.3. Siberian sturgeon hyperimmune antisera and SbSHV identification in**
219 **serum neutralization test.** No virus neutralizing antibodies were found in fish sera before
220 infection (the antibody detection threshold = 1:8), while high antibody titres were detected in
221 the survivors. At day 113 post-infection neutralizing titres varied between 1:600 – 1:3000.
222 The first reimmunization increased antibody titres in 3 out of 5 fish, which persisted for as
223 long as about 5 months and dropped down 3- to 4-fold one month later – to levels prior to

224 reimmunization. Within that period the maximal antibody titres (approximately 1:3500 –
225 1:4500) were found at the day 63 post reimmunization. The second reimmunization carried
226 out at about one year post infection (p.i.) brought the antibody titre in one of the fish up to its
227 highest level of 1:5400 forty days later. That serum, selected as a reference antiserum specific
228 to SbSHV strain SK1/0406, was successfully used later on for serological identification of
229 new field virus isolates. Other antisera to the same virus produced according to the above
230 mentioned protocol were also used in the study when needed.

231 When a routine SNT was used with the reference antiserum, positive neutralization
232 index was obtained (1585) clearly demonstrating that the isolate SIz6/0311 belonged to the
233 SbSHV group. However, results of neutralization were more variable when that isolate was
234 compared with the reference strain SK1/0406 by using three different hyperimmune antisera.
235 In particular, while NIs for the homologous virus were found to be strongly positive with each
236 of the three antisera used (from 10000 to $\geq$17783), the same antisera neutralized the isolate
237 SIz6/0311 not so readily showing clear individual neutralization patterns with a range of NIs
238 varying from positive value of 1585 (antiserum №1) through moderately positive 158
239 (antiserum №2) down to weakly positive 89 (antiserum №3) (Table 2).

240

241 **3.4. Comparison of the two virus types in pathogenicity to Siberian sturgeon.**
242 No signs of disease and mortality were observed in negative control groups of fish. In
243 SK1/0406 infected two- and three-month-old Siberian sturgeon fingerlings, the disease
244 developed as typical integumental necro-haemorrhagic syndrome described for this virus in
245 detail earlier (Shchelkunov et al., 2009). It started with lethargy and anorexia 7 – 10 days. p.i.
246 The affected fish became emaciated and pale. An attributive sign of the disease was multiple
247 small (1 – 4 mm in diameter) smoky-bluish semi-translucent raised plaques of hyperplastic
248 epidermis scattered all over the body surface. Those plaques were transient and necrotized in

249 a few days. Progressing fin necrosis developed starting from the distal ends. One – two days
250 before death, areas of hyperemia and petechial haemorrhages appeared on the skin,
251 particularly around the mouth, ventral part of rostrum, fin bases, below eyes, as well as other
252 locations. Many moribund animals showed mottled or camouflage-like skin appearance. In
253 fingerling fish the infection was acute and cumulative mortality usually approached 100% in
254 2 – 3 weeks p.i.

255 In 2-year-old Siberian sturgeon, the incubation period was about 25 days. A distinctive
256 clinical sign of the disease at that age of fish was the extensive haemorrhagic ulceration of
257 scutes and skin areas of various locations, but predominantly on the caudal trunk. The disease
258 broke out and ceased within 8 days showing cumulative mortality of about 36%.

259 At necropsy, virus infected fingerlings had an overall paleness of internal organs and
260 the liver was almost white in colour. The hind gut often showed signs of haemorrhagic
261 inflamation. In 2-year-old fish, the liver was unevenly coloured, the heart was knobby, flaccid
262 and mottled and the swim bladder enlarged.

263 The clinical signs of disease in 4-month-old Siberian sturgeon exposed to isolate
264 SIz6/0311 were essentially the same as those observed in SK1/0406 infected fish. The signs
265 appeared 32 days p.i. and ceased a few days before the experiment was terminated. The fish
266 began to die 38 days p.i. and finished 52 days p.i. with a final cumulative mortality of 93%.
267 The mean time to death calculated for the reference isolate SK1/0406 infected fish were as
268 follows: 10.6, 16.2, and 31.5 days in 2-month-old, 3-month-old and 2-year-old fish,
269 respectively, while that in SIz6/0311 infected 4-month-old fish was found to be 42.5 days.

270 The principal *in vivo* difference between the two isolates was the substantially
271 prolonged incubation period and MTD in virus-infected host found for the isolate SIz6/0311
272 in comparison with those of SK1/0406. Those were at least twice as long as observed in 2-

273 month-old or 3-month-old fish group mortality data. In contrast, the course of mortality from
274 both isolates was equally acute (the slope of the curves) and high (Figure 3).
275 The daily probability of surviving infection with the reference SK1/0406 isolate
276 indicated that there was a significant difference among all age classes of fish, wherein
277 survival appeared to increase/improve with age. Comparison of the two different isolates -
278 SK1/0406 and SIz6/0311 - in the closest age-matched groups of fish showed that survivorship
279 was delayed but not improved in the SIz6/0311 exposed fish. Due to the difference in
280 kinetics, the two viruses survivorship was different, but the final fraction of surviving animals
281 was not different. These findings were strongly supported by low p-values obtained with two
282 different statistical methods used (Figure 4, Table 3).
283
284 **4. Discussion**
285 During surveillance of sturgeon farms, two types of SbSHV were isolated, which
286 differed from each other in phenotypic and genotypic features. The major type I SbSHV,
287 represented by the isolate SK1/0406, was found to be widely distributed in European and
288 Asian parts of Russia and also in Kazakhstan. The minor type II SbSHV, represented by the
289 isolate SIz6/0311, was found so far in one Russian fish farm only. Both of the isolates were
290 found to be genetically related to the AciHV-2 species from the North America.
291 Several studies have been reported on the molecular comparison of different AciHV-2
292 isolates (Kelley et al., 2005; Doszpoly et al., 2008; Kurobe et al., 2008; Waltzek et al., 2009;
293 Doszpoly and Shchelkunov, 2010; Doszpoly et al., 2011a; Doszpoly et al., 2011b). However,
294 only one paper was published in the mid-1990s which involved serological differentiation of
295 the first acipenserid herpesviruses 1 and 2 isolated from white sturgeon (Watson et al., 1995).
296 Here we report on cell culture, genetic, *in vitro* serum neutralization, and *in vivo* experimental

297 infection data to characterize and discriminate between two different types of Siberian
298 sturgeon herpesvirus found in Russia.
299 Since the routine diagnostic PCR developed to detect and identify the type I SbSHV
300 failed to recognize the type II virus, new primers were designed to amplify and sequence new
301 genome regions of the latter. The sequenced genome fragments of the type II SbSHV showed
302 similar gene arrangement and 86 and 92% nt sequence homology to the type I SbSHV
303 (Doszpoly and Shchelkunov, 2010) and the AciHV-2 SRWSHV isolate (Doszpoly et al.,
304 2011b), respectively. Basically, genome organization of the sequenced regions of type II
305 SbSHV and the other Russian and American isolates are similar with discrepancies between
306 them: 1) a 50 amino acid gap~~deletion~~ was found in the major capsid protein of the type I
307 SbSHV (Figure 2); 2) ORF83 is 90 bp longer in type I SbSHV genome compared to that of
308 the other two viruses; and 3) the 472 bp long intergenic region between ORF44 and ORF83 is
309 missing~~deleted~~ from type I SbSHV. Interestingly, the type II SbSHV shows higher similarity
310 to the American AciHV-2 (California, Idaho, Oregon) isolates from white sturgeon than to the
311 Russian type I SbSHV. At the same time, type I SbSHV has 98% nucleotide identity (within
312 the 8 kb region between DNA polymerase and terminase genes) with the Canadian AciHV-2
313 strain (Doszpoly and Waltzek unpublished) isolated from shortnose sturgeon (LaPatra et al.,
314 2014).
315 Genomic peculiarities were not the only differentiating features of the two SbSHV
316 types. Their biological properties *in vitro* and *in vivo* were also different. Using serum
317 neutralization test with reference Siberian sturgeon hyperimmune antisera to type I SbSHV, it
318 was shown that all the virus isolates, independent of their types, belong to a single SbSHV
319 group. However, testing three different high titre type I SbSHV antisera against type II
320 SbSHV showed distinct neutralization patterns, with neutralization indices varying from
321 simply positive through moderately positive to weakly positive. These results suggest that

additional individual antisera might show negative NIs. We believe that in general little attention has been paid to this phenomenon in virology literature. Its mechanism may lie in different accessibility of neutralizing virus epitopes to the respective immunocompetent cells of individual animals. Taking this into account, we suggest that when low NIs are produced in serological virus identification work, more individual antisera should be tested before making the final conclusion about establishing a different serotype or finding a distinct virus.

In general virology as well as in fish virology, the character of CPE in a cell line has been usually considered a rather virus-specific feature, which was used for a tentative identification of isolated virus (Wolf, 1988). Two different types of CPE in the same cell line in different isolates of the same virus is a surprising event, at least in fish virology.

It is well known from general biology of herpesviruses that the two above mentioned phenomena (virus neutralization and type of cell pathology) are both mediated by viral glycoproteins, which form peplomers (or spikes) on the surface of the viral envelope. Herpesviruses possess 10 or more different glycoproteins whose functions are not yet well understood. Besides host cell specificity, which is also glycoprotein-mediated, these are also essential for herpesvirus infectivity and production of neutralizing antibodies by host. Thus, changes in glycoprotein genes might compromise manifestation of these phenomena. Based on this presumption, we believe that the present study suggests that unidentified differences exist in one or more glycoprotein genes of type II SbSHV relative to type I. This working hypothesis needs further experimental verification.

Also, fusion of herpesviral envelope with cell plasma membrane is initiated by attachment of viral glycoproteins to specific cell surface located receptors is a known early event in herpesvirus infection, as well as a trigger of syncytia formation. Since type II SbSHV is unable to trigger syncitia formation but still replicates and causes disease, fusion and cell entry may be mediated in a different way relative to type I. Recent publications suggest two

alternative modes of entry in the cell observed in herpesviruses: 1) by the above mentioned pH-independent membrane fusion or 2) alternatively, via endocytic pathway that may be phagocytosis-like (Akhtar and Shukla, 2009). Both of these are triggered by glycoprotein binding and finish with viral DNA released in cell cytoplasm, either directly or via endocytic vesicles. We believe that the "granulation and vacuolization of cell cytoplasm" observed in WSS-2 cells infected with type II SbSHV may be such endocytic vesicles. This may provide another example of herpesviruses using alternate modes of cell entry to secure their replication.

When proceeding to work on *in vivo* characterization of SbSHV, two tasks were set. First, to establish the age-dependent mortality/survival pattern the reference strain produces in its principal host – the juvenile Siberian sturgeon. Second, to determine the difference (if any) between the two virus types in experimentally infected host mortality/ survival kinetics. Since no fish of different age could be available from the same fish farm at the same time, experimental virus challenges were performed in different time and then combined and analyzed. Survival analysis clearly showed that survivorship was significantly different among all age classes of fish infected with the reference SK1/0406 isolate and that survival appeared to increase with age. Comparison of the two different isolates – SK1/0406 and SIz6/0311 - in the closest age-matched groups of fish showed that survival appeared delayed but not improved in the SIz6/0311 exposed fish of similar age. However, using different fish stocks to compare mortality patterns is not reliable enough. A direct comparison would require that the challenges with the two viruses be done on the same stock of fish and the same age. There may be differences in nutritional status, life history or subclinical infections that may cause a different response. So, unless they were the same stock of fish or more challenges were done with the type II virus, in-depth evaluation is not valid. At the same time,

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we believe that these data may be still presented here at least as observations if not as those with statistical significance implied.

Mortality manifested as: a) a short period between the onset and the end of outbreaks (acute disease course converting to more chronic one in older fish); b) high cumulative mortality (90 – 100%) in young fish decreasing with age; c) short incubation periods, and MTD to increased with fish age. Both types of SbSHV were highly pathogenic to their hosts and caused the same clinical signs of Siberian sturgeon herpesviral disease.

The mechanisms behind substantial increase of incubation period and MTD in type II vs type I SbSHV infected fish are not clearly understood and need further investigation. It is interesting that considerable delay in disease manifestation did not actually facilitate development of protection in fish against the virus challenge.

Acipenserid herpesvirus - 2 (AciHV-2) was discovered in North-America in the mid-1990s (Watson et al., 1995). In Europe (Russia) a closely related virus was found for the first time about 12 years later (Shchelkunov et al., 1997), suggesting that the Russian isolates may have originated from North America. This study shows that two Russian types of SbSHV (I and II) differ from each other in four principal marker traits and each of the two has close genetic relationship with one or another strain of North American AciHV-2 species. It is possible that two direct or indirect introductions of this virus to Russia occurred. A Canadian strain from shortnose sturgeon shows very high genetic similarity to type I SbSHV (reference isolate SK1/0406) and type II SbSHV (reference isolate SIz6/0311) from white sturgeon may be a novel AciHV-2 strain. Interestingly, these fish species have different natural origins. The shortnose sturgeon's range is located in eastern North America, while that of the Siberian sturgeon is the Asian part of Russia (Siberia). So, the latter host does not have natural contact with the North-American host species, and to the best of our knowledge there has been no official direct trade in live sturgeon between North American countries and Russia, at least

within the last fifteen years. For the sake of completeness we need to mention that these findings did not exclude the possibility that these viruses had originated from Eurasia and were introduced to North America, only they were discovered a decade later.

Taking into consideration the data from cell culture, viral genetics, serum neutralization, and in vivo experiments, the authors believe that the Russian type I and type II SbSHV may represent two different strains or genotypes of the *Acipenserid herpesvirus 2* species.

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6. Compliance with Ethical Standards

All animal procedures were approved by the Russian Agricultural Academy Procedures and Ethics Committee and performed in strict accordance with Russian Federation Home Office guidelines. All efforts were made to minimize suffering of experimental animals.

7. Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Two different types of cytopathic effect of SbSHV in WSS-2 cell line caused by the isolates SK1/0406 (A) and SIz6/0311 (B). A: extensively large syncytia B: granulation and vacuolization of cell cytoplasm, diffuse rounding of cells. C: uninfected cell line WSS-2. Unstained preparations (100x).

Figure 2. Amino acid alignment of the capsid proteins (partial) of the type I and II SbSHV and the North-American AciHV-2 isolate SRWSHV. There is a 50 amino acid deletion in the type I SbSHV protein.

Figure 3. Mortality kinetics in juvenile Siberian sturgeon of four different ages infected with SbSHV isolates SK1/0406 or SIz6/0311 by 1-hr-long water bath (approximately 10^4 $TCID_{50}$/ml) and further held at 14 – 17^{o}C.

Figure 4. Kaplan-Meier survivorship. Survivorship, or the estimate of instantaneous probability of survival, was calculated for each experimental challenge. The three experiments with SK1/0406 virus in 2-month-old (red), 3-month-old (purple), or 2-year-old (black) fish are shown at left. The two age-matched fish groups exposed to either SK1/0406 (purple) or Slx6/0311 (black) virus are shown at right. Dashed lines are 95% confidence interval (CI) around solid line estimate of instantaneous probability of survival. Tests for significant differences between survivorship among all three age classes of fish challenged with SK1/0406 virus, and the two closest age-matched fish challenged with either SK1/0406 or

Slx6/0311 virus, p-values from Log-rank and Wilcoxon tests (see Table 3). Analyses performed in R statistical software, using survival package.

Table 1. The primers used in the PCRs. Ambiguities are marked with the code recommended by IUPAC.

Table. 2. The difference between two SbSHV isolates in neutralization by three individual hyperimmune Siberian sturgeon antisera to reference type I SbSHV isolate SK1/0406.
NI – neutralization index.

Table 3. Tests for significant difference between pairs of survivorship functions, p-value of difference from either Log rank test (top) or Wilcoxon test (bottom). Comparisons in grey cells represent tests comparing either the same experiment or comparisons described in the lower half of the matrix and were not tested. n.a. 'not applicable'. Only the two closest age-matched fish challenged with two viruses were compared.