First detection of anguillid herpesvirus 1 (AngHV1) in European eel (*Anguilla anguilla*) and imported American eel (*Anguilla rostrata*) in Poland

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Abstract

Herpesvirus anguillae (HVA, AngHV1) belongs to the biggest group of DNA viruses known to induce disease in eel and poses a real threat to both farmed and wild eel species. Mortality rates in eels caused by AngHV1 range from 1% to 10%, and may increase up to 50% under stressful conditions. Since 2007, the European eel has been placed in the CITES list, and restoration of its population has been conducted, among other methods, by active restocking and introducing protective fishing regulations. The aim of the present work was to detect AngHV1 in eel and to evaluate the risk connected with its presence. This study was based on 60 specimens belonging to the genus *Anguilla* (European eel n = 50, Japanese eel n = 8 and American eel n = 2), collected from north-western Polish waters (Dąbie Lake, Szczecin Lagoon), China (food product for direct human consumption) and Denmark (breeding material). It is the first detection of AngHV1 in eel (*Anguilla anguilla*) in the Polish territory and in American eels (*A. rostrata*) from China at Polish fish processing plants. However, in imported Japanese eels (*A. japonica*) from China, AngHV1 was not detected.

Introduction

Herpesvirus anguilla (AngHV1) was first observed in skin lesions of the European eel (*Anguilla anguilla*) by electron microscopy (Békési et al., 1986). Its detailed systematic infection was established by Sano et al. (1990), who conducted studies on two eel species: *A. anguilla* and *A. japonica*. According to Ueno et al. (1992), the morphology of the virus was always identical and did not show any modifications (isolate differences) from one eel species to another. In Europe, AngHV1 (*Alloherpesviridae*) was for the first time described in an eel farm in the Netherlands, in 1998 (Davidse et al., 1999), and then its presence was confirmed both in fish farms and in natural waters i.e. in Japan, Taiwan, Italy, France and Germany (van Ginneken et al., 2004; Jakob et al., 2009; Haenen et al., 2010; Van Beurden et al., 2012). AngHV1 belongs to the most numerous group of DNA viruses encountered in fish and because of its

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pathogenicity and wide distribution it poses a serious threat to the eel (Haenen et al., 2002). On the basis of protein sequence analyses of the DNA-dependent DNA polymerase it has been established that the virus displays the highest level of homology to Cyprinid herpesvirus (CyHV-3) followed by Ranid herpesvirus 1 (RaHV1) and Ictalurid herpesvirus 1 (IcHV1) (Rijsewijk et al., 2005).

Characteristic clinical symptoms of AngHV1 may vary among and within outbreaks (Van Beurden et al., 2012). The most common include skin and fin haemorrhages (Ueno et al., 1992, Davidse et al., 1999; Haenen et al., 2002), varying degrees of erythema (Sano et al., 1990) or pale and swollen gills (Chang et al., 2002). The most affected regions included operculum, head, mouth (Davidse et al., 1999) and gills (Haenen et al., 2002). Infection with AngHV1 can also induce behavioural changes such as a loss of appetite (Lee et al., 1999) or apathy (Haenen et al., 2002). According to gross pathology, findings ranged from clinically normal (Chang et al., 2002) to severely affected. The most affected organs included liver (multifocal haemorrhages), kidney (swelling) and gallbladder (Ueno et al., 1992; Davidse et al., 1999; Haenen et al., 2002). Mortality rates range between 1% and 10%, but under stressful conditions can increase up to 50% (Sano et al., 1990; Haenen et al., 2002; Van Beurden et al., 2012). Due to their ability to enter into a latent state, herpesviruses may be present in clinically healthy individuals (Whitley, 1996). Therefore, there is a serious risk that dormant AngHV1 forms will become active leading to an outbreak of the disease when they spread among other, healthy individuals. The European eel is considered as a critically endangered species worldwide and is therefore included in the Red List of Threatened Species by the International Union for Conservation of Nature (IUCN) (Freyhof and Kottelat, 2010). Since 2007, export of the European eel has also been restricted worldwide and listed as an endangered species under CITES (2007, Appendix II/Annex B). The European Commission, following the findings of scientists from the Committee on Trade in Wild Fauna and Flora, proposed regulation and measures on export and import of European eel by member countries of the European Union that came into force in March 2009 (CITES, 2007). Among other reasons, the ban was introduced in order to make it impossible to sell the so-called montée (glass eel) to China and other Far East countries. Glass eels (elvers) are a stage in the development of eel larvae originating from the Sargasso Sea. In Poland, on the basis of Council Regulation (CE) No. 1100/2007 from September 18th 2007, the "Polish Eel Management Plan" has been developed in order to establish measures to enable to restore European eel stocks in the country. The following measures were taken: 1) reduce mortality due to harvesting (including fishing and angling), 2) restock with juvenile eel kept and fed in a farm until they were at least 20 cm long, 3) make river migration routes passable, and finally, 4) reduce the number of cormorants at eel sites.

Unfortunately none of these measures mention virological monitoring of the restocking eels used to restore the eel population. Previously in Poland, carp distributed as restocking fish did not undergo virological control, resulting in a rapid spread of koiherpes virus (KHV) throughout Poland and Europe. It led to the bankruptcies of many fish farms located in the Vistula and Oder rivers.

To avoid the danger of spreading another unwanted virus throughout Polish waters that may pose a threat to the European eel, we commissioned an AngHV1 screening program of restocking and imported eels. This would facilitate assessing the risk connected with the restoration program (managed by IRŚ, Poland) and the import of frozen eels, taking into account the potential risk of accidentally releasing sewage from a fish processing plant into the wild eel waters.

Material and methods

This study focused on adult specimens of the European eel (*A. anguilla*) obtained from Polish fishermen working with eel fyke nets in Dąbie Lake (n=14 53°27′49″N 14°40′29″E) and the Szczecin Lagoon (n=14 53°47′00″N 14°20′00″E). The second batch of samples were elvers (*A. anguilla*) imported from Denmark and bought from a Polish importer (n=6), who intended to keep them for human consumption. The third batch of specimens consisted of frozen and eviscerated eel (*A. anguilla*, *A. japonica*, *A. rostrata*) imported from China for direct human consumption (in total n = 26: *A. anguilla* n = 16, *A. rostrata* n = 8, *A. japonica* n = 2). It is important to mention that every specimen was packed individually, therefore gill samples were collected without any risk of cross contamination. The numbers of specimens representing respective species are given in Table 1. The study was conducted in the years 2009-2012 in the genetic laboratory of the Department of Aquaculture, West Pomeranian University of Technology in Szczecin (Poland). All of the analysed fish were free from clinical signs previously associated with AngHV1 infection.

From each of the 60 collected eel specimens, one gill arch was excised and then 30 mg of gill fragments without mucus were aseptically transferred to 1.5 ml Eppendorf tube and used for DNA isolation using peqGOLD Tissue DNA Mini Kit (PeqLab, Germany). The procedure of nucleic acid isolation was conducted according to manufacturer recommendations. Qualitative and quantitative evaluation of the obtained

Table 1. Origin, species identification and degree of AngHV1 PCR positive eels, Anguilla species from our study.

Country	Species	AngHV1-PCR-positive number of the eels from total no. (% of PCR positive eels)
Poland (Dąbie Lake)	*A. anguilla* (n = 14)	7/14 (50%)
Poland (Szczecin Lagoon)	*A. anguilla* (n = 14)	4/14 (28.6%)
Denmark	*A. anguilla* (n = 6)	6/6 (100%)
China	*A. anguilla* (n = 16)	8/16 (50%)
	A. rostrata (n = 8)	5/8 (62.5%)
	A. japonica (n = 2)	0/2 (0%)

DNA was performed using electrophoresis on a 1.5% agarose gel (Prona Agarose GQT, EU). Species affiliation of the studied specimens was identified by analysing the nucleotide sequence of COI: cytochrome c oxidase subunit 1. Amplification was conducted using FishF2_t1 and FishR2_t1 primers according to the methodology (PCR mixture components and thermal-temporal profile) described by Ivanova et al., (2007). As for the detection of the gene coding DNA-dependent DNA polymerase characteristic for AngHV1, it was conducted using HVAPOLVPSD and HVAPOLOOSN primers according to the procedure developed by Rijsewijk et al. (2005). Both the aforementioned primer pairs used to detect AngHV1 and to confirm eel species identification are characterised in Table 2. The results of every PCR were verified by separating the analysed samples in 1.5% agarose gel, after which, each PCR product was sequenced bi-directionally according to Sanger's method (Genomed, Poland). The results of sequence analysis were then analysed with the following software: BLAST 2.2.29, MEGA5 and BioEdit 7.2.5 (Altschul et al., 1990; Hall, 1999; Tamura et al., 2007).

Results

From the data generated by the bioinformatic analyses it was possible to identify the species affiliation of all 60 studied samples, confirming that the collected samples belonged to three species: *A. anguilla*, *A. rostrata* (Lesueur) and *A. japonica*. As a percentage of the total sample number this equated to 83.33% (n=50), 13.33% (n=8) and 3.33% (n=2), per species respectively. Additionally, our study revealed that AngHV1 genome was not detected in the samples from Japanese eels (*A. japonica*). The presence of the viral genome was confirmed in 50% (n=25) of the studied European eel (*A.* anguilla) specimens and in 62.5% (n=5) of the studied American eel (*A. rostrata*) specimens. A detailed list featuring respective species and percentages of AngHV1 PCR-positive individuals is given in Table 1. All nucleotide sequences of the DNA polymerase gene analysed in this study revealed 100% homology to the sequences that had been described previously in European and Japanese eel (Chang et al., 2002). In this study AngHV1 virus was detected for the first time in samples from American eel (*A. rostrata*). So far only two rhabdoviruses, Eel Virus American (EVA) and Eel Virus European X (EVEX) have been detected in *A. rostrata* (Sano et al., 1977).

Discussion

Global transportation of live fish renders obscure the boundaries along which diseases and pathogens are spreading among aquatic organisms (Boeger et al., 2002). In order to conduct restocking programs and to fatten eel

Table 2. Primer pairs used in the study to identify *Anguilla* species and detect AngHV1 genome.

Primer name	Primer sequence	Reference
FishF2_t1	5-TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC-3′	Ward et al. 2005
FishR2_t1	5-CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA-3′	
HVAPOLVPSD	5-′GTG TCG GGC TTT GTG GTG C-3′	Rijsewijk et al., 2005
HVAPOLOOSN	5′-CAT GCC GGG AGT CTT TTT GAT-3′	

for commercial purposes, fish farms have to import wild glass eel or elvers. However, there is no obligatory nor diagnostic examination to detect virulent eel viruses. According to Haenen et al. (2002), gill samples are the best tissues to detect AngHV1. Additionally, the AngHV1 isolates obtained in the Netherlands had been proven to be genetically highly homogeneous. The following diagnostic methods have been proven to be effective: in situ hybridization (Shih et al., 2003), PCR using specific primers (Shih, 2004; Rijsewijk et al., 2005; Jacob et al., 2009), electron microscopy (Jørgensen et al., 1994) and genome sequencing (van Beurden et al., 2010). However according to this study, detection of the AngHV1 genome using PCR method provided a high level of sensitivity and also keeps laboratory costs at a sustainable level level (Remigiusz Panicz, pers comm.). Viral infections in eel often can be double infections, but the absence of characteristic symptoms makes a quick differential diagnosis difficult and without tests like PCR even impossible. Instances of AngHV1 infections confirmed in European countries such as Germany (Jakob et al., 2009), Greece (Varvarigos et al., 2011), the Netherlands (Haenen et al., 2002), France (Jørgensen et al., 1994) and presently also in Poland, raise well justified fears connected with introducing eel to open waters. As there is no constant viral monitoring program it is impossible to evaluate the scale of potential danger of AngHV1. Also, importing non-eviscerated frozen eel from China to Polish fish processing plants (data obtained from the Customs Chamber in Szczecin) for further processing poses a potential threat that the virus will be released to the natural environment together with by-products from the fish processing factories. A lack of knowledge with respect to dormant and latent forms of HVA makes prevention of herpesvirus infections difficult. Additional problems arise when information on the exact origin of eel specimens is missing. In this study, it was impossible to detail the exact geographical origin within China where the analysed eels were cultured. Samples were imported to Poland as frozen, eviscerated and individually packed fish and every effort was made to collect the maximum amount of data. Unfortunately, the Customs Office could not provide us with additional information on sample origin because they are protected by international trade law.

There is no information available on the presence of possible vectors for AngHV1 in open waters, which may cause virus transmission in the natural environment. According to the most recent studies on herpesvirus infection (CyHV-3) in carp (*Cyprinus carpio*) the list of species which can transmit the virus includes 18 fish species other than carp (Kempter et al., 2008; Kempter et al., 2009). Furthermore, it was confirmed that a KHV infection may be caused through cohabitation of vector fish with healthy carp (Kempter et al., 2012). We assume that in the case of restocking with eel infected with AngHV1, particles of the virus are released into the environment. Possible vector fish species might transmit the virus, like with KHVD, thus contributing to the quick dispersal of the virus across Polish waters. This hypothesis would need more research.

Until recently, there was a common perception that AngHV1 may cause infections only in the European eel (*A. anguilla*) and the Japanese eel (*A. japonica*) (van Beurden et al., 2010). Results of the present study indicate that the spectrum

of affected species includes also the American eel (*A. rostrata*). Further intensification of research efforts is required in order to gain insight into the mechanisms of transmission of herpesviruses among hydrobionts. It ought to be seriously questioned whether introducing eel to open waters without restrictive virological control is justifiable, and stocking centres and other institutions involved in stocking ought to be warned against the potential threat that infected young eels pose to wild eel stocks.

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References

Altschul S, Gish W, Miller W, Myers EW and Lipman DJ (1990). Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403-410.

Békési L, Horváth I, Kovács-Gayer E and Csaba G (1986). Demonstration of herpesvirus like particles in skin lesions of European eel (*Anguilla anguilla*). *Journal of Applied Ichthyology* **4**, 190-192.

Boeger WA, Piasecki W and Sobecka E (2002). Neotropical Monogenoidea. 44. *Mymarothecium viatorum* sp. n. (Ancyrocephalinae) from the gill of *Piaractus brachypomus* (Serrasalmidae, Teleostei) captured in a warm-water canal of a power plant in Szczecin, Poland. *Acta Ichthyologica et Piscatoria* **32**, 157–161.

Chang PH, Pan YH, Wu CM, Kuo ST and Chung HY (2002). Isolation and molecular characterization of herpesvirus from cultured European eels *Anguilla anguilla* in Taiwan. *Diseases of Aquatic Organisms* **50**, 111–118.

CITES (2007). Notification to the Parties: Amendments to Appendices I and II of the Convention adopted by the Conference of the Parties at Its 14th Meeting, The Hague (Netherlands), 3–15 June 2007, No. 2007/022 (26 July 2007); http://www.cites.org/eng/notif/2007/E022.pdf.

Davidse A, Haenen OLM, Dijkstra SG, van Nieuwstadt AP, van der Vorst TJK, Wagenaar F and Wellenberg GJ (1999). First isolation of herpesvirus of eel (Herpesvirus Anguillae) in diseased European eel (*Anguilla anguilla* L.) in Europe. *Bulletin of the European Association of Fish Pathologists* **19**, 137-141.

Freyhof J and Kottelat M (2010). *Anguilla anguilla*. In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013; www.iucnredlist.org.

Haenen OLM, Dijkstra SG, van Tulden PW, Davidse A, van Nieuwstaadt AP, Wagenaar F and Wallenberg GJ (2002). Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in the Netherlands since 1996. *Bulletin of the European Association of Fish Pathologists* **22**, 247-257.

Haenen OLM, Lehmann J, Engelsma MY, Stürenberg F-J, Roozenburg I, Kerkhoff S and Klein Breteler J (2010). The health status of European silver eels, *Anguilla anguilla,* in the Dutch River Rhine watershed and Lake IJsselmeer. *Aquaculture* **309**, 15-24.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Ivanova NV, Zemlak TS, Hanner RH and Hebert PDN (2007). Universal primer cocktails for fish DNA barcoding. *Molecular Ecology Notes* **7**, 544–548.

Jakob E, Neuhaus H, Steinhagen D, Luckhardt B and Hanel R (2009). Monitoring of Herpesvirus anguillae (HVA) infections in European eel, *Anguilla anguilla* (L.), in northern Germany. *Journal of Fish Diseases*

32, 557–561.

Jørgensen P, Castric J, Hill B, Ljungberg O and De Kinkelin P (1994). The occurrence of virus infections in elvers and eels (*Anguilla anguilla*) in Europe with particular reference to VHSV and IHNV. *Aquaculture* **123**, 11-19.

Kempter J, Kielpinski M, Panicz R and Sadowski J (2008). Determination of the carrier species and the infection susceptibility for Koi-Herpes-Virus infections among selected cyprinid fish species and their hybrids acquired from open waters and culture sites of the Odra River drainage, 62pp. Akademia Rolnicza, Szczecin [in Polish with English summary].

Kempter J, Sadowski J, Schütze H, Fischer U, Dauber M, Fichtner D, Panicz R and Bergmann SM (2009). Koi herpes virus: do acipenserid restitution programs pose a threat to carp farms in the disease-free zones? *Acta Ichthyologica et Piscatoria* **39**, 119–126.

Kempter J, Kielpinski M, Panicz R, Sadowski J, Myslowski B and Bergmann SM (2012). Horizontal transmission of koi herpes virus (KHV) from potential vector species to common carp. *Bulletin of the European Association of Fish Pathologists* **32**, 212-219.

Lee NS, Kobayashi J and Miyazaki T (1999). Gill filament necrosis in farmed Japanese eel, *Anguilla japonica* (Temminck and Schlegel), infected with Herpesvirus anguillae. *Journal of Fish Diseases* **22**, 457–463.

Rijsewijk F, Pritz-Verschuren S, Kerkhoff S, Botter A, Willemsen M, van Nieuwstadt T and Haenen O (2005). Development of polymerase chain reaction for the detection of Anguillid herpesvirus DNA in eels based on the herpesvirus DNA polymerase gene. *Journal of Virology Methods* **124**, 8887-8894.

Sano T, Nishimura N, Okamoto N and Fukuda H (1977). Studies on viral diseases of Japanese fishes. VII. A rhabdovirus isolated from European eel, Anguilla anguilla. *Bulletin of the Japanese Society for the Science of Fish* **43**, 491–495.

Sano M, Fukuda H and Sano T (1990). Isolation and characterization of a new herpesvirus from eel. *In* **"Pathology in Marine Sciences"** (Perkins F.O., Cheng T.C., Eds) Academic Press, San Diego.

Shih HH, Hu CW and Wang CS (2003). Detection of Herpesvirus anguillae in eel using in situ hybridization. *Journal of Applied Ichthyology* **19**, 99-103.

Shih HH (2004). A polymerase chain reaction for detection Herpesvirus anguillae in asymptomic eels. *Taiwania* **49**, 1-6.

Tamura K, Dudley J, Nei M and Kumar S (2007). MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596-1599.

Ueno Y, Kitao T, Chen S-N, Aoki T and Kou G-H (1992). Characterization of a herpeslike virus isolated from cultured Japanese eels in Taiwan. *Fish Pathology* **27**, 7-17.

van Beurden SJ, Bossers A, Voorbergen-Laarman MHA, Haenen OLM, Peters S, Abma-Henkens MHC, Peeters BPH, Rottier PJM and Engelsma MY (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *Journal General Virology* **91**, 880-887.

van Beurden S, Engelsma MY, Roozenburg I, Voorbergen-Laarman MA, van Tulden PW, Kerkhoff S, van Nieuwstadt TP, Davidse A and Haenen OLM (2012). Viral diseases of wild and farmed European eel (*Anguilla anguilla*) with particular reference to the Netherlands. *Diseases of Aquatic Organisms* **101**, 69-86.

van Ginneken V, Haenen O, Coldenhoff K, Willemze R, Antonissen E, van Tulden P, Dijkstra S, Wagenaar F and van den Thillart G (2004). Presence of eel viruses in eel species from various geographic regions. *Bulletin of the European Association of Fish Pathologists* **24**, 268–271.

Varvarigos P, Vendramin N, Cappellozza E and Bovo G (2011). First confirmation of Herpes Virus Anguillae (HVA) and Infectious

Pancreatic Necrosis (IPN) virus infecting European eel *Anguilla anguilla* farmed in Greece. *Bulletin of the European Association of Fish Pathologists* **31**, 101-111.

Ward RD, Zemlak TS, Innes BH, Last PR and Hebert PDN (2005). DNAbarcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **360**, 1847–1857.

Whitley RJ (1996). Cercopithecine herpes virus 1 (B virus). *In* **"Fields Virology"** (B. N. Fields, D. M. Knipe, P. M. Howley, Eds). Lippincott-Raven, Philadelphia.