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Original article

Outbreak of ranavirus infection in sheatfish, *Silurus glanis* (L.), in Poland

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Abstract

Ranavirus was detected in adult sheatfish, with clinical signs, on a Polish fish farm in February. Farm isolates induced a strong cytopathic effect *in vitro* and were identified by electron microscopy and PCR amplification of the ranavirus specific gene fragment. Restriction analysis with the Acc I enzyme showed that isolated ranaviruses were different from the epizootic heamatopoietic necrosis virus (EHNV). We sequenced a fragment of the major capsid protein (MCP) gene and found that isolates were similar to other strains of ranaviruses, available in GenBank.

Key words: sheatfish, *Iridoviridae,* ranavirus, phylogeny

Introduction

Ranaviruses are an emerging group of viral pathogens (Chinchar et al. 2005) belonging to the *Ranavirus* genus, in the family *Iridoviridae*; a group of large, icosahedral, double-stranded DNA viruses (Chinchar et al. 2005). They have a wide susceptible host range, infecting fish, amphibians and reptiles, and have been implicated in the increase of epidemics in these animals.

There are currently 6 ranavirus species recognized by the International Committee on Taxonomy of Viruses (ICTV 2009); 3 of which infect fish: Epizootic heamatopoietic necrosis virus (EHNV), European catfish virus (ECV) (including both ECV and European sheatfish virus [ESV]) and Santee-Cooper ranavirus (SCRV). SCRV has caused epidemics in North America, affecting wild, largemouth bass (*Micropterus salmoides*) (Plumb et al. 1996). This ranavirus also appears to be impacting wild populations, with losses of larger and/or older fish in particular (Whittington et al. 2010). Two strains of SCRV, Doctor fish virus (DFV) and Gutapo virus 6 (GV6) have also been isolated in ornamental fish imported into America from South East Asia (Hedrick and McDowell 1995). Ranavirus species which infect amphibians include Ambystoma Tigrinum virus (ATV), Bohle iridovirus (BIV) and Frog virus 3 (FV3).

However, the taxonomy of the genus is still being debated, and further studies are needed, particularly with regard to its molecular aspects (Gobbo et al. 2010). Since recognition of the disease due to the presence of EHNV in Australia in 1986, similar systemic necrotising iridovirus syndromes have been reported in farmed fish in Europe. These include catfish (*Ictalurus melas*) in France (ECV) (Pozet et al. 1992, Bigarre et al. 2008), sheatfish (*Silurus glanis*) in Germany (ESV) (Ahne et al. 1989, 1990), turbot (*Scoph-*

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thalmus maximus) in Denmark (Bloch and Larsen 1993) and others in Finland (Ariel et al. 1999). Other ranavirus isolates, such as pike perch iridovirus (PPIV) and short-finned eel ranavirus (SERV) have been occasionally isolated in symptomless fish from both freshwater and marine environments (Tapiovaara et al. 1998, Jansen et al. 2009).

EHNV shares 98% nucleic acid identity in the Major Capsid Protein (MCP) gene with both ECV and ESV (Marsh et al. 2002) and was considered under EU Directive 2006/88 (Council Directive 2006/88/EC and Commission Regulation (EC) No1251/2008) as an exotic, notifiable disease.

In this report, we describe the first isolation and identification of ESV-like isolates, from sheatfish on a farm in Poland and developed a rapid detection method based on the polymerase chain reaction (PCR) and restriction enzyme analysis (REA) for differentiating these viruses. This method is recommended by the Office International des Epizooties (OIE) in the current Manual for Diagnostic Tests for Aquatic Animals (2012) and is routinely used for diagnosis by the Fish Diseases Department in Pulawy. Phylogenetic analysis of the sequences, in the MCP region, enabled us to exclude the presence of EHNV in our country.

Materials and Methods

Adult sheatfish (2400-3100 g) were diagnosed in a fish disease laboratory in February 2014. At this time, water temperature at the farm was between 4°C and 5°C. All infected fish showed clinical and anatompathological signs; swelling of the anus, bright red gills, increased swabs in skin, fluid in the body cavity and bleeding within internal organs.

Tissues from the liver, kidney and spleen of 6 fish were homogenized and subsequently suspended in the original transport medium. After 15 minutes centrifugation at 1500 × *g* at 4°C, the supernatant was filtered and used for virus isolation. For virus propagation, epithelioma papulosum cyprinid (EPC) and bluegill fry (BF-2) cell lines were inoculated and incubated at 15°C.

The cells were inoculated with supernatants from cultures with cytopathic effect. The cell culture medium was removed and monolayers were fixed in glutaraldehyde (3% in 0.1 M sodium cacodylate buffer, pH 7.4), post fixed in 2% osmium tetroxide, dehydrated through graded ethanol and embedded in Lx-112 embedding medium. Ultra-thin sections cut in parallel with the monolayer were contrasted in uranyl acetate and lead citrate, and examined in a Zeiss electron microscope, LIBRA 120.

For genetic identification, total DNA was extracted from the supernatant of virus-infected cells. Specific primers M153 and M154 (MCP-2, 625 bp) were used (OIE, 2012). The reaction was carried out using a GoTaq DNA Polymerase (Promega). Briefly, 45 µL of the reaction mixture, containing 5 µl of extracted DNA, 10 µM of each primer, were subjected to following thermal conditions: 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute and the final extension at 72°C for 5 minutes. The amplified products were separated by electrophoresis in 2% agarose gel with ethidinum bromide (1 µl/mL) in 1 × TAE (tris-acetate-EDTA) buffer and visualised by UV transillumination using EC3 Chemi HR 410 Imaging System (Ultra-Violet Products Ltd., UK). A GeneRuler™ 100bp DNA Ladder Plus (Fermentas) was used as a molecular size standard. PCR amplicons were subjected to restriction endonuclease analysis (REA) with the Acc I enzyme for 4 hours at 37°C, the products were separated by electrophoresis in 3% agarose gel with ethidinum bromide and visualised by UV transillumination

A second PCR with different primers MCP-1S (5'ACGCAGTCAAGGCCTTGATG 3') and MCP-2S (5'AGACCCGTTTTGCAGCAAAC 3') amplifying a 585 bp region (Bigarre et al. 2008) was used for sequencing. The following cycles were applied: 95°C for 5 minutes, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 40 seconds and the final extension at 72°C for 5 minutes.

Genetic material of EHNV (AJ316246) and ECV (FJ358608) strains was used as reference material for PCR. The negative control was the DNA isolated from non-infected cells.

Nucleotide sequences were deduced using the MEGA 6 program. One thousand bootstrapped alignments were constructed and adjoining trees were built.

Results

During virus isolation we observed a strong cytopathic effect in the first passage, after 4 days. Then, the next passage was performed, with a similar effect. In an attempt to visualize the morphology of the virus, a cell culture with CPE were fixed and prepared for electron microscopy. Icosahedral particles were observed in the cytoplasm of the cells, indicating the presence of a iridovirus (Fig. 1). PCR with specific primers allowed amplification of a 625 bp region in the ranavirus MPC gene. PCR amplicons were subjected to REA with the Acc I enzymes. Restriction analysis gave a product indicative of the presence in the sample of (625 bp) ESV, ECV, BIV or WV and

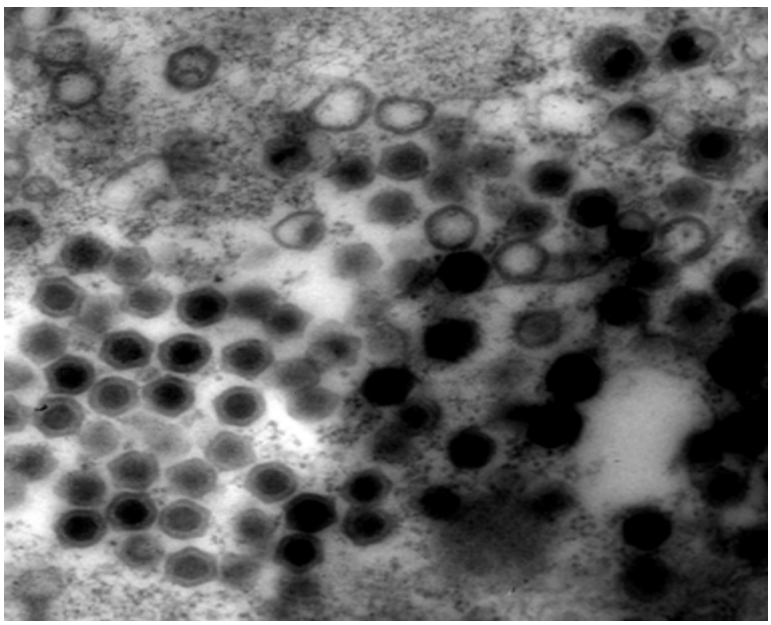

Fig. 1. Icosahedral viral particles in sections from infected EPC cell. Bar = 500 nm

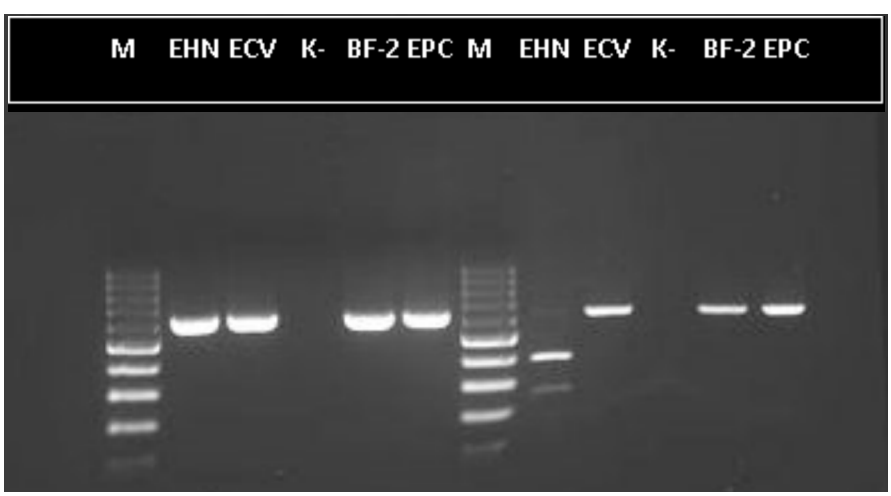

Fig. 2. Amplification for sequencing of DNA extracted from supernatant of cell culture of cells inoculated with isolate from sheatfish (M- marker 100bp, reference EHNV, reference ECV, negative control, test samples products – 585 bp) and REA with the Acc I enzymes (M- marker 100 bp, reference EHNV product 238 and 387 bp, reference ECV product 625 bp, negative control, test samples – 625 bp).

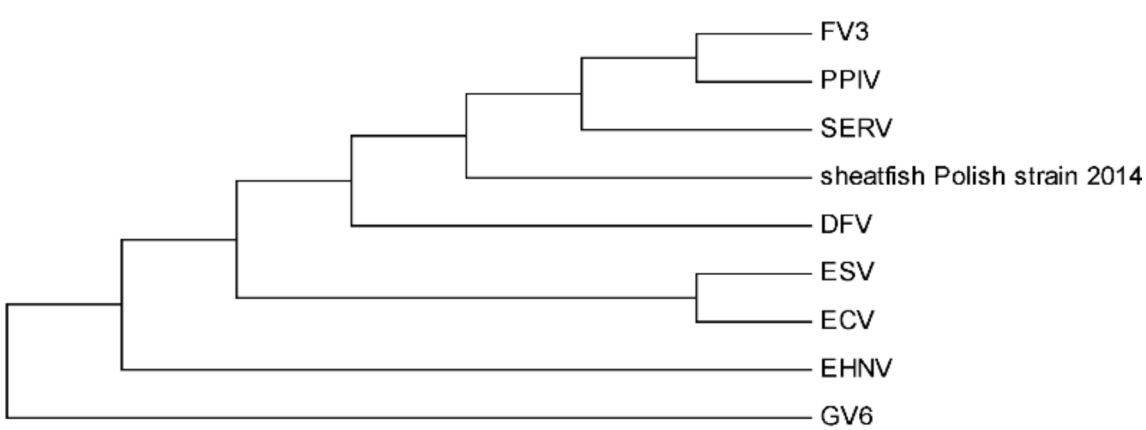

Fig. 3. Phylogenetic relations between sheatfish Polish isolates and other irodoviruse isolates. GV6 Gutapo virus 6 (AF157649), EHNV Epizootic hematopoietic necrosis virus (AJ316246), ECV European catfish virus (FJ358608), ESV European sheatfish virus (FJ358609), DFV Doctor fish virus (AF157665), SERV Short-finned eel ranavirus (FJ358612), PPIV Pike perch iridovirus (FJ358610) and FV3 Frog virus 3 (AY548484).

excluding the presence of EHNV (238 and 387 bp) in the sample (Fig. 2). Finally, phylogenetic analysis showed that Polish isolates are highly similar to ECV from France, ESV from Germany and, surprisingly, to DFV from Asia (Fig. 3).

Discussion

This study revealed, for the first time, the presence of ranavirus in sheatfish in Poland. The Polish strain is highly similar to ther fish ranaviruses.

Several comparative studies have been carried out on ranaviruses revealing a high degree of similarity in the MCP and other genomic regions (Marsh et al. 2002). Due to the genetic similarity of ranaviruses, many known isolates could be considered to be of the same species. However, classification of the members of the genus *Ranavirus* into different species has been favoured, as ranaviruses have clearly different hosts and geographical ranges.

Currently, EHNV is the only ranavirus listed by the OIE as notifiable for fish. In addition to EHNV there are also other known pathogenic ranavirus isolates, e.g. ESV and ECV (Ahne et al. 1989, Pozet et al. 1992, Bovo et al.1993). The common denominator linking these events together is the relatively high water temperature under which, the fish were cultured. Several other ranavirus isolations were done, many of which appear to be haphazard isolations from symptomless fish, indicating that ranavirus isolates are not always virulent (Chinchar et al. 2005).

The MCP gene has been commonly used to define ranavirus taxonomy and to differentiate between virus isolates and the complete MCP sequences of some of the isolates included in this study are already known, because the MCP is very stable. The stability of the MCP (over time and species) indicates that small differences in the sequence of the coding region are significant. This coding region may therefore be a suitable target for the future identification of ranaviruses (Hyatt et al. 2000).

Identification of the sheatfish ranavirus in Poland led us to suspect introduction of the virus was due to the international trade of contaminated fish or stocking material. The high density is likely a factor in explaining the virus outbreak which benefited from a natural down-regulation of an excessive sheatfish population.

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