Ranaviruses associated with high mortalities in catfish in France

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Abstract

Two ranaviruses have been detected in dead catfish collected in France in two lakes highly affected by mortality episodes during summer 2007. The present isolates induce cytopathic effects *in vitro* and are recognized by a serum against Epizootic haematopoietic necrosis virus (EHNV). A portion of the Major Coat Protein gene has been PCR amplified and sequenced, indicating that both isolates are identical and likely strains of European Catfish Virus (ECV) isolated in France 15 years ago.

Introduction

During summer 2007, two wild populations of bullhead catfish (*Ictalurus melas*) in two lakes in the west and east of France were affected by massive mortalities in a few weeks. These episodes did not affect other fish species and coincided with a particularly dense population of catfish. A mortality episode occured about 15 years ago, affecting the same host in a little pond in the east of France. At this time, a virus related to Sheatfish iridovirus and Epizootic haematopoietic necrosis iridovirus (EHNV), and now identified as European catfish virus (ECV), was found as the causative agent of the outbreak (Pozet et al., 1992).

Iridoviridae are cytoplasmic viruses containing a large dsDNA genome. They have been the cause of serious losses in aquaculture and wildlife (Ahne et al., 1989; Hedrick et al., 1990; Langdon et al., 1986). Currently, 5 genus are recognized, among which the ranavirus genus including European catfish virus, Doctor fish virus (DFV), Frog virus 3 (FV3), European sheatfish virus (ESV) and Epizootic haematopoietic necrosis virus. European catfish virus and ESV are likely strains of the same virus and are different from EHNV as revealed by restriction of the full genome (Mao et al., 1997). However, EHNV and ESV/ECV have related antigens and cannot be distinguished by an immunological method (Hedrick et al., 1992). While the complete genome sequence of FV3 is available (Tan et al., 2004), only partial DNA sequences of EHNV, ESV and ECV have been obtained to date. EHNV shares 98% of nucleic acid identity in the Major Coat Protein (MCP) gene with both ESV and ECV (Marsh et al., 2002). The high similarity in this region of the genome is challenging for an accurate method of typing, either by PCR/restriction targeting the MCP gene as proposed (Marsh et al., 2002) or by PCR/sequencing. The distinction between EHNV and ECV/ESV is important since the former is notifiable to the OIE, but not the latters.

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We have demonstrated the presence of a ranavirus in the 2 present outbreaks affecting catfish and obtained sequences in the MCP regions indicating the implication of ECV, while excluding the presence of EHNV.

Materials and methods

Fish and virus

From June to August 2007, a high mortality affecting all sizes of catfish in the lake Le Bourget, east of France was reported. A high mortality was also observed during the same period in the lake of Apremont, west of France, from which 150kg of dead fish were collected during three weeks.

The distance between the two lakes is 800km. Dead fish from both sites were sent to the regional laboratory in Poligny (France) for autopsies and cell culture tests. Organs from seven fish were ground in PBS (1/10), centrifuged at 2,600 g for 15 min and inoculated on the *Epithelioma papulosum cyprini* (EPC) cell line at 14, 20 and 29°C, and on the Rainbow Trout Gonad cell line (RTG2) at 14 and 20°C. A cytopathic effect (cpe) was observed after two days on both cell lines and the cell culture supernatant from EPC cells was subsequently forwarded to AFSSA for confirmation of the cpe and further immunological and PCR analysis. The presence of three rhabdoviruses, viral haemorrhagic septicaemia virus (VHSV), infectious hematopoietic necrosis virus (IHNV) and spring viremia of carp virus (SVCV), as tested by immunofluorescence on cell culture was excluded. Isolate 7892 originates from lake Le Bourget and isolate 9537 from lake of Apremont.

Immunofluorescence (IFAT)

For immunological identification, the following procedure was applied: 24 h after inoculation of the isolates at 24°C, EPC cells cultivated in 4 well-plates were fixed with acetone/alcool (3v/7v) for 15 min at room temperature and rinsed 3 times with PBS-tween 20 (0.05%). Then, 250 µl of an anti-EHNV rabbit serum (M708, University of Sidney) diluted 1/1500 was distributed in the wells. The plates were incubated for 1h at 36°C, rinsed with PBS-T, and stained for 1 h at 36°C with 250 µl of a 1/300 solution of goat anti-rabbit immunoglobulin serum conjugated with fluorescein isothiocyanate (Sigma). The cells were rinsed with PBS-T and observed for fluorescence (Olympus TX50 inverted fluorescence microscope).

Amplification by PCR

DNA was purified from 200µl of cell culture supernatant displaying a CPE using a Nucleospin blood kit (Macherey-Nagel) and eluted in a final volume of 80µl. Amplification of DNA was performed according to the OIE protocole with minor modifications. Two primers oPVP29 (5'ACGCAGTCAAGGCCTTGATG) and oPVP30 (5'AGACCCGTTTTGCAGCAAAC) target a 585bp region of the MCP gene of ranaviruses. 5, 10 or 20 µl of DNA were mixed with 2,5 units of TaqGold polymerase (Applied Biosystems), 0.4 µM of each primer, 200 µM of dNTP, and 2,5 mM of $MgCl_2$ in Taqgold bufferII. The following cycles were applied: 1 step of 5 min at 95 °C followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 40 sec, and a last step at 72 °C for 5 min. After electrophoresis, the PCR product was gel-purified and either cloned in TOPO-

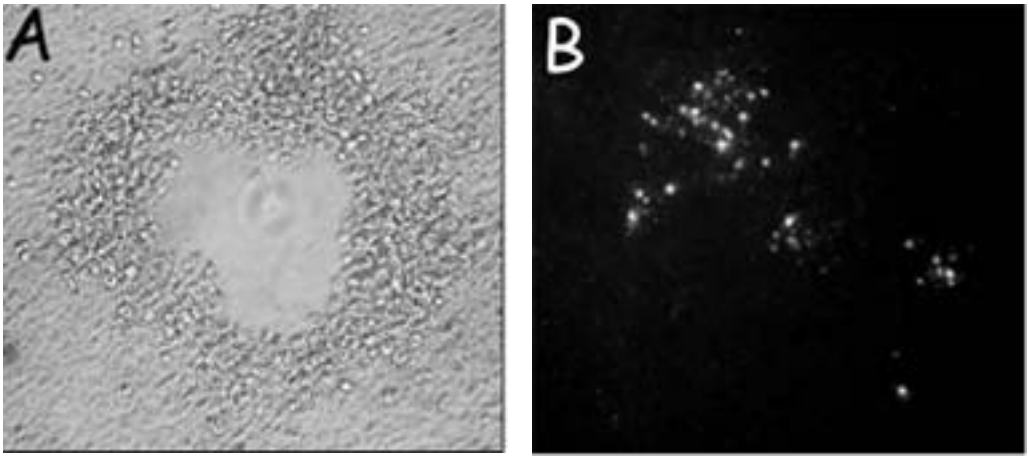

Figure 1. Detection of the isolate 7892 on EPC cells. **A.** Cytopathic effect observed 48h after inoculation. **B.** Immunofluorescence produced with a serum anti-EHNV.

TA (Invitrogen) for sequencing (isolate Le Bourget) or directly sequenced with primers oPVP29/30 (isolate Apremont). All sequences were analysed with VNTI9.0 (Invitrogen). The alignment and the phylogenic tree were obtained with MEGA 4 (http://www.megasoftware.net/) with a neighbor-joining method and a bootstrap of 1000 replicates.

Results

Post-mortem examination of dead fish from both sites revealed petechial haemorrhages at the basis of the pectoral and abdominal girdles. Skin decoloration, fin necrosis, abdominal haemorrhagic ascitis, kidney hypertrophy, pale liver and petechiae on viscera were also observed.

Fourty-eight hours after inoculation of cells with the homogenate from organs, a cpe was observed on EPC cells at 14°, 20° or 29° C and on RTG2 cells at 14° or 20° C.

Plaques surrounded by a ring of compacted cells were observed (Figure 1A). This effect was identical to the one initially described on EPC cells inoculated with ECV (Pozet et al., 1992). The cpe became evident after a second passage of the supernatant collected from the first EPC cell culture.

Twenty-four hours after inoculation of cell culture monolayers, a strong fluorescent signal appeared on groups of inoculated cells stained with an antiserum raised against EHNV (Figure 1B), indicating the presence of a ranavirus antigenically related to this virus

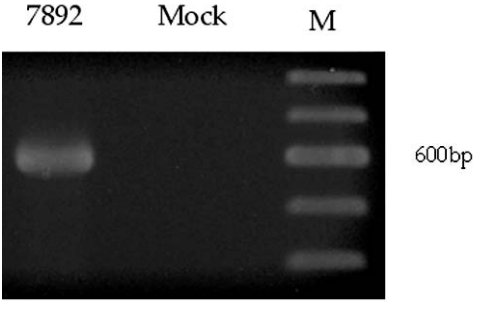

Figure 2. Amplification of DNA extracted from supernatant of cell culture of cells inoculated with isolate 7892 or mock- inoculated. M, marker 100bp.

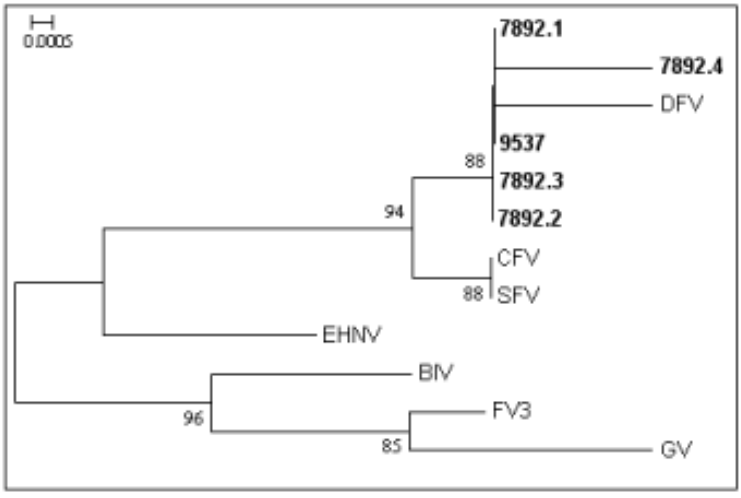

Figure 3. Phylogenetic relations between the two isolates and other iridoviruse isolates. BIV Bohle Iridovirus (AF157651), GV Gutapo virus (AF157649), FV3 Frog virus 3 (AY548484, EHNV Epizootic Hematopoietic Necrosis virus (AF157667), ESV European Sheatfish virus (AF157679), ECV European Catfish virus (AF157659), DFV Doctor fish virus (AF157665). The 2 isolates of this study are in bold (4 clones sequences for isolate 7891). Bar gives the genetic distance.

from Australia. No signal was obtained on mock-inoculated cells or on inoculated cells probed with antisera against three rhabdoviridae, VSHV, IHNV and SVCV (not shown).

The presence of a ranavirus was further confirmed by PCR targeting a 585bp portion of the MCP gene of ranaviruses. A product of the expected size was detected in DNA extracted from supernatant of EPC cells inoculated with isolate 7892 (Figure 2). The analysis by gel electrophoresis revealed that the signal was less intense as the volume of DNA increased from 5 to 20 µl suggesting an inhibition effect of the reaction (not shown). The amplification product of the isolate 7892 was cloned, and four clones sequenced. These four clones were almost identical, differing only by a few mutations that could have originated from the presence of variants in the inoculum or during the PCR amplification. A similar PCR product was obtained with isolate 9537 and directly sequenced without cloning. The consensus sequence of isolate 7892 is identical to the sequence obtained by direct sequencing of the second isolate (9537), except for one nucleotide deleted in 9537 (position 98303 in FV3, Genbank AY548484). Since this single deletion is within the MCP open reading frame, it would affect the product of the gene and is therefore likely a mutation produced early during PCR, or during the first cell culture passage.

The two present isolates are highly similar (>99%) to ECV, ESV and DFV, the latter being geographically distinct. The similarity with EHNV is also high, albeit more nucleotide differences appear which induces a separate grouping in the phylogenetic tree (Figure 3).

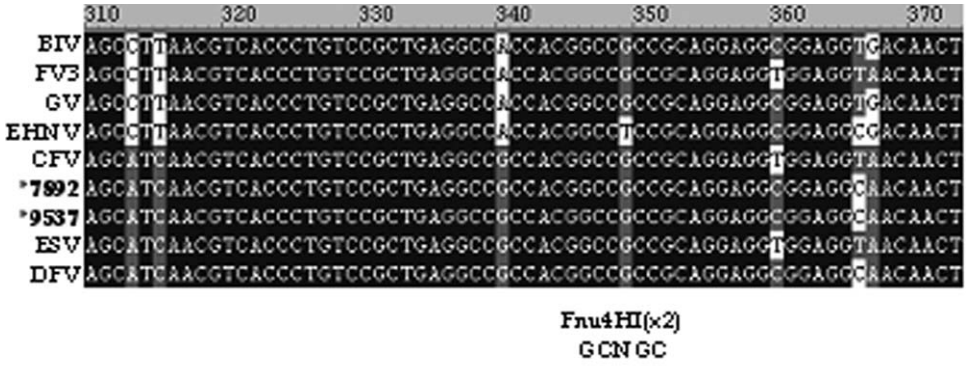

Figure 4. Alignment of a portion of the sequences of isolates 7892 and 9537 with other iridoviruses. Only EHNV does not contain two Fnu4HI restriction sites in this region. Position 310 corresponds here to nt 98572 in FV3 (Genbank AY548484).

Discussion

Two mortality episodes of catfish in France were associated with the presence of a ranavirus strain highly similar in the MCP gene to the cluster ECV/ESV, and sligthly distinct from EHNV. The two present isolates 7892 and 9537 are highly similar to ECV from France, ESV from Germany and surprisingly to DFV from Asia. The original ECV strain was isolated about 15 years ago in a pond 100kms away from isolate 7892. It is intriguing that the two isolates ECV and 7892 from the same host species and the same geographical region are less similar than 7892, 9537 and DFV isolated on different hosts from different continents. More genomic data is needed to clarify if the four European ranavirus isolated to date are variants of a single viral species, and to determine their genetic relationship with DFV from Asia.

The high similarity between the two isolates of this study, collected at two sites separated from 800km with no hydrographic connection, suggest a strong selection pressure on this portion of the genome or less lileky a recent common origin. Indeed, a 100% identity was also observed between two isolates of EHNV from Australia collected in two sites and from two different fish species, rainbow trout and redfin perch. The conservation of this region of the genome is thus an advantage for a diagnostic test but seems poorly informative for genotyping.

Presently, the distinction between EHNV and ECV is based on PCR with two primers sets, followed by restriction with specific endonucleases (Marsh et al., 2002). This test is moderately satisfactory because the two-steps procedure is time-consuming, and more importantly because a single G→T mutation in ECV can modify one of the key restriction sites and lead to a false diagnosis of EHNV (Figure 4). Since these two viruses have clear genomic specificities (Mao et al., 1997), it should be possible to target a genomic domain specific to one or the other. Therefore, complete sequences of the genomes of ECV and EHNV are needed to set up a simple PCR test able to differentiate efficiently ECV and EHNV, bypassing restriction or sequencing.

The bullhead catfish originates from America and was introduced in France in 1871. Since then, it successfully colonized numerous fresh waters sites, where it is often considered a problem for other fish populations. Since 2003, Le Bourget has been a site of uncontrolled proliferation of catfish. This high density is likely a factor to explain the virus oubreak, which benefited in the natural down-regulation of an excessive catfish population.

It is not known if catfish is the unique carrier of ECV in France or if other fish species can be hosts. Since ranaviruses usually have a large host range among piscine and amphibian hosts, ECV is considered as a threat to other fishes and should be surveyed.

Acknowledgements

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