Megalocytivirus infection in orbiculate batfish *Platax orbicularis*

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ABSTRACT: Megalocytiviruses cause systemic disease in both marine and freshwater fishes, negatively impacting ornamental and food fish aquaculture. In this report, we characterize a megalocytivirus infection in a captive marine ornamental fish, the orbiculate batfish *Platax orbicularis*. Histologic examination revealed cytomegalic cells characterized by strongly basophilic granular intracytoplasmic inclusions within various organs. Transmission electron microscopy revealed icosahedral virus particles within the cytoplasm of cytomegalic cells consistent with an iridovirus infection. Analysis of the major capsid protein gene sequence confirmed that the orbiculate batfish virus is a member of the family *Iridoviridae* and is identical to the only other megalocytivirus reported from a marine ornamental fish, the Banggai cardinalfish *Pterapogon kauderni* iridovirus.

KEY WORDS: Iridovirus · Megalocytivirus · Orbiculate batfish · Phylogeny

INTRODUCTION


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Epizootics may result in up to 100% mortality under intensive aquaculture conditions (Fraser et al. 1993, Rodger et al. 1997, He et al. 2000, Sudthongkong et al. 2002). These viruses can spread horizontally from fish to fish by cohabitation, exposure to water or equipment carrying the virus, and ingestion of infected fish or feed items (He et al. 2002, Go & Whittington 2006, Yanong & Waltzek 2010).


Phylogenetic analyses support 4 separate species within the genus *Megalocytivirus* (Kurita & Nakajima 2012, Waltzek et al. 2012). Megalocytiviruses related to red sea bream iridovirus (RSIV) cluster into 1 of 2 genotypes and have been associated with mass mortality epizootics in more than 30 maricultured species in Japan, Korea, China, and Southeast Asia (Inouye et al. 1992, Jung & Oh 2000, Kawakami & Nakajima 2002, Wang et al. 2003). Infectious spleen and kidney necrosis virus (ISKNV), originally isolated from mandarin fish *Siniperca chuatsi* raised for food in China, has also resulted in epizootics in more than 10 species of freshwater ornamental fishes (He et al. 2000, Paperna et al. 2001, Sudthongkong et al. 2002, Yanong & Waltzek 2010). A second ISKNV genotype was recently recognized following disease episodes in ornamental and food fish species (Kurita & Nakajima 2012), which included Banggai cardinalfish iridovirus (BCIV) isolated from a marine ornamental species (Banggai cardinalfish *Pterapogon kauderni*) and marbled sleepy goby iridovirus (MSGIV) isolated from a freshwater species (marbled sleepy goby *Oxyeleotris marmorata*) cultured for food in China (Weber et al. 2009, Wang et al. 2011). The third species, turbot reddish body iridovirus (TRBIV), has primarily been associated with disease in a cultured Asian flatfish species, the turbot *Scophthalmus maximus* (Shi et al. 2004). A fourth megalocytivirus species was recently proposed, threespine stickleback iridovirus (TSIV), from an epizootic that occurred in a Canadian collection of threespine stickleback *Gasterosteus aculeatus* (Waltzek et al. 2012).

Orcibulate batfish *Platax orbicularis* are tropical marine ornamental species belonging to the order Perciformes, family Ephippidae. They can be found throughout the Western Indo-Pacific region (Capuli & Orantez 2011). Orcibulate batfish occur in a variety of habitats: mangroves in shallow coastal waters, other sheltered waters, deep seaweed reefs, and open waters. They prefer water temperatures ranging from 22 to 28°C (71.7 to 82.4°F) (Capuli & Orantez 2011). Orcibulate batfish have only recently been successfully bred in captivity, and thus information regarding viral diseases in this species is limited (David et al. 2010). Only 2 viral infections, lymphocystis and betanodavirus, have previously been reported in *P. orbicularis* (Lawler et al. 1978, David et al. 2010).

In the present study, we report a megalocytivirus infection in *Platax orbicularis* that was associated with mortality in recently imported wild-caught fish acquired by a public aquarium in Belgium. The objective of this study was to compare clinical aspects of the disease as well as microscopic and ultrastructural features of the virus with previously described megalocytiviruses, including the virus associated with the only other marine ornamental epizootic reported in Banggai cardinalfish.

**MATERIALS AND METHODS**

**Clinical history**

A mortality event occurred in March 2010 among a group of 10 oribiculate batfish quarantined at a public aquarium in Belgium. These wild-caught juvenile
orbiculate batfish (average of 12 cm in total length) from Indonesia had been purchased by an ornamental fish import facility in the UK and sent to a quarantine facility in the UK, where they were acclimatized and quarantined prior to delivery to the aquarium in Belgium in February 2010. At the aquarium, the batfish were maintained in a quarantine system equipped with an external power filter. Water quality parameters were maintained at a temperature of 24°C (75°F), pH 7.8, nitrite <0.3 mg l−1, nitrate 0 to 12.5 mg l−1, salinity 32, 96% oxygen saturation, and undetectable total ammonia nitrogen. The orbiculate batfish were fed mysid (Mysidopsis) and brine shrimp (Artemia) 5 to 6 times per day.

On arrival, several fish had moderately frayed fins. Two days after arrival, one fish became anorexic, displayed an increased respiration rate, and was noticeably darker than other batfish in the same tank. A wet mount examination of skin mucus of this batfish was negative for parasites. This fish was dipped in freshwater for 3 min d−1 for 2 d to help remove any parasites not detected by wet mount examination. All fish in the same tank were treated with a formalin bath at 100 mg l−1 for 6 h to control a suspected parasite infection. Fish were also treated prophylactically with an oxytetracycline (Aquafet®, PHARMAQ) bath at 100 mg l−1 daily for 7 d. Mortality began during the treatment and a total of 8 fish died during the 4 wk quarantine period. Gross necropsy findings were unremarkable; however, fleshy white nodules were noted on the dorsal fins of all fish that died and were also observed on the remaining 2 fish in the tank. Following necropsy, specimens were fixed in 10% neutral buffered formalin and sent to the International Zoo Veterinary Group (IZVG) Pathology Laboratory, UK, for histological processing and examination.

Histopathology

For 2 separate formalin-fixed fish that had been necropsied, 5 transverse sections and 1 sagittal section of the head were cut and dehydrated through a series of water and alcohol mixtures to full alcohol. Tissues were then cleared through a clearing agent and embedded into paraffin blocks (2 blocks per individual). The 4 paraffin blocks were sectioned at 3 µm, mounted on glass slides, and stained with hematoxylin and eosin (H&E). In addition to examination by IZVG Pathology Laboratory, these blocks were also submitted to the University of Florida Marine Animal Disease Laboratory, Gainesville, FL, USA.

Transmission electron microscopy

One paraffin block was selected by evaluating an H&E-stained slide for features consistent with megacytivirus pathology. Heart tissue was selected from this block for TEM evaluation and sent to the Electron Microscopy Laboratory (EML), Department of Medical Pathology and Laboratory Medicine, School of Medicine, University of California at Davis. An area with characteristic megacytivirus pathology in the heart was removed from the paraffin block and placed in 100% xylene; after clearing overnight, the tissue was rehydrated and processed using a standard protocol (Johannessen 1977) as previously described (Hayat 1989). Ultrathin sections (45 to 60 nm) were post-stained with 2% aqueous uranyl acetate followed by Reinold’s lead citrate and examined using a transmission electron microscope at the EML. These samples were also examined with an electron microscope at the University of Florida Electron Microscopy and Bio-imaging Laboratory.

DNA extraction, PCR amplification, and sequencing

At the University of Florida Marine Animal Disease Laboratory, DNA was extracted from 50 µm sections of formalin-fixed, paraffin-embedded tissues using a commercial extraction kit (DNeasy® Blood and Tissue Kit, Qiagen) following the manufacturer’s instructions for paraffin-embedded tissues. PCR amplification of the viral full-length major capsid protein (MCP) gene sequence was performed using 14 primer pairs designed from the complete MCP gene sequence of BCIV (which is also known as the Pterapogon kauderni iridovirus or PkIV; GenBank accession no. AB669096.1) (Table 1).

The 20 µl PCR mixtures consisted of 0.1 µl of Platinum Taq DNA Polymerase (Invitrogen), 2.0 µl of 10× PCR buffer, 0.8 µl of 50 mM MgCl₂, 0.4 µl of 10 mM dNTPs, 1.0 µl of 20 µM of forward and reverse primers, 11.7 µl of molecular grade water, and 3 µl of DNA template. The PCR conditions used for all reactions included an initial denaturation of 5 min at 95°C, followed by 50 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min, followed by a final elongation step at 72°C for 10 min. After electrophoresis, bands were gel extracted using a QIAquick gel extraction kit (Qiagen). Purified DNA fragments were then submitted to the University of Florida Interdisciplinary Center for Biotechnology Research for sequencing on ABI 3130 DNA sequencers (Applied Biosystems).
Following sequence assembly and removal of primer sequences, general BLASTN searches (www.ncbi.nlm.nih.gov/blast/Blast.cgi) of the full-length viral MCP gene sequence were conducted (Altschul et al. 1997). Megalocytiviral taxa identified by these analyses were combined with taxa from recent comprehensive phylogenetic analyses (Kurita & Nakajima 2012, Waltzek et al. 2012) to build the final data set. Sequence alignments were performed using Mafft 5.8 (Katoh et al. 2005) followed by minor manual adjustments in ClustalW (Thompson et al. 1994). The E-INS-I alignment strategy was used with the following parameters: scoring matrix BLOSUM62, a gap open penalty of 1.53, and an offset value of 0. The final aligned data set was trimmed to the first conserved nucleic acid at the 3’ end of the MCP sequence due to incomplete data being available for certain important taxa. The aligned data set was imported into jModelTest version 0.1.1 (Guindon & Gascuel 2003, Posada 2008) and Akaike’s information criterion was used to select a best-fit model of evolution for phylogenetic analysis. Maximum likelihood analyses were conducted using MEGA version 5 (Tamura et al. 2011) with 1000 bootstrap replicates selected for determining node support.

### RESULTS

#### Histopathology

Histopathologic examination revealed multiple areas of necrosis with pyknotic and karyorrhectic cellular debris in the renal hematopoietic tissue (with occasional associated minimal epithelial necrosis) (Fig. 1A), liver, and spleen. Numerous cytomegalic cells containing granular basophilic intracytoplasmic inclusions were commonly observed in the renal interstitium and glomeruli of both specimens (Fig. 1A). Similar cytomegalgy with associated inclusions were also observed within the spleen, liver, gill lamellae (Fig. 1B), lamina propria and submucosa of the esophagus, stomach (Fig. 1C), intestine, and heart (Fig. 1D).

#### Transmission electron microscopy

TEM revealed numerous icosahedral virus particles arranged in paracrystalline arrays consistent with an iridovirus within the cytoplasm of enlarged cardiac myocytes (Fig. 2). The observed viral assembly sites appeared to disrupt the myocyte myofibrils (Fig. 2B). Virus particles were naked with an electron-dense nucleic acid core surrounded by a translucent zone and an outer nucleocapsid layer (Fig. 2C,D). The mean diameter of virus particles, measured from apex to apex, was 158 nm (n = 50, SD = 7.5 nm), and from side to side was 128 nm (n = 50, SD = 5.6 nm).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5’−3’)</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>MCPF1</td>
<td>Forward</td>
<td>CTGTTGGTCTTGTGACGTGC</td>
<td>158</td>
</tr>
<tr>
<td>BeginR</td>
<td>Reverse</td>
<td>GCGTACAGTGCCAGTGACGTGC</td>
<td>177</td>
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<tr>
<td>Angelnew1F</td>
<td>Forward</td>
<td>GCCACCGTGACACTAAGCTC</td>
<td>194</td>
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<tr>
<td>Angelmcp2R</td>
<td>Forward</td>
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<td>150</td>
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<td>Angelmcp3R</td>
<td>Reverse</td>
<td>GCATGCCAAATCATCTTGT</td>
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Table 1. Primers used to amplify fragments of the major capsid protein gene sequence from the orbiculate batfish iridovirus (OBIV). Note that amplicon size includes primers.
The sequenced PCR amplicons generated in this study ranged in size from 121 to 214 bp (Table 1) and when assembled resulted in 1470 contiguous bp of the viral genome that contained the full-length MCP sequence (1362 bp; BankIt no. 1592328). The BLASTN search of the viral MCP sequence from the infected batfish revealed highest sequence identity with ISKNV genotype 2 megalocytiviruses, including BCIV (GenBank accession no. AB669096; 100%) and MSGIV (GenBank accession no. HM067835; 99%). The final aligned MCP data set after truncation of the 3' end of the sequence contained 1357 nucleic acid characters for 90 taxa. jModelTest identified the TrN+G model to be the most suitable model for phylogenetic analyses.

The phylogenetic analysis of the MCP gene supported the recognition of 4 species within the genus Megalocytivirus (Fig. 3). The Maximum Likelihood analysis demonstrated with a high level of confidence that the identical virus from orbiculate batfish (hereafter referred to as the orbiculate batfish iridovirus, OBIV) and Banggai cardinalfish (BCIV) is the sister group to MSGIV. These 2 megalocytiviruses form the ISKNV genotype 2 clade (Fig. 3).
Fig. 3. Phylogram depicting the relationship of the orbiculate batfish iridovirus (OBIV) to representatives from each of the 4 megacystivirus species (red sea bream iridovirus [RSIV], infectious spleen and kidney necrosis virus [ISKNV], turbot reddish body iridovirus [TRBIV], and threespine stickleback iridovirus [TSIV]) in the family Irido- viridae, based on the sequence of the major capsid protein (MCP; 1337 nucleic acid characters including gaps). Numbers above or below each node represent bootstrap support of the maximum likelihood analysis (values >80 shown). Branch lengths are based on the number of inferred substitutions, as indicated by the scale. The red arrow shows the position of OBIV. Accession numbers, host, and location of isolation are listed for each operational taxonomic unit. *Note: OBIV and the Banggai cardinalfish iridovirus (BCIV, which is also known as the Pterapogon kauderni iridovirus or PkIV_AB669096_Banggai cardinalfish_SouthEast Asia) were treated as a single taxon in the phylogenetic analysis given their identical MCP sequences.
DISCUSSION


The OBIV full-length MCP sequence was found to be identical to the only other megalocytivirus report-Kueh et al. 2003, Weber et al. 2009). (Paperna et al. 2001, Sudthongkong et al. 2002, Gib-ornamental fishes, including Banggai cardinalfish tivirus infections in tropical freshwater and marine virions is also consistent with reports of megalocy-ivirus species (Kurita & Nakajima 2012). The size, icosahedral shape, and intracytoplasmic location of virions is also consistent with reports of megalocy-ivirus infections in tropical freshwater and marine ornamental fishes, including Banggai cardinalfish (Paperna et al. 2001, Sudthongkong et al. 2002, Gib-son-Kueh et al. 2003, Weber et al. 2009).

Interestingly, concurrent with the orbiculate batfish mortality event, recently imported wild Banggai cardinalfish that had been kept at the same quarantine facility in the UK also died after delivery to another public aquarium in Denmark. Those fish were found to be positive for BCIV by histopathology and PCR (M. F. Stidworthy & T. B. Waltzek unpubl. data). Taken together with the genetic evidence, these data suggest that OBIV and BCIV are the same virus. This agent may be capable of infecting other species, similar to what has been reported for other megalocytivirus species (Kurita & Nakajima 2012).

It cannot be determined whether the imported wild orbiculate batfish and Banggai cardinalfish spread the virus to each other following importation into the UK or whether these species had already acquired the virus in the wild or at the export facilities. Although the Banggai cardinalfish is restricted to a small region in Indonesia known as the Banggai archipelago, the orbiculate batfish occurs sympatrically, and both species are sometimes cultured on the same Indonesian ornamental fish farms for sale in international ornamental fish markets (T. B. Waltzek pers. obs.). Future surveillance efforts are needed to determine where marine ornamental fishes become infected with megalocytiviruses as well as to assess the overall impact on international trade (e.g. frequency of epizootics and number of susceptible species).

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