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Characterization of a ranavirus isolated from cultured largemouth bass (*Micropterus salmoides*) in China

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ABSTRACT

During June through October 2008, numerous cultured largemouth bass (*Micropterus salmoides*) died in the Foshan area of Guangdong Province, China. Affected fish had ulcerations on the skin and muscle. Epithelioma papillosum cyprini (EPC) cell cultures inoculated with filtrates of muscle homogenates from the diseased fish developed the cytopathic effect (CPE) 5 days after inoculation at the primary passage. Transmission electron microscopy of the ulcerative muscle tissue and the infected EPC cells revealed a cytoplasmic, icosahedral virion that averaged 145 nm in diameter. Intramuscular injection of the virus ($\geq 10^{7.82}$ $TCID_{50}$ ml^{-1}) resulted in clinical signs of the disease and caused 100% mortality of healthy largemouth bass. In addition, the mandarinfish (*Siniperca chuatsi*) was susceptible to the virus after intramuscular injection of the virus (0.2 ml of $10^{9.82}$ $TCID_{50}$ ml^{-1}), and the mortality was 20%. However, no death or clinical signs were found in grass carp (*Ctenopharyngodon idella*), bighead carp (*Aristichthys nobilis*), Nile tilapia (*Oreochromis niloticus*), crucian carp (*Carassius carassius*), common carp (*Cyprinus carpio*), and fancy carp (*C. carpio*) after experimental infection. DNA fragments of the viral major capsid protein (MCP) and DNA methyltransferase (MTase) were amplified and sequenced. Subsequent multiple alignment and phylogenetic analysis revealed that the virus is identical to doctor fish virus (DFV) and closely related to largemouth bass virus (LMBV).

1. Introduction

The largemouth bass (*Micropterus salmoides*), which is native to freshwater areas in the southeastern United States, was introduced into China in 1983. It has been widely cultured in China because of its fast growth rate, wide temperature tolerance, adaptive plasticity to new environments, and appeal as a food source. In recent years, the annual output in China reached 100,000 t (Wu et al., 2006; Bai et al., 2008). Recently, the aquaculture industry in China has faced increases in epizootic diseases (Huang et al., 1996; Jin et al., 2005). An ulcerative syndrome in largemouth bass broke out in 2006 and progressed severely from June to October 2008. In a 0.3 ha pond in Huangsha Village of Foshan, about 5200 cultured largemouth bass died of the disease. Affected fish had a swollen spleen and kidneys, extensive ulcerations on the body surface, local haemorrhages and necrosis of naked muscle, and tumefaction and ulceration on the fin base.

Epizootic ulcerative syndrome is a common disease that affects both cultured and wild fish. The skin is the most susceptible target organ for infectious agents in fish (Noga, 1996). Bacteria, fungi, parasites, and viruses are considered to be the causative agents of infectious skin ulcerative syndrome (Frerichs, 1995; Andrew et al., 2008; Grove et al., 2008; Hemanand et al., 2008; Løvoll et al., 2009). For example, *Aeromonas hydrophila* and *Pseudomonas sp.* infection in largemouth bass in Lochloosa Lake, Florida, caused deep cutaneous ulcers and killed adult fish (Francis-Floyd et al., 1993). In China, strains of *A. hydrophila* and *Aeromonas sobria* were isolated from the ulcerative tissue of affected largemouth bass (Li et al., 1994). The fungus *Fusarium fusarioides* was detected in diseased largemouth bass, and experimental infection caused slight skin ulcerations (Huang et al., 1996). However, until now there have been no reports of a viral ulcerative disease in largemouth bass.

Iridoviruses are well known as causative agents of serious diseases in cultured fish, and they have been identified from at least 20 fish species (Hyatt et al., 2000; Zhang et al., 2004). The family *Iridoviridae* is divided into five genera: *Iridovirus*, *Lymphocystivirus*, *Chloriridovirus*, *Ranavirus*, and *Megalocytivirus*. Members of the family are icosahedral virions 120–300 nm in diameter that contain a double stranded linear DNA genome 100–210 kb in length. The viral genome encodes about 100 proteins (Chinchar et al., 2005). Major capsid protein (MCP) is the most abundant structural protein in the viral particle (40–45%). The MCP gene is highly conserved but distinct enough to allow identification of different iridovirus genera (Mao et al., 1997; Tidona et al., 1998).

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Ranaviruses have been isolated from aquatic vertebrates at different taxonomic levels, including the redfin perch (*Perca fluviatilis*), largemouth bass (*M. salmoides*), ornate burrowing frog (*Limnodynastes ornatus*), pig frog (*Rana grylio*), green python (*Chondropython viridis*), and leopard tortoise (*Geochelone pardalis pardalis*) (Langdon et al., 1986; Speare and Smith, 1992; Hengstberger et al., 1993; Plumb et al., 1996; Mao et al., 1999; Zhang et al., 2001; Hyatt et al., 2002; Benetka et al., 2007). The largemouth bass virus (LMBV), a member of the genus *Ranavirus* (Mao et al., 1999), was first isolated in Florida and since has been found in several other locations in the southeastern United States (Plumb and Zilberg, 1999a; Grizzle et al., 2002).

In this study, we detected the presence of a ranavirus that caused ulcerative syndrome in largemouth bass in China. We isolated, propagated, and characterized the virus, and we tested its pathogenicity in experimentally infected largemouth bass and seven other freshwater fish species. Comparison of the predicted amino acid sequences of major capsid protein (MCP) and DNA methyltransferase (TMase) indicated that the virus is closely related to doctor fish virus (DFV) and LMBV.

2. Materials and methods

2.1. Necropsy of largemouth bass

Twenty moribund largemouth bass (mean weight 200 g) were collected from Huangsha Village on June 18, 2008, placed in 20 plastic bags containing crushed ice, and shipped to Pearl River Fisheries Research Institute within 2 h for necropsy. Each fish was dissected to allow observation of pathological changes in the viscera.

2.2. Virus preparations

The virus sample was isolated from a pool of infected largemouth bass muscle (1 g) of five individuals. Muscle tissue was dissected from infected fish, pooled, homogenized in 10 ml 199 medium (Gibco, USA) without fetal bovine serum (FBS), and centrifuged at 9600 g for 30 min at 4 °C. The supernatant was filtered through a sterile 0.22 μm filter, and the filtrate was collected for cell inoculations. In addition, 50 μl of filtrate was cultured on nutrient agar plates for bacteriological examination.

2.3. Virus infection, replication, and titration

Epithelioma papillosum cyprini (EPC) cells (Fijan et al., 1983) were cultured in 199 medium supplemented with 10% FBS (Gibco, USA) in 25 cm^2 cell culture flasks at 28 °C for 24 h. Next, serial 10-fold dilutions of the stock virus filtrate were made in Hanks' balanced salt (Gibco, USA) solution. Dilutions were prepared from 10^{-1} to 10^{-4}. One milliliter of filtrate dilution was inoculated onto a monolayer of EPC cells and allowed to absorb for 1 h. The inoculum then was removed and the monolayer cells were incubated in fresh culture medium containing 2% FBS at 28 °C. Cell cultures then were examined daily. For virus propagation, the monolayer cell culture in each flask was infected with 1 ml of virus isolates and incubated at 28 °C until the cytopathic effect (CPE) was detected. Infected cells exhibiting the CPE were examined by transmission electron microscopy.

The viruses were titrated using a limiting dilution assay on EPC cell monolayers grown in 96-well plates. After EPC cells were cultured for 24 h, the medium was drained. Serial 10-fold dilutions (10^{-1} to 10^{-10}) of virus suspension in 199 medium with 2% FBS were inoculated into EPC cell monolayers and incubated at 28 °C. Each dilution was repeated in six wells, and two wells without virus inoculation served as controls. The virus titers were expressed as the $TCID_{50}$ according to the method of Reed and Muench (1938).

2.4. Pathogenicity assay

2.4.1. Intramuscular injection trials in largemouth bass

One hundred and forty healthy largemouth bass (50–80 g) with no previous history of disease were collected from the culture farm at Pearl River Fisheries Research Institute located in Guangzhou city of Guangdong province. The sampled fish were randomly divided into thirteen experimental groups and one control group and transferred into 100 l tanks (10 specimens per tank as one group) with cycling water at 28 °C. Serial 10-fold dilutions of propagated virus isolates of the third and the fifth passage were prepared for injection. Largemouth bass in the experimental groups were challenged by intramuscular (IM) injection with 0.2 ml of propagated virus. Fish in the control groups received IM injection of an equivalent volume of normal cell culture. Ulcerative muscle of infected fish was examined by electron microscopy.

2.4.2. Intramuscular injection trials in seven fish species

Healthy mandarinfish (*Siniperca chuatsi*) (average weight 40 g), grass carp (*Ctenopharyngodon idella*) (average weight 52 g), bighead carp (*Aristichthys nobilis*) (average weight 99 g), Nile tilapia (*Oreochromis niloticus*) (average weight 48 g), crucian carp (*Carassius carassius*) (average weight 46 g), common carp (*Cyprinus carpio*) (average weight 36 g), and fancy carp (*C. carpio*) (average weight 43 g) were obtained from freshwater fish farms in central Guangdong Province, China. For each of the eight species tests, twenty individuals were divided into two groups, a group received the IM injection and a group served as control. Each group was kept in a 100 l tank at 28 °C. After 24 h starvation, largemouth bass (average weight 38 g) as the positive control and the seven fish species in the experimental groups received an IM injection of 0.2 ml of propagated virus at a concentration of $10^{9.82}$ $TCID_{50}$ ml^{-1}. Fish in the negative control groups were injected with an equivalent volume of cell culture medium. The mortality was calculated 20 days after infection.

2.5. Transmission electron microscopy

For transmission electron microscopy analysis, 0.5–1.0 mm^3 samples of the ulcerative muscle from naturally and experimentally infected fish were cut out and fixed in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4 °C for 3 h. For analysis of the monolayers of EPC cells grown in 25 cm^2 cell culture flasks that were inoculated with the virus isolate, after the appearance of 70% CPE, infected cells were harvested and pelleted by centrifugation at 2400 g for 15 min. The cell pellets were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h. After several rinses with 0.1 M phosphate buffer, each sample was post-fixed with 1% OsO_4 for 3 h. Both the tissue samples and the EPC cells were subsequently dehydrated in an ethanol series and transferred to acetone before being embedded in Spur's resin. Ultrathin sections were prepared using an RMC-MTX ultramicrotome (Tucson, Arizona, USA). The sections were stained with lead citrate and uranyl acetate and observed under a Philips-CM10 transmission electron microscope (Eindhoven, Netherlands).

2.6. Molecular analysis

2.6.1. Isolation of DNA

DNA was extracted from infected EPC cells and necrotic fish muscle using the TIANamp Genomic DNA Kit (Beijing, China) and then used as a PCR template. DNA quality was assessed by electrophoresis on 0.7% agarose gel with ethidium bromide staining.

2.6.2. PCR amplification and cloning

Primers targeting conserved regions of the ranavirus MCP and MTase genes were designed following Mao et al. (1999). The sequences of individual primers were as follows: MCP forward