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Full Length Research Paper

Ranavirus (family *Iridoviridae*) detection by polymerase chain reaction (PCR) in Chinese giant salamander (*Andrias davidianus,* Blanchard, 1871), China

Z. Y. Zhou[1], Y. Geng[1,2]*, S. Y. Ren[1], K. Y. Wang[1,2], X. L. Huang[3], D. F. Chen[3], X. X. Liu[1] and W. M. Lai[1]

[1]College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, Sichuan 625014, People's Republic of China.
[2]Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, Sichuan 625014, People's Republic of China.
[3]Department of Aquaculture, Sichuan Agricultural University, Ya'an Sichuan 625014, People's Republic of China.

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A disease in farmed Chinese giant salamander (*Andrias davidianus*) was a common event, being an economically important threat for Chinese farms. Based on the clinical signs, epizootiology and pathogens belonging to the genus, *Ranavirus* was suspected as the possible etiology. Although in a cultured Chinese giant salamander (*Andrias davidianus*) farm in Hanzhong County, Shanxi Province, China, a ranavirus infection case had been reported, the presence in other farms was never mentioned so far. The objective of this study was to detect the presence of ranaviral agents in affected Chinese giant salamanders using polymerase chain reaction (PCR) assay. Major capsid protein (MCP) gene was selected as the targets to amplify the high conserved fragment. Positive PCR results were obtained when sick Chinese giant salamanders from Sichuan and Gansu Province were analyzed. Purified and sequenced PCR products showed high degree of identity with several members of *Iridoviridae*, mostly with those belonging to the genus *Ranavirus* in GenBank BLAST searches. Obtained sequences were registered in the GenBank with accession numbers HQ684750, JN590256 and JN651174. This report indicated that *Ranavirus* should be considered a common disease etiology throughout these geographical regions.

Key words: Polymerase chain reaction (PCR), *iridovirus*, *Ranavirus*, *Andrias davidianus*, Chinese giant salamander.

INTRODUCTION

The Chinese giant salamander (*Andrias davidianus*, Blanchard, 1871), which belongs to the order Caudata and family Cryptobranchidae, is the largest ancient animal with the longest life among the exiting amphibians in the world. It was listed in CR threaten category by IUCN/SSC in 2004, and was listed as top of the 10 oldest endangered amphibians in the world by London animal association of UK in 2008. Due to its good meat quality, high medical value and wide market acceptance in China, the Chinese giant salamander culture is an expanding activity in China. Improvements obtained in farming techniques allowed Chinese giant salamander farmers to increase densities and as a consequence disease, susceptibility also had arisen. The occurrence of various diseases has limited the sustainable development of the Chinese giant salamander culturing.

Since determining that ranaviruses were an etiologic agent in amphibian die-offs in the early 1990s, ranavirus-associated mortality had been reported on 5 continents, at all latitudes and elevations that amphibians inhabit, and especially in most of the major families of Anura and Urodela (Cunningham et al., 1996; Docherty et al., 2003; Daszak et al., 2003; Ariel et al., 2009; Balseiro

*Corresponding author. E-mail: gengyisicau@126.com. Tel: +86 0835 2885753. Fax: +86 0835 2885302.

et al., 2009; Geng et al., 2011). *Ranavirus* is classically described as causing systemic disease, with marked tissue necrosis and mortality ranging from low to nearly 100% (Gray et al., 2009). Recognizing the potential threat of ranaviruses to global amphibian biodiversity, ranavirus infections had been added to the list of “notifiable” diseases by the world organization for animal health.

This means that international trade of live amphibians and related products now require applying the health certifications according to OIE standards, making it obligatory for both the public and the OIE to be notified about the detection of ranavirus infection. Although a ranavirus infection case had been reported about a cultured Chinese giant salamander (*Andrias davidianus*) farm in Hanzhong County, Shanxi Province, China (Geng et al., 2011), the presence in other farms was never mentioned so far. From May 2010 to September 2011, some diseased Chinese giant salamanders were collected from Sichuan and Gansu Province. Based on clinical signs and epizootiology, pathogens belonging to the family *Iridoviridae* were suspected as the possible etiology. The objective of this work was to detect the presence of ranaviral agents in affected Chinese giant salamanders using polymerase chain reaction (PCR) assay.

MATERIALS AND METHODS

Sample collection

From May 2010 to September 2011, eighteen sick Chinese giant salamanders with similar clinical signs and gross lesions were collected from three farms which respectively are located in Longnan County of Gansu Province, Leshan and Gongxian County, Sichuan Province, in China. All the three farms bred the giant salamanders in caves, fed with fresh fish or frogs, used the subsoil water and changed regularly. The samples were transferred alive to the key laboratory of animal disease and human health of Sichuan Province to determine if the cause of the disease was *Ranavirus* infection.

DNA extraction and primers

Tissues (typically kidney and liver) were collected from the remaining carcasses and frozen at -20°C for *Ranavirus* examination. Genomic DNA was extracted from tissues using a commercially available kit (TaKaRa, Dalian, China). DNA from tissues was used as a template and amplified by PCR using primers (forward primer: 5′-GACTTGGCCACTTATGAC-3′, 5′-GTCTCTGGAGAAGAAGAA-3′) targeted to highly conserved regions of the major capsid protein (MCP) gene of Ranaviruses (Mao et al., 1997; Gray and Miller, 2009). These primers were used to yield an expected fragment size of approximately 500 bp.

PCR reaction and sequencing

PCR reaction was performed in a Biorad Thermal Cycler. For each reaction, mixture was 0.5 µM of each primer, 50 µM of each nucleotide (dATP, dTTP, dGTP, dCTP), 10× PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3), 2 mM $MgCl_2$ and 2.5 U of Taq polymerase (TaKaRa, Dalian, China) 2 µl of DNA template and distilled water to complete a 25 µl total volume. PCR amplification conditions were 1 cycle at 94°C for 4 min, followed 30 cycles of 95°C for 1 min, 48°C for 1 min and 72°C for 1 min, and finally elongated at 72°C for 10 min. The PCR products were resolved by 0.8% agarose gel electrophoresis for determination of the presence/absence of the expected fragment size of approximately 500 bp. One distinct PCR-positive band was randomly and respectively chosen from three farms, cut from the gel, and purified with the Gel DNA Purification Kit (TaKaRa, Dalian, China). PCR-positive bands were sequenced using the AMI PRISM 377 automated sequencer (Shanghai Invitrogen Biotechnology Co. Ltd., Shanghai, China). A sequence-similarity search of the National Center for Biotechnology Information (NCBI) database was conducted using the Basic Local Alignment Search Tool (BLAST) algorithm. The program Clustal × 1.8 with default parameters was run for multiple sequence alignment. Phylogenetic and distance analysis of the aligned sequences were performed using the program MEGA 4.1 (Tamura et al., 2007). The amino acid sequence was deduced and a comparison was performed using Blast search tool (http:// www.ncbi.nlm.nih.gov/BLASTp/).

RESULTS

The clinical signs and gross lesions of sick Chinese giant salamanders included lethargy, slow movement, anorexia, bloody stools, vomiting (occasionally bloody), small pale raised foci in the skin, and cutaneous erosions and ulcers; Ecchymosis and swollen areas were noted on the head, ventral surface and limbs, and in some cases necrosis and rotting occurred in the limbs. Liver and kidney tissues collected from all diseased Chinese giant salamanders were PCR positive for *ranavirus*. The 500 bp fragment of the *ranavirus* major capsid protein (MCP) gene in each test sample was amplified (Figure 1), and then one of each farm was randomly selected for direct sequencing. A phylogenetic tree was constructed based on the MCP gene sequences (Figure 2), investigated in this study and the homologous sequences of other virus of the family *Iridoviridae*.

The sequences (GenBank accession numbers HQ684750, JN590256 and JN651174) investigated in this study together with *Rana grylio* virus (AY294406), Ambystoma tigrinum virus(AY552611), Soft-shelled turtle iridovirus (DQ335253) and Frog virus3 (U36913) formed a cluster with 95 to100% sequence similarities. The corresponding protein sequences GenBank accession numbers ADZ47912, AEO92417 and AEX93338), compared with other *ranavirus* MCP sequences by Blast search tool (http:// www.ncbi.nlm.nih.gov/BLASTp/) revealed 100% identity with Testudo hermanni ranavirus MCP (gb|AAP47820.1), Rana grylio virus MCP (gb|AEK81557), Epizootic haematopoietic necrosis virus MCP (gb|ACO25204), Pike-perch Iridovirus MCP (gb|ACO90019), Frog virus 3 MCP (gb|ACR15869); 99% identity with Largemouth bass ulcerative syndrome virus MCP (emb|CBW45582) and Singapore grouper Iridovirus MCP (gb|AAS18087); and 98% identity with Ambystoma tigrinum virus MCP (gb|AAS49505, gb|AAS49511, gb|AAT57923).

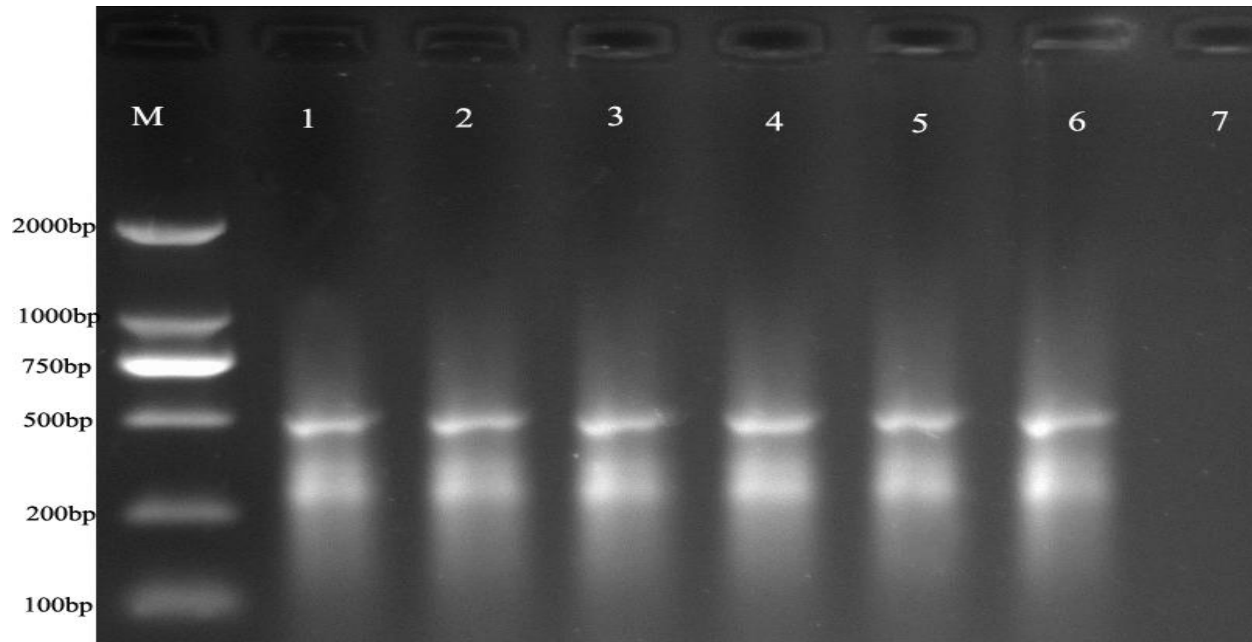

Figure 1. *Ranavirus* detection by PCR in Chinese giant salamander. Lane M: Standard marker; lanes 1 to 2: Samples from Lashan; lanes 3 to 4: Samples from Gongxian; lanes 5 to 6: Samples from Longnan (*Ranavirus* infection); lane 7): negative control.

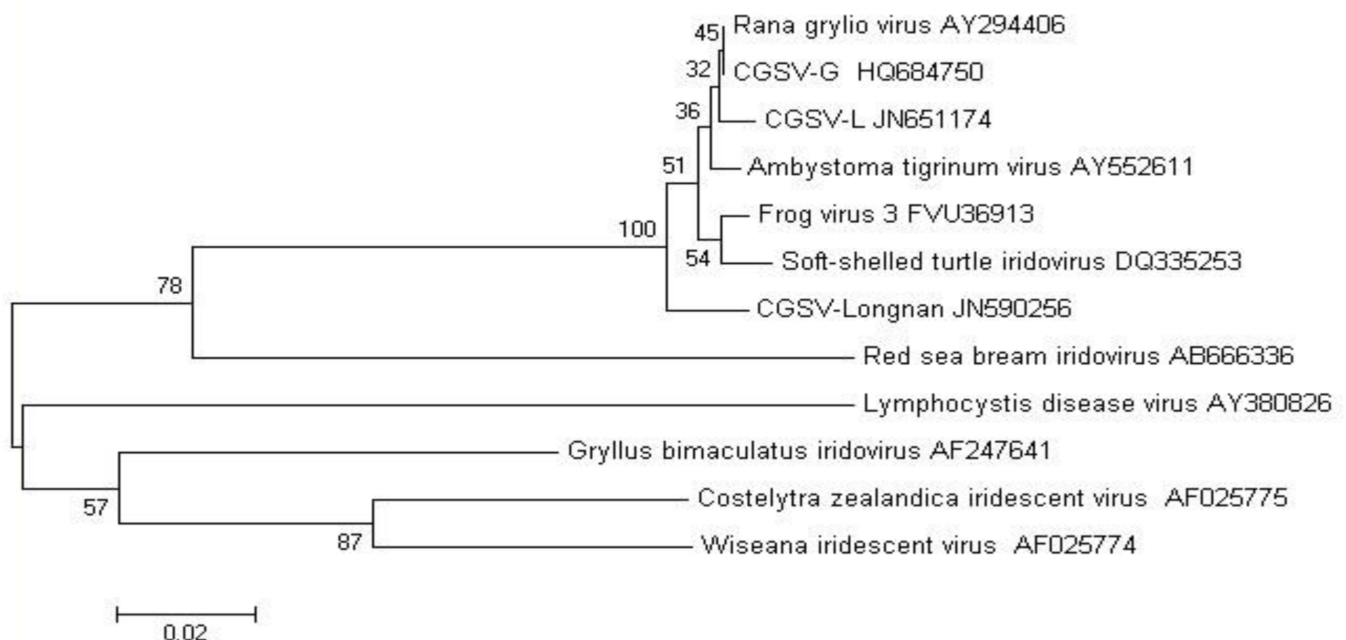

Figure 2. The phylogenetic tree was generated based on MCP gene sequences by using the neighbour-joining method; Maximum Composite Likelihood; 1000 bootstrap replicates. The bootstrap values (%) were shown besides the clades, accession numbers were written besides the name of virus, and scale bars represented distance values.

DISCUSSION

Amphibian populations are declining globally (Houlahan et al., 2000; Stuart et al., 2004). While there are a number of factors that have contributed to these declines, emerging infectious diseases have been regarded as one main factor (Daszak et al., 2003). *Ranaviruses* infections have become more prevalent and are increasingly associated

with mass amphibian dies-off of wild as well as cultured populations (Ariel et al., 2009; Green et al., 2002; Gray et al., 2009; Geng et al., 2011). *Ranavirus* have been regarded as emerging cold-blooded killers, which have a significant impact on diverse populations of ectothermic animals (Chinchar, 2002; STORFER et al., 2007). Muths et al. (2006) reported that 43% of the reported amphibian die-offs in the United State from 2000 to 2005 due to ranaviruses (Muths et al., 2006). Similarly, Green et al. (2002) reported that 57% of the mortality events investigated by the United States Geological Survey National Wildlife Health Center from 1996 to 2001 were wholly or partially caused by ranaviruses.

The first ranavirus infection in China was reported in a mass die-off of the pig frog, *Rana grylio*, in 2001 (Zhang et al., 2001), and then ranavirus infection of cultured Chinese giant salamander in Hanzhong County, Shanxi Province was reported in 2011 (Geng et al., 2011). As far as we know, ranavirus infection of cultured Chinese giant salamander in other regions was never mentioned. In the present study, ranavirus infection of cultured Chinese giant salamander was detected by PCR in some farms of Sichuan and Gansu Province. The results indicate that *Ranavirus* should be considered a common disease etiology throughout these geographical regions. And the association of these viruses with diseases and mortality events is actually being studied.

Ranaviruses were highly virulent, with reported mortality rates often greater than 50% (Green et al., 2002; Bollinger et al., 1999; Gray et al., 2009). Risk factors for acquiring ranavirus infection in amphibians are associated with some natural stressors and anthropogenic factors, such as changes in ambient temperature (Gray et al., 2007), overcrowding (Green et al., 2002), and that appeared to be important regulating mechanisms in ATV-salamander systems (Greer and Collins, 2008), food limitation, threat of predation, or decreases in water quality from agricultural operations (Gray et al., 2009). Density-dependent infection and mortality associated with ranaviruses have been suggested based on field observations at die-off sites (Brunner et al., 2004; Green et al., 2002), and appear to be important regulating mechanisms in ATV-salamander systems (Greer and Collins, 2008; Brunner et al., 2007). Chinese giant salamanders are cultured under severely stressful conditions in China, especially overcrowding. Therefore, further study on the risk factors for acquiring ranavirus infection in Chinese giant salamanders should be conducted.

The relationships between the three isolations in this study and other iridoviruses of fish, insects and amphibians emerged in the phylogenetic analysis. The CGSV-L and CGSV-G were more similar with Ambystoma tigrinum virus and Forg virus 3, than CGSV-Longnan which was also on the same basal branch. It was suggested that the isolated virus belong to the *Ranavirus*. Nevertheless, more information about the isolates should be unequivocally to determine their taxonomic status. Although there is a remarkable identity in encoded proteins for MCP obtained sequences with other ranaviruses from reptilian, piscine and salamanders, there are some diversity of encoded proteins for MCP obtained sequence from Gansu Province and Sichuan Province. The encoded proteins for MCP obtained sequence from Longnan County, Gansu Province had 30 amino acids missing in the N-terminal. This data suggested that the Chinese giant salamander ranavirus might have different biological characteristics from different regions. An epidemiological survey and more information on genomic sequences should be performed to determine whether the Chinese giant salamander ranaviruses have diversity in Sichuan and Gansu Provinces.

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