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FV3-like ranavirus infection outbreak in black-spotted pond frogs (*Rana nigromaculata*) in China

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ABSTRACT

In April 2016, an outbreak emerged in a cultured population of black-spotted pond frog tadpoles in Shuangliu County, China, whereas tadpoles were suffering from substantial mortality (90%). Principal clinical signs of diseased tadpoles were comprised haemorrhage on their body surface, swollen abdomen with yellow ascites, congestion and swelling of the liver. The diseased tadpole's homogenates tissue were inoculated into epithelioma papulosum cyprini (EPC) cells at 25 °C for 4 days which caused typical cytopathic effect, and the viral titer $TCID_{50}$ reached 10^7/0.1 mL. In pathogenicity tests, tadpoles were immersed in 2‰ virus fluid for 8 h, the clinical signs were observed similar to those recognized in naturally infected tadpoles and mortality rate were reached up to 80%, which affirms that the virus was the main cause for this disease. In addition, transmission electron microscopy of EPC cells infected with isolated virus reflected that the virus was in a regular hexagon way (shape) with capsule like structure. The diagonal diameter was recorded 135 ± 8 nm, wherever virus particles were arrayed in crystalline manner in the cytoplasm. The electrophoresis of MCP gene PCR-product showed that the samples of diseased tadpoles, aquaculture water source and isolated virus were all positive. The sequence of the isolate revealed more than 99% similarities to ranavirus based on homology and genetic evolution analysis of the whole MCP gene, and the isolate belongs to FV3-like virus group. This study confirmed that ranavirus was the causative agent of this outbreak, and named the virus as *Rana nigromaculata* ranavirus (RNRV).

1. Introduction

Ranavirus belongs to the family *Iridoviridae*, which composed of a unique DNA double-stranded linear molecule with variable size ranging from 105 to 212 kbp depending on the species [1]. At the beginning of mid-1980s continuing to till present, cases of the ranavirus infection and disease have been documented in six continents and at least 175 species of ectothermic vertebrates [2]. Ranaviruses are now viewed as pathogenic agents capable of infecting all classes of ectothermic vertebrates (fish, reptiles, and amphibians) and triggering significant morbidity and mortality [3,4].

The first ranavirus was isolated from northern leopard frogs (*Lithobates pipiens*) from the Midwest USA in the 1960s [5]. Although in the following decades, reports of ranavirus-related mortality and infection in amphibians have grown rapidly, and observed 22 genera infected ranavirus just in *Ranidae*. In China, the first ranavirus was isolated from the cultured frogs *Rana grylio* in 1996 [6], at the same time within the following years the reports about ranaviruses occurred rapidly. In 2002, Outbreaks of ranavirus in cultured tiger frog (*Rana tigrina rugulosa*) were reported in Southern China [7]. Similarly in 2009, ranavirus infection associated with mass mortality in cultured Chinese giant salamanders (*Andrias davidianus*) was recognized in Shanxi Province, China [8]. Next in 2010, the free-ranging *Rana dybowskii* was detected ranavirus at seven sites throughout Heilongjiang Province of China [9], which confirmed that in China, ranavirus is a serious threat to ectothermic vertebrates, especially amphibians.

As an Asian water frog, black-spotted pond frogs are mainly distributed in China, Japan and Korea. It is not only a typical and useful material in amphibian researches [10], but also a kind of delicious food which has been loved by the people of China. In recent years many frogs farms have been set up among Sichaun, Chongqing, and Hunan Provinces of China, however, increasing diseases plagued the development of this industry. In April 2016, a sudden disease outbreak occurred in black-spotted pond frogs farms in Shuangliu, Sichuan Province. The estimated mortality in the farms was about 90% of the cultured tadpoles. According to the bacteriological, parasitological and virological

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examinations, a FV3-like ranavirus was confirmed the causative agent of this natural epizootic incident. To the best of our knowledge, this is the first report on FV3-like ranaviral infection in black-spotted pond frogs in China.

2. Materials and methods

2.1. Sample collection and examination

Twenty diseased tadpoles along with part breeding frogs' eggs were collected alive, rinsed with sterile water and placed in individual glass jars, respectively. Three water samples of 2 L specification were collected in sterilized beaker from the intake water which was using to raise tadpoles. All samples were brought into the laboratory for further detection and 2 L of water sample was concentrated into 1 mL for viral testing with NaCl-$AlCl_3$•$6H_2O$ precipitation method [11]. The animals were humanely euthanized by transdermal exposure to MS-222 (Wanke, Chengdu, China) before necropsies were performed.

Parasitological examination: skin section was observed under an optical microscope for the parasite analysis. Bacteriological examination: The samples were obtained from the kidney, liver and spleen of diseased tadpoles and streaked directly onto the brain heart infusion agar (BHIA, Difco, and Detroit, MI, USA), and incubated at 28 °C for 24–48 h. Virological examination: Kidney and spleen samples were homogenized and diluted to 1:10 with Hanks' balanced salt solution (HBSS) and then filtered directly onto confluent monolayers of EPC cells at 25 °C. A cultures demonstrating viral cytopathic effect were harvested and isolates were verified by the subsequent electron microscopy and molecular testing.

2.2. Electron microscopy

Monolayers of EPC cells were inoculated in the virus culture at the rate of 2.8×10^6 PFU mL^{-1}. The infected cells were collected at 48 h after post incubation and fixed with 2% glutaraldehyde in phosphate buffer solution (pH 7.3, 0.1 M) at 4 °C for 1 h, then post-fixing with 1% osmium tetroxide (OsO_4). The samples were then dehydrated using a series of graded alcohols and embedded in epoxy resin and the blocks were sectioned at 50 nm, stained with uranyl acetate, lead citrate, and analyzed through transmission electron microscope.

2.3. Pathogenicity tests

Healthy black-spotted pond frog tadpoles with average body weight of 3.0 ± 0.5 g were used for the pathogenicity test. Each group consisted of 20 individuals in a 10 L plastic tank while water temperature was kept constant 22 ± 2 °C. After seven days of acclimatization, tadpoles for the experimental group were exposed to 5 L viral suspension with a 2‰ dilution for 8 h, beside this the aquarium water were renewed completely, whereas the tadpoles of negative control group were treated similarly except replacing the viral suspension from the cell culture medium (M199). Tadpoles were checked on daily basis for clinical signs and mortality. Dead tadpoles were collected and their tissues (liver, kidney and spleen) were attained for PCR detection.

2.4. PCR detection

DNA was extracted by using the DNA Tissue Kit (Takara, Dalian, China) from tissue homogenates, infected cell culture supernatants and water sample. The extracted DNA was utilized as a template and amplified by PCR using primers (forward primer: 5′-GACTTGGCCACTTA TGAC-3′ and reverse primer: 5′-GTCTCTGGAGAAGAAGAA-3′) targeted to highly conserved regions of the major capsid protein (MCP) gene of ranaviruses [12]. These primers were used to obtain an expected fragment size of approximately 500 base pairs (bp). The PCR products were confirmed by electrophoresis using 1% (w/v) agarose gels that were stained with 0.1 μL mL^{-1} Agerviw nucleic acid dyestuff (Bionova, China), followed by direct sequencing (Tsingke, Chengdu, China) and sequence identification using a GenBank BLAST search.

2.5. Phylogenetic analysis

Two primers (forward primer: 5′-ACAGTCACCGTGTATCTTA-3′ and 5′- GGAAAAGACTTTGCGCTGAA-3′) were used to amplify the complete MCP gene of ranavirus [13]. The resulting PCR products were cloned into a pMD19-T vector (Takara, Dalian, China), followed sequenced at Tsingke (Chengdu, China). A phylogenetic tree based on the complete MCP gene of ranavirus was constructed by the neighbor-joining method in MEGA 5.1 software.

3. Results

3.1. Examination of diseased tadpoles

Tadpoles started to show field signs of disease and death beginning in April 2016 at black-spotted pond frogs farms in Shuangliu, Sichuan Province, at water temperatures of 22 °C. The cumulative mortality of tadpoles was reached to approximately 90% in this case. Typically, infected tadpoles stopped feeding and become lethargic and then died within few days. Macroscopic lesions included haemorrhage in the underjaw and abdomen of tadpoles, and swollen abdomen with yellow ascites were highlighted (Fig. 1). During the necropsy, congestion and swelling of the liver was also observed, and the intestine was noticed empty and filled with a lot of mucus.

No parasites were observed on the body surface of the diseased tadpoles and no bacteria were isolated from the kidney, spleen or liver. Nevertheless, tissue filtrates from kidney and spleen produced CPE in EPC cells (Fig. 2A and B). Virus isolated from the tissue homogenates grew on EPC cells at 25 °C, and CPE was observed on the third day post-inoculation. The isolated virus was provisionally designated as MWH421017.

Examination of electron microscopy showed the presence of

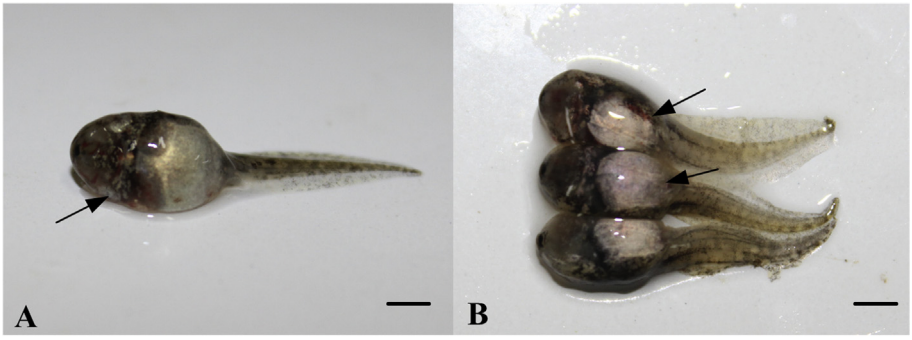

Fig. 1. Gross lesions in diseased tadpoles. A. Haemorrhage in the underjaw of tadpoles. B. Haemorrhage and edema in the abdomen of tadpoles. Bar = 0.25 cm.