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First Report of a Ranavirus Associated with Morbidity and Mortality in Farmed Chinese Giant Salamanders (*Andrias davidianus*)

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Summary

From February to May 2010, an outbreak of disease occurred amongst farmed Chinese giant salamanders (*Andrias davidianus*) in Hanzhong County, Shanxi Province, China. Clinical signs included anorexia, lethargy, ecchymoses and swollen areas on the head and limbs, and skin ulceration. The aim of this study was to determine the cause of this disease. Necropsy examination revealed subcutaneous and intramuscular oedema, swollen and pale livers with multifocal haemorrhage, swollen kidneys with multifocal haemorrhage and distended fluid-filled intestines with areas of haemorrhage. Light microscopy revealed intracytoplasmic inclusions suggestive of a viral infection in a variety of organs, as well as degeneration and necrosis of these organs. Electron microscopy of ultrathin sections of the same tissues revealed iridovirus-like particles within the inclusions. Of the six specimens tested, all were positive for ranavirus major capsid protein (MCP) gene. Sequence alignments of the ranavirus MCP gene from these specimens showed 95–98% similarity with published ranavirus data. The virus, provisionally designated as Chinese giant salamander virus (CGSV), was isolated from tissue homogenates of diseased salamanders following inoculation of epithelioma papilloma cyprini cells. Sequence analysis of the MCP genes showed that the isolated virus was a ranavirus with marked sequence identity to other members of the genus Ranavirus. Koch's postulates were fulfilled by infecting healthy Chinese giant salamanders with the CGSV. These salamanders all died within 6–8 days. This is the first report of ranavirus infection associated with mass mortality in Chinese giant salamanders.

Keywords: *Andrias davidianus*; Chinese giant salamander; iridovirus; pathology; ranavirus

Introduction

Iridoviruses are large enveloped viruses that contain a linear double stranded DNA genome. The family Iridoviridae currently contains five genera: Iridovirus and Chloriridovirus are associated with insects; Lymphocystivirus and Megalocytivirus infect fish species; and Ranavirus members infect fish, reptiles and amphibians and are associated with clinical illness that ranges in severity from inapparent to fulminant (Williams *et al*., 2005b). Ranaviruses are receiving increasing attention due to the severe losses they can cause to both wild and farmed fish and amphibian populations (Pozet *et al*., 1992; Cullen and Owens, 2002; Bigarré *et al*., 2008). Mass mortality of amphibians caused by ranaviruses has been reported in the Americas, Europe and Asia (Ariel *et al*., 2009; Gray *et al*., 2009; Une *et al*., 2009b). The first ranavirus infection in China was reported in an episode of mass deaths of pig frogs (*Rana grylio*) in 2001 (Zhang *et al*., 2001). There is evidence that ranaviruses may be an emerging infectious disease (Storfer *et al*., 2007), possibly due to emergence of a novel strain (Picco and Collins, 2008) or increased occurrence of anthropogenic stressors on the landscape (Forson and Storfer, 2006; Gray *et al*., 2007). Recognizing the potential threat of ranaviruses to global amphibian biodiversity, ranavirus infections have been added to

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the list of 'notifiable' diseases by the World Organization for Animal Health (OIE; http://www.oie.int/eng/maladies/en_classification2010.htm?e1d7). This means that international trade of live amphibians and related products now requires health certification to be applied according to OIE standards, making it obligatory for both the public and the OIE to be notified about the detection of ranavirus infection.

Ranaviruses negatively impact on amphibian populations throughout the world and have been associated with population fluctuations and mass mortality events (Collins and Storfer, 2003; Daszak *et al.*, 2003). Ranaviruses have been identified in tissues obtained from a variety of wild and captive salamander species, including the Sonoran tiger salamander (*Ambystoma tigrinum stebbinsi*) (Jancovich *et al.*, 1997), tiger salamander larvae (*Ambystoma tigrinum diaboli*) (Bollinger *et al.*, 1999), the spotted salamander (*Ambystoma maculatum*) (Douglas *et al.*, 2003), the Japanese clouded salamander (*Hynobius nebulosus*) (Une *et al.*, 2009a) and 10 species of lungless salamanders (such as *Desmognathus conanti*, *Desmognathus imitator*, *Desmognathus monticola*, *Desmognathus quadramaculatus* and *Eurycea wilderae*) (Matthew and Debra, 2009) that died in Canada, the USA and Japan. The Chinese giant salamander (*Andrias davidianus*), which belongs to the order Caudata and family Cryptobranchidae, is the largest extant species of amphibian. There are only three species in this family: the Chinese giant salamander (*A. davidianus*) in China, the Japanese giant salamander (*Andrias japonicus*) in Japan and the hellbender (*Cryptobranchus alleganiensis*) in North America (Regal, 1966). The Chinese giant salamander is classified as critically endangered by the International Union for Conservation of Nature and Natural Resources, is a class II state major protected species in China and is included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora. This species is in peril because of habitat loss, relatively slow growth to sexual maturity (6 years), poaching for human consumption, medicinal use and infectious disease (Wang *et al.*, 2004).

From February to May 2010, an outbreak of an infectious disease occurred in farmed Chinese giant salamanders in Hanzhong County, Shanxi Province, China. Symptoms included skin ulceration, anorexia, lethargy and occasionally oedema, and the mortality rate was high. The Key Laboratory of Animal Disease and Human Health of Sichuan Province performed diagnostic evaluations of this outbreak. Pathological and molecular studies confirmed that the infectious agent was a ranavirus. This is the first report of ranavirus infection associated with mass mortality in Chinese giant salamanders.

Materials and Methods

Sample Collection

From February to May 2010, a disease outbreak occurred in farmed Chinese giant salamanders in Hanzhong County, Shanxi Province, China. Larval, juvenile and adult salamanders were affected. During the outbreak, approximately 350 of 570 salamanders died. Twelve sick salamanders (four larvae, five juveniles and three adults) were collected and transferred alive to the Key Laboratory of Animal Disease and Human Health of Sichuan Province to determine the cause of the disease.

Necropsy Examination

Necropsy examinations were performed on all 12 Chinese giant salamanders. Samples for microscopical examination, including major viscera (liver, lung, heart, kidney, spleen, stomach and intestine), brain and any gross lesions, were collected from all 12 salamanders and fixed in 10% neutral buffered formalin. Formalin-fixed tissues were processed routinely and embedded in paraffin wax. Sections (4 μm) were stained with haematoxylin and eosin (HE).

For electron microscopical examination, selected areas of liver, kidney and spleen from five affected Chinese giant salamanders (two larvae, two juveniles and one adult) were cut into 1 mm^3 pieces and fixed in 2% glutaraldehyde in phosphate buffer (pH 7.3, 0.1 M) at 4°C. After post-fixation with 1% osmium tetroxide, the pieces were dehydrated through a series of graded alcohols, embedded in epoxy resin, sectioned at 50 nm, stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (TEM; JEM-1200EX, JEOL, Tokyo, Japan).

Bacteriological Examination

The body surface of each salamander was swabbed with 70% ethanol to prevent contamination. Samples from the kidney, liver, spleen and ascites fluid of each sick salamander were streaked directly onto sheep blood tryptone soy agar (TSA) and inoculated at 30°C. All isolates were identified using the API 20E system (BioMerieux, La Balm, France).

PCR for Ranavirus in Tissues

Tissues (typically lung, liver and spleen) were collected from the remaining carcasses and frozen at −20°C for virus examination. The previous epizootiological and clinical observations suggested that an iridoviral agent may have been the primary cause of the disease. Therefore, a conventional polymerase chain reaction (PCR)