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Detection of goldfish haematopoietic necrosis herpes virus (Cyprinid herpesvirus-2) with multi-drug resistant *Aeromonas hydrophila* infection in goldfish: First evidence of any viral disease outbreak in ornamental freshwater aquaculture farms in India

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ABSTRACT

This outbreak report details of a mortality event where Cyprinid herpes virus-2 (CyHV-2) was detected in association with multidrug-resistant *Aeromonas hydrophila* infection in goldfish, *Carassius auratus*, from commercial farms. The goldfish exhibited large scale haemorrhages on the body, fins and gills, lepidorthosis, necrosed gills, protruded anus and shrunken eyes. White nodular necrotic foci in spleen and kidneys were noticed, along with necrosis and fusion of gill lamellae. Transmission electron microscopy of affected tissues revealed the presence of mature virus particles. Involvement of CyHV-2 was confirmed by PCR, sequencing and observed cytopathic effect in koi carp fin cell line along with experimental infection study. A bacterium isolated from the internal organs of affected fish was found to be pathogenic *Aeromonas hydrophila* having resistance to more than 10 classes of antibiotics. We postulate that CyHV-2 was the primary etiological agent responsible for this outbreak with secondary infection by *A. hydrophila.* The experimental infection trials in *Labeo rohita* and koi carp by intraperitoneal challenge with CyHV-2 tissue homogenates failed to reproduce the disease in those co-cultured fish species. This is the first report of a viral disease outbreak in organised earthen ornamental fish farms in India and bears further investigation.

1. Introduction

Ornamental aquaculture has increased in popularity in India as a result of its economic benefits due to a large internal market and the potential for exports. The goldfish (*Carassius auratus*) is one of the most popular species both in terms of production and trade value in India. Aquaculture of goldfish in ponds, ranging in size from 0.01 ha to more than 1.0 ha, either alone or in combination with Indian Major Carps (IMC) is very common in some pockets of West Bengal, India. While some of the broodstock are sourced from home-grown stock, the vast majority are imported from neighbouring countries. Despite the large scale culture, no major viral disease outbreak has been reported earlier in India, either from ornamental farms or the IMC farms that are the mainstay of Indian aquaculture. However, viral disease problems have been reported and characterized elsewhere in goldfish (Groff et al., 1998). Recently, George et al. (2015) isolated and characterized a ranavirus from koi, *Cyprinus carpio* L., experiencing mass mortalities in India and mortality due to viral nervous necrosis were reported in an experimental lot of goldfish and zebrafish in India (Binesh, 2013).

Herpes viral haematopoietic necrosis (HVHN) is a fatal disease of goldfish, leading to 100% mortality. It is caused by Cyprinid herpesvirus-2 (CyHV-2) (Goodwin et al., 2009), a member of the Cyprinid herpesvirus group that includes carp pox (CyHV-1) and koi herpesvirus (CyHV-3) (Goodwin et al., 2006b). CyHV-2 has mostly

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been detected from goldfish (Goodwin et al., 2006b), although there are reports on mortalities in Prussian carp (*Carassius gibelio*) (Wu et al., 2013) and crucian carp (*Carassius carassius*) (Bergmann et al., 2010; Fichi et al., 2013).The disease has been reported world-wide including USA, UK, Australia, New Zealand, Taiwan, China, Italy, Hungary, Czech Republic and most recently in France (Groff et al., 1998; Stephens et al., 2004; Goodwin et al., 2006a,b, 2009; Jeffery et al., 2007; Fichi et al., 2013; Wu et al., 2013; Xu et al., 2013; Boitard et al., 2015) following its first report in juvenile goldfish (*C. auratus auratus* L.) in Japan in 1992 (Jung and Miyazaki, 1995). Common characteristics of the disease include high mortality in juveniles at temperature ranging from 15 to 25 °C, with the fish exhibiting lethargy, anorexia, pale gills, and swollen and necrotic spleen and kidney tissues (Goodwin et al., 2006a,b; Jeffery et al., 2007). Besides histology and detection of the virus in tissues under ultrastructure examination (Wu et al., 2013), isolation of virus using goldfish fin and koi fin cell lines (Xu et al., 2013; Ito and Maeno, 2014) and PCR-based methods have been well developed for the identification and characterization of CyHV-2 (Goodwin et al., 2006a,b, 2009; Waltzek et al., 2009; Fichi et al., 2013).

There is little evidence to suggest the presence of this virus in aquaculture systems in tropical countries like India. However, bacterial disease outbreaks have been widely reported from aquaculture establishments, particularly due to motile aeromonad septicaemia involving *Aeromonas hydrophila* (Mohanty et al., 2008) and other bacterial pathogens (Swann and White, 1991; Nielsen et al., 2001; Rahman et al., 2002; Yesmin et al., 2004; Wahli et al., 2005). Previously, Fichi et al. (2013) have reported the association of *A. sorbia* with CyHV-2 mortality in crucian carp *C. carassius* from Italy. This case report details a mortality event where CyHV-2 was detected in association with multidrug-resistant (MDR) *A. hydrophila* infection in goldfish from commercial farms based on clinical signs, histology, electron microscopy, cell line infectivity, experimental infection and PCR-based assay. It is not known whether this virus infects other Cyprinids, or other ornamental species being cultured with goldfish in the same ponds. The results obtained here, independently verified at two different labs, suggest the complexity of the infection process and lay a foundation for further studies on the epidemiology, pathology and prevention of newly emerging freshwater viral diseases in India.

2. Materials and methods

2.1. Case history and analysis

Late in the rainy season in August 2013, large scale mortalities of goldfish, *C. auratus* and koi carp *C. carpio* in ornamental fish farms of Hooghly district, West Bengal, India were reported by the Fishery Extension Officer of the Department of Fisheries, Government of West Bengal to the Faculty of Fishery Sciences (FFSc), West Bengal University of Animal and Fishery Sciences, Kolkata, India. The cumulative mortalities of goldfish and koi carp in the affected ponds were in the range of 70–90%. Preliminary bacteriology and microscopy revealed the etiological agents to be motile *Aeromonas* spp. along with *Microcystis* intoxication. No viral involvement was looked into during that outbreak period. Treatment for bacterial infection was attempted with the use of water sanitizer, benzalkonium chloride and enrofloxacin in the diet, which led to partial control of mortality (Abraham, T.J., personal communication). In early winter (November 2014), the ornamental fish farmers reported mass mortality of goldfish in few of the ponds in the same locality. In the affected farms, goldfish, koi carp and barbs were reared along with other Indian major carps (IMCs). However, no mortality was observed in the IMCs and barbs. The mortality rate was recorded at 1–5% daily in different goldfish ponds, with a cumulative mortality above 90% during this period in goldfish. The affected fish showed clinical signs such as sluggish movement, erratic swimming, anorexia, spinning, fin rot, focal cutaneous haemorrhages, reddish lateral line, protrusion and sloughing off of scales, enophthalmia, peeled skin, corneal opacity, blind eye, etc. Bacteriological analysis of goldfish samples at FFSc, Kolkata revealed *Aeromonas veronii* biovar *sobria* from kidney and *Flavobacterium* sp. from the skin ulcers (Abraham, T.J., personal communication). Water disinfection along with antibiotic treatment had no apparent effect on disease control this time, with increased and continuous mortality. The authors here were called in following a subsequent disease outbreak in goldfish from other farms in the same locality in mid-December 2014.

2.2. Fish samples

The ornamental fish farms where the outbreak occurred are located on both sides of and fed by sewage and waste water channels draining into the River Hooghly. The details of geographical location of three farms from which samples were collected are as follows: Piyarapur/Chakpathakoria, Serampore Block, Hooghly District, West Bengal, India; Latitude-22^{0}46′27.1″N, Longitude-88^{0}17′47.0″E; Latitude-22^{0}46′40″N, Longitude-88^{0}18′6″E and Latitude-22^{0}47′49.5″N, Longitude-88^{0}18′1.8″E. The ponds were mostly stocked with goldfish (*C. auratus*) along with few koi carp (*C. carpio*), barbs (*Barbus barbus*) and few IMCs, acquired as seeds from Howrah, West Bengal, India, at a density of 20,000–40,000 numbers per acre. Only goldfish, approximately 3 months old, were affected, and symptoms included protrusion of scales, dropsy, enophthalmia, necrotized gills, haemorrhagic patches on the body surface particularly towards the base of the caudal fin, sluggish movement and gulping of air followed by large scale mortality. The diseased fish were transported to the laboratories both on ice (n = 15) and alive, in bags with oxygen (n = 10). According to the laboratory standard operating procedures, live fish were euthanised with clove oil and all samples underwent necropsy, parasitological, bacteriological, histological and virological examinations. For experimental infection, apparently healthy goldfish, koi carp and rohu carp (*Labeo rohita*) juveniles were obtained from fish farms located at Bhubaneswar, Odisha, India and Kochi, Kerala, India. The fish were kept in 500 L capacity FRP tanks with aerated water at a temperature of 25–27 °C and fed with commercial pellet feed for one week of acclimatization before the challenge experiment.

2.3. Post mortem findings

All the affected fish showed gill necrosis, splenomegaly with large white granular nodules, pale liver, swollen kidneys with small white focal necrosis and empty intestine.

2.4. Bacteriology and parasitology

Inocula from the blood, gill and kidney of infected goldfish was streaked on to tryptone soy agar (TSA, HiMedia, India) and single colonies obtained were purified and used for taxonomic analysis according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994), with biochemical reactions performed following standard protocols. Sequences of the 16s rRNA gene following PCR were used to screen for the pathogen, following Nayak et al. (2013). Based on the results, further PCR for aerolysin and beta-haemolysin genes specific to *A. hydrophila* were carried out for reconfirmation following Mohanty et al. (2008). OmpTs and AHCYTOEN gene based primers were also used to determine whether the strain involved was pathogenic or not (Cagatay and Sen, 2014).The mucus, gill and viscera samples were also screened for the presence of parasites.