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Identification and rapid diagnosis of the pathogen responsible for haemorrhagic disease of the gill of *Allogynogenetic crucian carp*

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A B S T R A C T

In recent years, an epizootic causing severe mortality among *Allogynogenetic crucian carp* (ACC), designated as haemorrhagic disease of ACC gill, occurred in Yancheng city of Jiangsu province of China. Obvious haemorrhage in the gills of moribund fish and a mortality rate of 100% were observed when ACCs were artificially infected with liver homogenate from diseased fish. A herpes-like virus, with enveloped virions ranged from 170 to 220 nm in diameter, could be observed in the tissues of challenged ACCs by examination with electron microscopy. Specific products representing the polymerase and helicase genes of Cyprinid herpesvirus-2 (CyHV-2) could be amplified from the challenged fish, suggesting that the haemorrhagic disease of ACC gill was caused by infection with CyHV-2. To rapidly diagnose CyHV-2-infected fish, an easy and effective detection assay with loop-mediated isothermal amplification (LAMP) was established. The LAMP assay was more sensitive than conventional PCR and the limit of detection was approximately 100 copies of target DNA. With this LAMP assay, CyHV-2 could be detected in some asymptomatic ACCs from the epidemic area and in eggs from the diseased ACCs, suggesting that CyHV-2 could infect ACCs latently and that the virus may be passed onto offspring by vertical transmission.

1. Introduction

Recently, in northern Jiangsu province, a new infectious disease in *Allogynogenetic crucian carp* (ACC) has appeared which is termed "beauty cheek" by the townsfolk for its most obvious feature of a ruddy spot on the operculum of some moribund fish. This disease, accompanied by mass mortalities, has occurred in many fish breeding ponds. Infected fish display anorexia and apathy, with skin lacking lustre and having necrotic spots. The gills of infected fish also present with necrosis of the lamellae. The operculum typically contains pooled blood and this disease had been designated as a haemorrhagic disease of the gills of ACC. To date, effective treatments have been identified for ACCs infected with two parasites, *Myxosporea* (Liu et al., 2012; Wang et al., 2003) and *Sinergasilus* sp. (Nie and Yao, 2000), and five bacterial pathogens, *Aeromonas sobria*, *Aeromonas hydrophila* (Sun et al., 1991), *Aeromonas punctata* (Die et al., 1993), *Plesiomonas shigelloides* (Lu et al., 2009), and *Shewanella putrefaciens* (Qin et al., 2012). However, control measures, such as water disinfection or oral administration of antibiotics, have not been effective in the control of haemorrhagic disease in ACC gills. Recently, a herpesvirus, Cyprinid herpesvirus-2 (CyHV-2), was found in diseased ACCs and regarded as a pathogen (Luo et al., 2013; Wang et al., 2012; Xu et al., 2013). It was also reported that a fatal disease of Prussian carp (*Carassius gibelio*) in mainland China was associated with CyHV-2 based upon histo- and ultra-pathological observations (Wu et al., 2013).

The purpose of this study was to identify the pathogen responsible for haemorrhagic disease in the gills of ACC and to develop a rapid and efficient method for the detection of this pathogen. A herpes-like virus was observed in the tissues of the challenged fish with liver homogenate from infected fish, and specific PCR products representing the polymerase and helicase genes of CyHV-2 were amplified simultaneously from the challenged fish. Furthermore, to rapidly detect and diagnose CyHV-2 infection, we developed an assay based on a loop-mediated isothermal amplification (LAMP) method. The LAMP assay detected CyHV-2 in some asymptomatic individuals and in eggs from infected ACCs. These results suggested that ACCs could be infected latently by CyHV-2 and that CyHV-2 could be transferred to descendants via vertical transmission.

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Table 1
Primers used in this study.

Name of gene	Accession number	Primers	Sequence (5′–3′)
CyHV-3 *Thymidine kinase*	JQ247183.1	CyHV3-TK1	GGGTTACCTGTACGAG
		CyHV3-TK2	CACCCAGTAGATTATGC
CyHV-3 ORF54	DQ657948.1	CyHV3-Sph-1	GACACCACATCTGCAAGGAG
		CyHV3-Sph-2	GACACATGTTACAATGGTCGC
CyHV-2 *Polymerase*	JQ067603	CyHV2-pol1	CAGCAACATGTGCGACGGAGG
		CyHV2-pol2	CACCACAGTCATACCCGGAGT
CyHV-2 *Helicase*	JQ815364	CyHV2-h1	ATGCTCACGGGTCCCATGCTG
		CyHV2-h2	CGCTCGTCCGGGTTCTGCACG

2. Material and methods

2.1. Fish samples

Moribund fish with gill haemorrhage were sampled at an aquaculture farm in Sheyang County in northern Jiangsu of China where an outbreak had occurred causing mass mortality.

Virus-free ACCs (weighing 50–75 g) were kindly supplied by the Aquaculture Institute of Suzhou Beauty of Tai Lake Medicine Co., Ltd. The pathogen free goldfish (weighing 8–10 g) were cultured in our laboratory for more than 1 year.

2.2. Artificial infections

Approximately 0.5 g of liver from an infected ACC was mixed with 50 ml of 0.01 M phosphate buffer solution (PBS; pH 7.2), the homogenate was centrifuged at 13,000 × *g* for 20 min and the supernatant was filtered to remove bacteria via a filter having a pore size of 0.45 μm. The filtered fluid was used to determine pathogenicity in ACC by artificial infection. Each healthy ACC received 5 μl of the filtered fluid by intraperitoneal injection. The control group received injections of PBS. Goldfish were treated with either 1 μl of the filtered fluid or PBS. All fish were kept in tanks with aerated water at 25–26 °C during the experimental period.

2.3. Electron microscopy

Tissues of the challenged fish were fixed in 2.5% glutaraldehyde and rinsed for 6 h in 0.1 M sodium cacodylate, which was replaced every 2 h, then post-fixed for 2 h in 1% osmium acid. The specimens were washed with 0.1 M sodium cacodylate twice, followed by dehydration through a graded series of ethanol, and embedded in epoxy resin (Epon 812). Sections were prepared using a microtome (Leica UC7, Solms, Germany) at a thickness of 50–60 nm, mounted on uncoated copper grids, and stained with 2% uranyl acetate and Reynolds's lead citrate. Grids were observed with a transmission electron microscope (HITACHI-H7650, Tokyo, Japan).

2.4. PCR identification

Suspecting that the virus might be CyHV-2 or CyHV-3, we designed different primer pairs based on the thymidine kinase (*tk*) and ORF54 (*sph*) genes of CyHV-3 and the polymerase (*pol*) and helicase (*hel*) genes of CyHV-2 (Table 1).

Approximately 0.5 g of liver from an infected ACC was homogenized in 50 ml of PBS, centrifuged at 13,000 × *g* for 20 min, and DNA was extracted from the supernatant. Supernatant (500 μl) was gently agitated with Tris–phenol (500 μl) for 10 min and centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant was transferred to a new tube and treated in sequence with Tris–phenol, Tris–phenol with chloroform (1:1, V/V), and chloroform. Precipitation of the DNA was performed by adding 50 μl of 3 M sodium acetate and 1.5 ml of 99.9% ethanol followed by incubation for 15 min at −20 °C. After centrifugation at 15,000 × *g* for 10 min at 4 °C, the DNA pellet was washed with 1.5 ml of 75% ethanol and dissolved in 30 μl of TE buffer.

The PCR reaction mixture contained 0.5 U of *Taq* polymerase, 2.5 μl of 10× PCR buffer, 2.5 μl of 25 mM $MgCl_2$, 1 μl of deoxynucleotide triphosphate mixture (2.5 mM each), 1 μl of genomic DNA template, 0.5 μl of each primer (100 pmol μl^{-1}), and distilled water to a total volume of 25 μl. Amplification was performed for 35 cycles: denaturation for 5 min at 94 °C, annealing for 40 s at 55 °C, and extension for 30 s at 72 °C. A final extension step was used at 72 °C for 10 min. The PCR products were stained with SYBR Green I and were visualized by electrophoresis on a 1% agarose gel.

The PCR products recovered from the gel were cloned into plasmid pMD19-T (Takara, Dalian, China) and sequenced. Homology search was performed using the BLAST program online (http://www.ncbi.nlm.nih.gov/BLAST). After the pathogen was identified as CyHV-2, the PCR reaction for diagnosis was performed using only the primer pair of CyHV-h1 and CyHV-h2.

2.5. LAMP assay for detection of CyHV-2

The FIP, BIP, F3, B3, and loop primers (LF and LB) used in the LAMP assay were designed based upon the CyHV-2 helicase gene sequence (Table 2, Fig. 1). To optimise the LAMP assay, varying concentrations of primers, betaine, and different reaction temperatures and times were tested with a pMD-hel plasmid containing partial sequence of CyHV-2 helicase gene. After the reaction conditions were optimised, the LAMP assay for detection of CyHV-2 was performed in a final volume of 25 μl, containing 8 U of Bst DNA polymerase (NEB, Massachusetts, USA), 2.5 μl of 10× reaction buffer (NEB, MA, USA), 3.75 μl of betaine (10 M), 1 μl of dNTPs (2.5 μM of each dNTP), 1 μl each of FIP and BIP (10 μM), 0.5 μl each of LB, LF, F3, and B3 primers (10 μM), 1 μl of template DNA. Each reaction was incubated at 61 °C for 30 min.

The template DNA for the LAMP assay was prepared using a boiling lysis. Briefly, 2 g of tissue from a diseased ACC was placed in 400 μl of LAMP reaction buffer. The tissues were shaken vigorously for 1 min prior to incubation in a boiling water bath for 10 min. After boiling, samples were placed into an ice water bath for 5 min prior to centrifugation at 12,000 × *g* for 1 min. The supernatant was used for the LAMP assay.

Additionally, we also detected CyHV-2 in the eggs from the diseased ACC. To prevent possible contamination on the surface of the eggs, the collected eggs (2 g) were washed with distilled water in a 1.5 ml micro centrifuge tube. After centrifugation at 5000 × *g* for

Table 2
Primers used in the LAMP assay.

Primers	Sequence (5′–3′)
F3	CAACACCTGGTGGCGATAG
B3	GTGCTAACGTTGTGCTGAAC
FIP	ACGTCCACAGACCGTCGTCGttttGTTCGGTCACCCAGCTACA
BIP	AATGGGATCAGGTCAAGTGCGCtttCGCTGTACTCTGGATCCTCT
LF	CCTGTCCCTGTCCTCGGG
LB	CTTTCAACCTACCCTTTAGCGTC