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Cyprinid herpesvirus-2 (CyHV-2): a comprehensive review

Raja Swaminathan Thangaraj[1], Sundar Raj Nithianantham[1], Arathi Dharmaratnam[1], Raj Kumar[2], Pravata Kumar Pradhan[2], Sumithra Thangalazhy Gopakumar[3] and Neeraj Sood[2]

1 Peninsular and Marine Fish Genetic Resources Centre, ICAR National Bureau of Fish Genetic Resources, Kochi, Kerala, India
2 ICAR National Bureau of Fish Genetic Resources, Lucknow, Uttar Pradesh, India
3 Marine Biotechnology Division, ICAR-Central Marine Fisheries Research Institute, Kochi, India

Correspondence
Raja Swaminathan Thangaraj, Peninsular and Marine Fish Genetic Resources Centre, ICAR-NBFGR, CMFRI Campus, P.O. Number 1603, Kochi, Kerala 682018, India. Email: rajanbfgr@gmail.com

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Abstract

Cyprinid herpesvirus-2 (CyHV-2) is a linear double-stranded DNA virus in the genus *Cyprinivirus* of family *Alloherpesviridae*. The virus is known to be highly pathogenic to ornamental goldfish (*Carassius auratus*), crucian carp (*C. carassius*) and Gibel carp (*C. auratus gibelio*), and also to the hybrids of goldfish and other carps. *Cyprinid herpesvirus-2*, having the smallest genome (290.3 kb) among *Cyprinivirus*, causes herpesviral hematopoietic necrosis disease (HVHND) that results in huge economic losses in aquaculture industry as the disease can cause high mortality (50–100%) among the affected fish. The disease was initially reported as the cause of epizootics in juvenile goldfish of Japan during 1992 and 1993. To date, this disease has been reported around the world including Europe, North America, Oceania and Asia. Huge economic losses due to the CyHV-2 infection among cultured gibel carp in China, during 2011–2012, mass mortality in crucian carp during 2012 in Italy, 95% mortality in goldfish during 2014 in France, 85% mortality in goldfish during 2016 in Poland had been reported. Strategies for controlling the spread of CyHV-2 are thus urgently required to limit economic damage. Furthermore, the review will shed light on lacunae in current knowledge as well as on the perspectives that merits further investigations on CyHV-2 research. The paper forms the first comprehensive overview of CyHV-2 causing a serious economically significant fish disease and, will be helpful for the researchers to get all related information from a single manuscript.

Key words: Cyprinid herpesvirus-2, CyHV-2, goldfish, Carassius sp., herpesviral haematopoietic necrosis, goldfish haematopoietic necrosis virus.

Introduction

Fish, crustaceans and mollusks represent the vital global aquaculture industry of 80 million tonnes in 2016 with an estimated total farm gate value of US$ 232 billion (FAO 2018). Further, the growing international aquaculture development and increasing global trade in live aquatic animals including food and ornamental fish and aquatic products facilitate wide geographical relocation of aquatic animal species and their pathogens. Ornamental fish industry is responsible for the movement of billions of live fish worldwide annually. This is a multibillion-dollar industry in more than 125 countries with an involvement of >2500 species and has an estimated wholesale and retail value of 1 billion and 3 billion USD, respectively (Dey 2016. High-density aquaculture and chronic stress provide opportunities for the emergence of new diseases. The common cause of infectious disease aetiological agents in aquaculture industry is bacteria (54.9%), viruses (22.6%), parasites (19.4%) and fungi (3.1%) (Reviewed in Kibenge *et al.* 2012). Generally, viruses affecting aquatic or terrestrial animals co-evolve with their hosts for their long-term survival within their natural range that negatively affect aquaculture. Viruses are the principal pathogens that negatively affect aquaculture worldwide. During 1960s, studies on aquatic animal virology such as establishment of fish cell lines for the isolation of fish viruses (Wolf & Quimby 1962) and demonstration of crustacean viruses using electron microscope (Vago 1966) were initiated. The viral diseases in aquatic animals are mainly detected and confirmed in an opportunistic manner, for example, herpesvirus was detected during mass mortalities of wild aquatic animal

species (Hedrick *et al.* 2000). Data on viruses of farmed aquatic animal species lag behind that of viruses terrestrial animal viruses. Consequently, there is a wide knowledge gap in many viral diseases of ornamental fish, even though viral diseases are recognized to cause significant economic losses with 100% mortality rates to the ornamental fish trade (Bernoth & Crane 1995; Cardoso *et al.* 2019).

The major viral pathogens that are considered potential rising threats to global aquaculture mainly include iridoviruses, reoviruses, rhabdoviruses, nodaviruses and herpesviruses (Murray 2013). Of these, herpesviruses are recognized as important pathogens and even though over 14 known herpesviruses are associated with disease outbreaks; there are still many more disease-causing fish herpesviruses that are yet to be characterized (Hanson *et al.* 2011). A general overview of herpesviruses that infect fish along with details of the two most characterized herpesviruses, namely *Cyprinid herpesvirus1* (CyHV-1) and *Cyprinid herpesvirus3* (CyHV-3) has been given by Hanson *et al.* (2011). However, scientific data on the disease caused by *Cyprinid herpesvirus2* (CyHV-2), the aetiological agent of a highly contagious viral disease, namely herpesviral haematopoietic necrosis disease (HVHND), are very scattered in spite of massive damage caused by the disease to production of goldfish (*C. auratus*) and many other Cyprinids (Panicz *et al.* 2019). Besides goldfish, recently, the disease has been reported from other species of the same genus like *C. gibelio* (Prussian carp) (Doszpoly *et al.* 2011; Xu *et al.* 2013) and *C. carassius* (crucian carp) (Fichi *et al.* 2016; Zhao *et al.* 2019), posing threat for food security also, especially in China where more than 2.5 M metric tons of crucian carp and Prussian carp are produced (FAO 2014). Thus, considering the significance of fish trade worldwide, and emerging data on CyHV-2 infections from various countries, improvements in its diagnosis and prophylaxis are called for to limit its occurrence and impacts to aquaculture. This necessitates a comprehensive knowledge of various characteristics of CyHV-2 such as aetiology, host range, distribution, transmission, pathology, immunology, diagnosis, prevention and control measures. The present paper delineates these aspects along with the recent advances by assembling and collating all available literature of this highly contagious and lethal viral disease. Furthermore, the information in this review will shed light on lacunae in current knowledge as well as on future perspectives on CyHV-2 research.

CyHV-2 infections in fish: early history and worldwide distribution

In the spring of 1992 and 1993, a new disease occurred causing severe mortality among cultured goldfish (*C. auratus)* in Japan. A herpesvirus was later isolated from these moribund fish and the pathogenicity of the viral isolate was confirmed through experimental infection (Jung & Miyazaki 1995). The disease was named as herpesviral haematopoietic necrosis (HVHN) or goldfish haematopoietic necrosis virus (GHNV) due to the characteristic manifestations of necrosis in the haematopoietic tissue of affected fish. Apart from HVHN, CyHV-2 has been recently associated with a new epizootic causing severe mortality among allogynogenetic crucian carp in China and it is designated as haemorrhagic disease of gill (Zhu *et al.* 2018). Since the first report in 1992-1993, CyHV-2 infections have been reported from various countries worldwide including USA (Groff *et al.* 1998; Goodwin *et al.* 2006a), Taiwan (Chang *et al.* 1999), Australia (Stephens *et al.* 2004), UK (Jeffery *et al.* 2007; Ito *et al.* 2013), Hungary (Doszpoly *et al.* 2011), China (Wang *et al.* 2012; Luo *et al.* 2013; Zhu *et al.* 2018; Jiang *et al.* 2020), Czech Republic (Danek *et al.* 2012), Italy (Fichi *et al.* 2013), Japan (Ito *et al.* 2013), India (Sahoo *et al.* 2016), Switzerland (Giovannini *et al.* 2016), Germany (Adamek *et al.* 2017), France (Boitard *et al.* 2016), Netherlands (Ito *et al.* 2017), Turkey (Kalaycı *et al.* 2018) and Poland (Panicz *et al.* 2019). Even though the disease was initially reported from *C. auratus,* CyHV-2 infections from other species like crucian carp (*C. carassius*), Prussian carp (*C. gibelio*) (Danek *et al.* 2012; Luo *et al.* 2013; Fichi *et al.* 2013; Ito & Maeno 2014) and allogynogenetic crucian carp (Wu *et al.* 2013) have been reported recently (Fig. 1). Details of CyHV-2 infections reported worldwide are given in Table 1. CyHV-2 outbreaks usually occur during spring and autumn season where the water temperature ranges from 15–25°C. The disease usually fades away when the temperature falls below 10°C or rises above 30°C. CyHV-2 decreases its pathogenicity when the temperature exceeds 27°C (Goodwin *et al.* 2009). Recently, Ouyang *et al* (2020) reported an outbreak of CyHV-2 disease in gibel carp, *C. auratus gibelio* at a non-permissive temperature of 10°C. The disease can cause severe mortality to all sizes of fish (Wu *et al.* 2013). Various stress factors like high stocking density, handling events, transport and holding at wholesalers can act as the predisposing factors for CyHV-2 infections (Goodwin *et al.* 2009; Davison *et al.* 2013).

Aetiology

Cyprinid herpesvirus-2 (CyHV-2) is the aetiological agent of HVHN/gill haemorrhagic disease in *Carassius* sp. It is the first and only herpesvirus causing infection in goldfish having a synonym as goldfish haematopoietic necrosis virus (GFHNV) (Jung & Miyazaki 1995). CyHV-2 is a member of the genus *Cyprinivirus* under the family *Alloherpesviridae* of order *Herpesvirales* (ICTV 2018). Other members of the genus *Cyprinivirus* are CyHV-1 (carp poxherpesvirus),

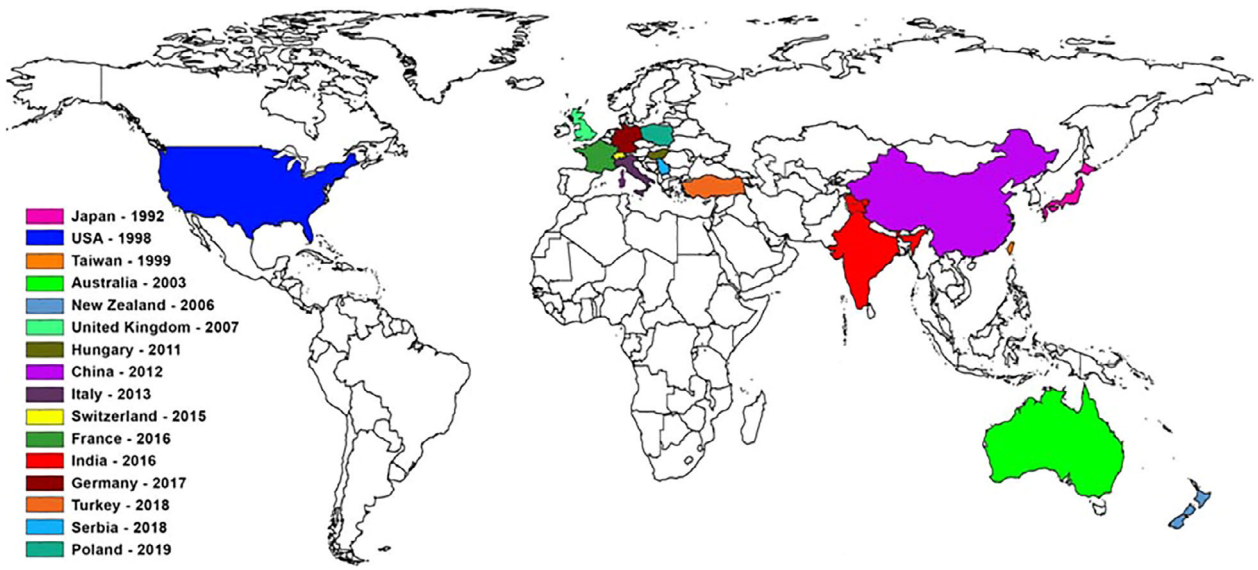

Figure 1 Global distribution of Cyprinid Herpesvirus -2 (CyHV-2) from 1992–2019. Japan – 1992; USA – 1998; Taiwan – 1999; Australia – 2003; New Zealand – 2006; United Kingdom – 2007; Hungary – 2011; China – 2012; Italy – 2013; Switzerland – 2015; France – 2016; India – 2016; Germany – 2017; Turkey – 2018; Serbia – 2018; Poland – 2019.

CyHV-3 (koi herpesvirus) and *Anguillid herpesvirus 1* (AngHV1/freshwater eel herpesvirus (ICTV 2018). Herpesviral Hematopoietic Necrosis Disease (HVHND) is caused by *Cyprinid Herpesvirus 2* (CyHV-2) is a member of the *Cyprinivirus* genus. It is highly pathogenic to goldfish, crucian carp and even the hybrids of goldfish and carp (Hedrick *et al.* 2006; Davison *et al.* 2013).

Virion: structure, composition and genome

Like other herpesviruses, CyHV-2 has an icosahedral capsid containing double-stranded DNA and a lipid envelope bearing viral glycoproteins. The virus multiplies and assembles in hematopoietic cells of spleen and kidney, and in gills of infected fish (Hedrick *et al.* 2000). Maturing process of CyHV-2 occurs in Golgi apparatus and final maturation occurs through budding into trans-Golgi network vesicles containing viral glycoproteins (Wu *et al.* 2013). Thus, matured virions can be seen as enveloped virions within a cellular vesicle inside the cytoplasm. Enveloped virions are round, having a size of 170–220 nm, and can be also seen in the extracellular spaces of infected cells (Jung & Miyazaki 1995). In infected cells, the virus forms characteristic spherical or hexagonal intranuclear inclusion bodies comprising of nucleocapsid having an edge-to-edge diameter of 115–117 nm (Jung & Miyazaki 1995). Very recently, Gao *et al.* (2020) have identified the structural proteins of CyHV-2 after purification of virions using a sucrose density gradient in combination with ultracentrifugation. The viral proteins were then separated by SDS-PAGE and identified by mass spectrometry. Results showed that CyHV-2 contained 74 proteins, including 3 capsid proteins, 18 membrane proteins and 53 other proteins.

Comparative analysis of nucleotide sequences among different CyHV-2 isolates was carried out based on partial sequence of DNA polymerase by Goodwin *et al.* (2006a) and Li *et al.* (2015) in the USA and China, respectively, which showed that CyHV-2 isolated from both countries were identical to ST-J1 isolated from Japan. However, Li *et al.* (2015) observed that there were clear differences in the predicted amino acid sequence of intercapsomeric triplex proteins from ST-J1 and CyHV-2 strains from China. Subsequently, Ito *et al.* (2017) pointed out that there are at least 6 different lengths for various CyHV-2 isolates in a region of viral genome namely; mA (marker A) region and the same can be amplified by using the primers designed by Boitard *et al.* (2016). The authors have also demonstrated that CyHV-2 can be divided into pleural genotypes based on the length of this mA region. Afterwards, the entire CyHV-2 genome was sequenced by different groups (Davison *et al.* 2013; Li *et al.* 2015; Zeng *et al.* 2016; Liu *et al.* 2018), and the results showed that the genomic DNA of CyHV-2 is about 290 kb in length comprising 150 protein-coding genes. The different strains of CyHV-2 namely, ST-J1, SY-C1 (Davison *et al.* 2013; Li *et al.* 2015), CaHV (Zeng *et al.* 2016), SY (Liu *et al.* 2018) contain many mutations, insertions, deletions and rearrangements in their genome, although all the strains share around 98% homological

Table 1 Details of CyHV-2 detection, isolation and confirmation in different countries during 1995 to 2020

S. No.	Country	Fish species	Clinical signs observed	Pond/tank water Temperature	PM lesions observed	Tests used for virus confirmation				*In vitro* isolation	Experimental challenge	References
						PCR	HP	TEM	Other tests			
1.	Japan	Goldfish *C. auratus*	No visible external signs except for listlessness	15–25°C	Softening and discoloration of the spleen and kidney and necrotic foci in the haematopoietic tissue	NM	Haematopoietic cells in the head and trunk kidneys, spleen, lamina propria and submucosa of the intestine showing necrosis, karyopyknosis and karyorrhexis	Hexagonal shape Enveloped virions size ranges from 170 to 220 nm diameter	NM	CPE on FHM, EPC and TO-2 cells, no CPE on in RTG-2, CHSE-214 and EK-1 cells	Goldfish die within 3–6 dpi and cumulative mortalities 100% within 13 days and not in *Cyprinus carpio* koi	Jung and Miyazaki (1995)
2.	USA	Goldfish *C. auratus*	50–100% mortality	20–22°C	NM		Hypertrophy and hyperplasia of the secondary lamellar gill epithelium and fusion of the adjacent lamellae and necrosis of the spleen and the hematopoietic cells	Enveloped intranuclear virion with spherical to hexagonal and size from 100–110 nm	NM	No CPE in the CHSE-214, EPC or FHM cell lines	NM	Groff *et al.* (1998)
3.	Taiwan	Goldfish *C. auratus*	There was yellowish discoloration of skin associated with natural infections of goldfish	22°C	NM	NM	Multiple focal necrosis in kidney with enlarged nuclei showing prominent chromatin margination	Enveloped virion with hexagonal shape virion of 100 to 110 nm in diameter	NM	NM	NM	Chang *et al.* (1999)
4.	Australia	Goldfish *C. auratus*	The gills had a striated appearance and pallor of primary lamellae and several white patches on the external surface of the fish.	12–15°C	Spleen was enlarged.	NM	Necrosis of haematopoietic tissue in the spleen, thymus and kidney and hyperplasia in the gill and nuclei with marginated chromatin and intranuclear inclusions.	Spleen and kidney tissue demonstrated hexagonal intranuclear virus particles 100 nm in diameter	NM	NM	NM	Stephens *et al.* (2004)

Table 1 (continued)

S. No.	Country	Fish species	Clinical signs observed	Pond/tank water Temperature	PM lesions observed	Tests used for virus confirmation				*In vitro* isolation	Experimental challenge	References
						PCR	HP	TEM	Other tests			
5.	USA	Goldfish *C. auratus*	fish appeared lethargic and remained at the bottom of the pond	Late summer 2003	Viscera appeared pale and soft and there were petechial haemorrhage in swim bladder.	Degenerate viral DNA polymerase gene PCR	Necrosis of the hematopoietic tissues of the trunk kidney, spleen, gills and GI tract with enlarged nuclei and peripherally displaced chromatin.	NM	NM	CPE on multiple passages on KF-1 FHMs and EPC	NM	Goodwin *et al.* (2006a)
6.	UK	Goldfish *C. auratus*	Fish became lethargic, gathered at the surface and died within 24 h and pale gill	19–21°C	Pale patches on the gills and skin	DNA polymerase gene	Necrotic lesions in the spleen and kidney and focal patches of necrosis in the gill lamellae with marginated chromatin and intranuclear inclusions	Spleen tissue revealed typical herpesvirus- like particles measuring 100 nm in diameter		No CPE in BF-2 cells and EPC cells But CPE in KF-1 cells		Jeffery, *et al.* (2007)
7.	Hungary	Prussian carp, *C. auratus gibelio*	NM	NM	NM	DNA polymerase gene	NM	NM	NM	NM	NM	Doszpoly *et al.* (2011)
8.	Czech Republic	Wild Prussian carp *C. auratus gibelio*	NM	16.1–20.5°C	NM	DNA polymerase gene	NM	Herpesvirus- like virions were observed in infected cell culture	NM	CPE on BF-2, EPC, FHM and RTG 2	NM	Danek *et al.* (2012)
9.	Italy	Female crucian carp, *C. carassius*	Haemorrhages at different points of the body and fins, gills and eyes.	Spring 2011	Haemorrhages in heart kidney swim bladder and ovary, Spleen granulomas	DNA polymerase gene	Necrosis of the epithelial cells of the lamellae and fusion. Necrosis of kidney and karyorrhectic nuclei scattered with cellular debris	Herpesvirus-like particles were demonstrated in the gills.		No CPE was observed in BF-2, EPC and CCB cells after three blind passages.	*Aeromonas sobria* by experimental infection was confirmed	Fichi *et al.* (2013)
10.	China	Prussian carp, *C. auratus gibelio*	Black body colour, lethargic and stayed on the bottom of the pond and gills were pale with striated appearance.	20–30°C	Spleens were enlarged.	DNA polymerase gene and DNA helicase gene	Necrosis with karyopyknosis and karyorrhexis in the haematopoietic tissues of the kidney and spleen In the sinusoids of the liver.	Hexagonal enveloped virus particles 170–220 nm in diameter in the cytoplasm kidney tissue			Silver Prussian carp began to die at 5 dpi, and the cumulative mortalities 100% within 7 days	Luo *et al.* (2013)

Table 1 (continued)

S. No.	Country	Fish species	Clinical signs observed	Pond/tank water Temperature	PM lesions observed	Tests used for virus confirmation				*In vitro* isolation	Experimental challenge	References
						PCR	HP	TEM	Other tests			
11.	China	Prussian carp, *C. auratus gibelio*	NM	NM Samples were collected during April to July in 2012	NM	DNA polymerase gene	Infiltration of hemocytes, hypertrophied nuclei, marginal chromatin and karyorrhexis, epithelial cell shedding, vacuolar degeneration and focal necrosis.	NM	FISH	NM	NM	Ding *et al.* (2014)
12.	Australia	Imported goldfish, *C. auratus*	NM	NM	NM	qPCR of DNA polymerase gene	NM	NM	NM	NM	NM	Becker *et al.* (2014)
13.	China	Silver crucian carp, *C. auratus gibelio*	High mortality and carriers displaying no clinical signs	NM		qPCR of ORF 121			ISH		Fish began to die at 5 dpi, and the cumulative mortalities reached 100% within 7 days	Wang *et al.* (2016)
14.	Netherland	Gibel carp, *C. auratus gibelio*	Whitish slime layer over their eyes and an erythema on their skin, haemorrhagic scales.	20–25°C	NM	qPCR	NM	NM	NM	No CPE on EPC and FHM cells at 15 and 20°C	NM	Haenen *et al.* (2016)
15.	India	Goldfish *C. auratus*	Large scale haemorrhages on the body, fins and gills, lepidorthosis, necrosed gills, protruded anus and shrunken eyes.	Early winter season	White nodular necrotic foci in spleen and kidneys were noticed, along with necrosis and fusion of gill lamellae	DNA polymerase gene and helicase gene	The gill lamellae and kidney showed massive necrosis and sloughing of epithelia. Spleen with necrosis of the lymphoid tissue, and hypertrophied nuclei with marginated chromatin material	Mature virus particles were demonstrated in the gills and spleen	NM	CPE on CCKF cells were first observed at 5 dpi	Typical signs of gill necrosis within 48 h of post-challenge in intraperitoneal group and within 72 h in immersion group	Sahoo *et al.* (2016)
16.	Switzerland	Goldfish, *C. auratus*	Fluid Accumulation, oedema of body and ascites	15–18°C	Enlarged pale kidneys and liver with multiple pinpoint haemorrhages	DNA polymerase gene	Multifocal necrosis within the hematopoietic tissue along with oedema	NM	ISH	No CPE on CCB and KF-1 cells	Koi showed no signs of CyHV-2	Giovannini *et al.* (2016)

Table 1 (continued)

S. No.	Country	Fish species	Clinical signs observed	Pond/tank water Temperature	PM lesions observed	Tests used for virus confirmation				*In vitro* isolation	Experimental challenge	References
						PCR	HP	TEM	Other tests			
17.	France	Goldfish, *auratus*	Simple loss of scale and discoloration to ulcerative lesions Gills were pale and covered with excess mucus	20–22°C	Organs were congested and petechiae on the liver, peritoneal fat and peritoneum. White nodules in the spleen and the kidney	DNA polymerase and helicase genes	Hyperplasia of gill branchial epithelium. The kidney and spleen showed necrosis of hematopoietic tissue and enlarged nuclei with margination of the chromatin	NM	NM	NM	NM	Boitard *et al.* (2016)
18.	Germany	Traded goldfish, *C. auratus*	Instant mass mortality of goldfish with ascites	NM	NM	DNA polymerase gene and qPCR of ORF 121	Gills show clubbing and fusion of secondary gill lamellae with occlusion of the interlamellar spaces. The kidney shows a focal necrosis	NM	NM	NM	NM	Adamek *et al.* (2017)
19.	Turkey	Goldfish, *C auratus*	Lethargy and anorexia high mortality (50–100%)	15-25°C	NM	PCR	NM	NM	NM	NM	NM	Kalaycı *et al.* (2018)
20.	Poland	Imported goldfish varieties, like Veiltail, Wakin, Red Cap Oranda and Ranchu in	lethargy and extremely pale gills and the final mortality rate exceeded 90%	19.9 to 33.2°C	NM	mA (marker A)	NM	NM	NM	NM	NM	Panicz *et al.* (2019)
21.	China	goldfish *C. auratus*	Inappetent, lethargic, strayed away from the schooling pond population; cumulative mortality rate was above 80%.	15 and 28°C	Severe necrosis of the gills, bleached appearance in gill filaments unilateral exophthalmia, splenomegaly.	Helicase gene	Marked acute necrotizing splenitis, nephritis and bronchitis, mild to severe necrotizing myocarditis and encephalitis, severe enteritis.	Virions with a diameters of 260–290 nm and nucleocapsids measuring approximately 100–117 nm in diameter. An electron-dense core, hexagonal nucleocapsid	NM	NM	NM	Jiang *et al.* (2020)

NM, Not mentioned; BF-2, Bluegill Fry; CCB, common carp brain; CCKF, *Cyprinus carpio* koi fin; CHSE-214, chinook salmon embryo; CPE, Cytopathetic effect; DNA, Deoxy ribonucleic acid; dpi, days post-inoculation; EK-1, eel kidney; EPC, epithelioma papulosum cyprinid; FHM, fathead minnow; FISH, Fluorescence in situ hybridization; HP, Histopathology; ISH -In situ hybridization; KF-1, Koi carp Fin; NM, not mentioned; ORF, open reading frame; PCR, polymerase chain reaction; PM, post-mortem; qPCR, quantitative Polymerase chain reaction; RTG-2, Rainbow trout gonads; TEM, Transmission Electron Microscopy; TO-2, tilapia ovary; UK, United Kingdom; USA, United States of America.

genome sequences (Liu *et al*. 2018). Overall, G + C content of the genome is around 52%. Based on the differences in genome Li *et al*. (2015) proposed that CyHV-2 can be divided into 2 different genotypes namely China genotype (C genotype) and Japan genotype (J genotype) according to their isolation loci. These two genotypes shared a homology of 98.8% in their genome. Furthermore, molecular epidemiological surveys indicated that dominant genotype of CyHV-2 circulating in mainland China is closer to C genotype than the J genotype (Li *et al*. 2015). Recently, Liu *et al*. (2018) showed that genome of the new CyHV-2 strain isolated from allogynogenetic crucian carp of China had many variations from C and J genotypes with overall sequence identity of 99.1% and 98.4%, respectively. Their study pointed out that ORF10, ORF107 and ORF156 can be used as the marks of SY strains. They also showed that 16 and 2 genes in the CyHV-2 genome may be transferred from the host and bacteria, respectively, through horizontal transfer analysis. Further, analyses of the amino acid sequence homology of the core ORFs from the alloherpesvirus family showed relatively higher similarity of 4 core ORFs (ORF33, ORF79, ORF92 and ORF107) among different viruses (Liu *et al*. 2018) within the family *Alloherpesviridae*.

Host range

Determining the host range and transmission of pathogens is extremely important for the prevention of any infectious diseases; however, literature related to the host range and vertical transmission of CyHV are scanty. Generally, herpesviruses are characterized by a high level of host specificity (Hanson *et al*. 2011). Historically, the host species for CyHV-2 was goldfish (*C. auratus)*. CyHV-2 can infect all the different life stages of goldfish such as egg, fry, fingerling and adult fish; of which, juvenile stages are more susceptible (Groff *et al*. 1998). An experimental challenge study showed that all the three varieties of goldfish viz., Ryukin, Edonishiki and Ranchu, were susceptible to CyHV-2 (Ito & Maeno 2014), whereas no disease was observed in *C. auratus langsdorfii*, *C. auratus buergeri*, *C. auratus grandoculis* and in common carp (*Cyprinus carpio*) (Ito & Maeno 2014). However, natural CyHV-2 infections are now reported from a wider range of cyprinid species like crucian carp (*C. carassius*), Prussian carp (*C. gibelio*) (Hedrick *et al*. 2006; Bergmann *et al*. 2010; Danek *et al*. 2012; Fichi *et al*. 2013; Luo *et al*. 2013; Ito & Maeno 2014) and allogynogenetic crucian carp (Wu *et al*. 2013). Further, in the spring season of 2015, Zhu *et al*. (2018) noted that diseased *Aristichthys nobilis* (Bighead carp), *Erythroculter ilishaeformis*, *Culter alburnus*, *Hypophthalmichthys molitrix* (Silver crap) and *Mylopharyngodon piceus* (Black carp) in the Jiangsu province of China, had similar clinical features of *C. auratus* suffering from gill haemorrhagic disease. Later, diagnosis by LAMP assay and electron microscopy examination confirmed that these species were positive for CyHV-2. These results suggested that the infection of CyHV-2 is not now limited to goldfish (Zhu *et al*. 2018) and the virus can cause cross-infection among different species of fish. Wei *et al*. (2019) proved that CyHV-2 can establish a persistent infection in some organs of asymptomatic goldfish, especially the spleen and trunk kidney in experimental infection studies.

Transmission

Horizontal disease transmission is the usual means of CyHV-2 transmission between fish populations. This transmission occurs either by direct fish to fish transmission or possibly through a vector. However, possible role of vectors in disease transmission studies has not been carried out for CyHV-2. Direct fish to fish transmission can be through contact with infected fish or fish asymptomatically carrying CyHV-2 (Goodwin *et al*. 2009). Ito and Maeno (2014) found that goldfish infected with CyHV-2 at 13–15°C water temperature neither died nor acquired resistance to the disease, but act as carriers to infect other fish. Experimental infection studies have revealed that spleen and trunk kidney act as the primary site for persistent infection of CyHV-2 in *C. auratus* (Wei *et al*. 2019). Vertical transmission is also not confirmed in CyHV-2; however, an epidemiological investigation documenting the occurrence of CyHV-2 in offspring seeds, breeding fish, disinfected eggs and fry of goldfish, suggested that vertical transmission is possible for CyHV-2 in goldfish (Goodwin *et al*. 2009). Further, results of different diagnostic methods namely, RT-PCR, LAMP assay and electron microscopic examination have revealed the presence of CyHV-2 in eggs of the diseased fish, further suggesting that CyHV-2 can be transmitted vertically to offspring (Zhu *et al*. 2018). The vertical transmission of CyHV-2 (Goodwin *et al*. 2009; Zhu *et al*. 2018) was poorly studied unlike the horizontal transmission of CyHV-2, so more research is needed to generate more information of the same. So with the available data on literature, CyHV-2 infection is being transmitted to other to goldfish, crucian carp, prussian carp and even the hybrids of goldfish by horizontal route than vertical route.

Impact of infection on fish

Clinical signs

Infection with CyHV-2 is most severe in goldfish, where it can cause 100% mortality in all ages with a daily mortality rate of 1–5% especially at water temperature of 15–20°C (Jung & Miyazaki 1995; Sahoo *et al*. 2016). Jung and Miyazaki (1995) reported only listlessness and staying at the pond bottom in the affected goldfish. However, in further

reports, typical signs and lesions were recorded. Chang *et al.* (1999) observed only yellowish discoloration of skin as a clinical sign in natural cases of CyHV-2 infection in goldfish. Jeffery *et al.* (2007) described pale skin, bilateral exophthalmia, pustules in fin and decaying white gill filaments with bleached appearance as clinical signs in CyHV-2 affected goldfish. Groff *et al.* (1998) recorded signs such as lethargy, pale gills and anorexia, often with elevated respiratory efforts. The most common sign of the disease is reported as anaemia as the virus attacks haematopoietic tissues (Goodwin, *et al.* 2006a). Thus, characteristic signs of CyHV-2-infected fish include lethargic behaviour, gasping at the surface with erratic swimming (spiralling/whirling), lying down at the tank bottom before death, pale gills, enophthalmos, patches of necrotic tissues on gills and gills, and mortality in all sizes of fish (Sahoo *et al.* 2016) (Fig. 2a, b,c,d). Recently, Adamek *et al.* (2017) described ascites in affected goldfish. Similar clinical signs were described in diseased Prussian carp *viz.*, anorexia, lethargy, exophthalmia, haemorrhagic spots on external surface, hyperaemia on submaxilla and abdomen, necrotic gills and gill filaments, petechial and ecchymotic haemorrhages on opercula, gills and around the base of fins, eyes, blood engorgement in the inner membranes of both opercula, swollen abdomen and vent inflammation (Danek *et al.* 2012; Wang *et al.* 2012; Xu *et al.* 2013; Wu *et al.* 2013). Clinical signs reported in CyHV-2-infected Crucian carp were haemorrhages at different points of body and fins, swollen anus and presence of haemorrhages in gills and eyes (Fichi *et al.* 2013).

Pathology-gross, histological and ultrastructural lesions

In contrast to clinical signs, there were several gross pathological changes in internal organs of affected goldfish during the first report itself *viz.*, pale coloration of gills and liver, ascites, splenomegaly with white nodular lesions, swollen pale kidney and empty intestine (Jung & Miyazaki 1995). Jeffery *et al.* (2007) reported necrotic gills, abdominal distension, pale kidney and liver as well as splenomegaly in affected goldfish. We have also observed severe, widespread necrosis of hematopoietic tissues of trunk kidney and spleen in CyHV-2 affected goldfish (Fig. 2e,f). Gross pathological changes described in affected Prussian carp included liver hyperaemia, splenomegaly, renal hypertrophy, empty intestine and petechial haemorrhaging of swim bladder (Wang *et al.* 2012). In affected Crucian carp Fichi *et al.* (2013) documented haemorrhages in heart, kidney, swim bladder and ovary, and granuloma in spleen.

Typical histological lesions reported in goldfish infected with CyHV-2 include extensive necrosis in spleen and kidney, enlarged nuclei of kidney hematopoietic cells with

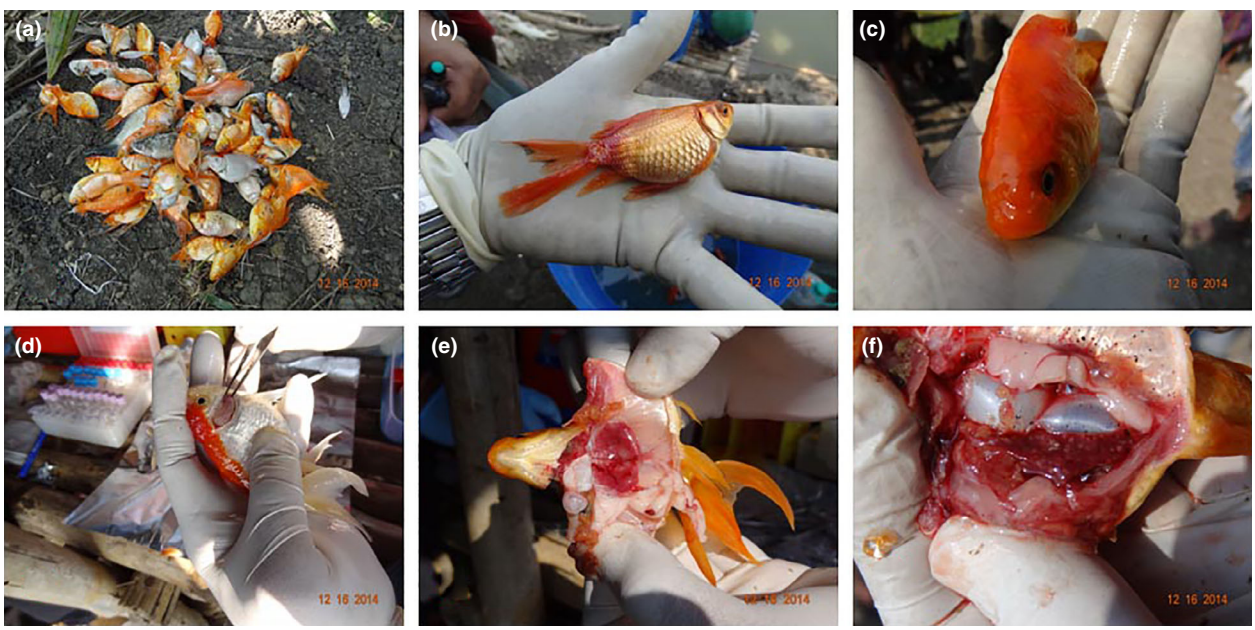

Figure 2 Cyprinid herpesvirus-2 (CyHV-2) infection in goldfish Carassius auratus. (a) Mass mortality of goldfish in a polyculture pond in a farm in India during 2014; (b) External clinical signs of CyHV-2 affected goldfish Carassius auratus including haemorraghes on the body, ascites and protrusion of scales on the body surface; (c) CyHV-2 affected goldfish Carassius auratus showing enophthalmia (sunken eyes); (d) Inflamed and swollen gills in CyHV-2 affected goldfish Carassius auratus; (e) swollen and enlarged kidney in CyHV-2 affected goldfish Carassius auratus; (f) enlarged liver with white necrotic foci in CyHV-2 affected goldfish Carassius auratus.

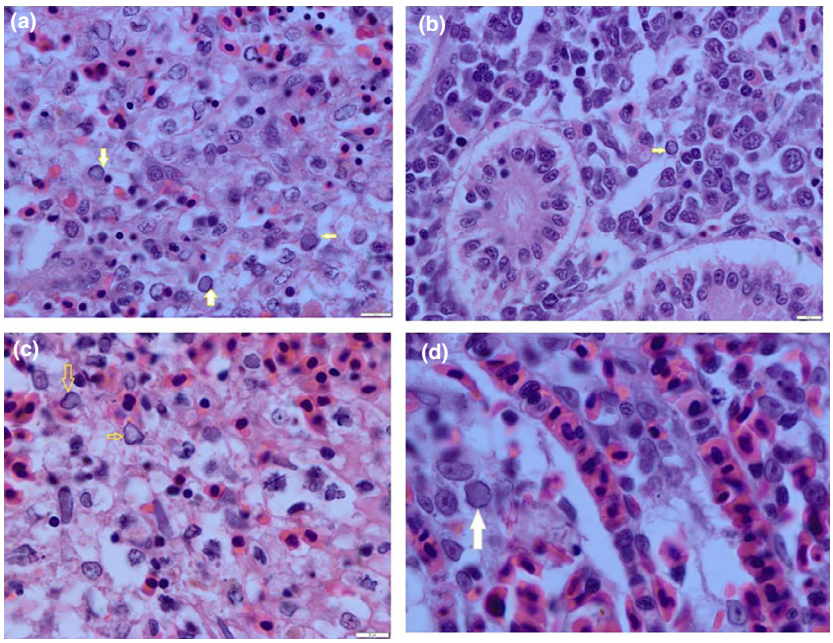

Figure 3 Histopathological lesions in affected tissues of a goldfish with Goldfish Herpesviral Hematopoietic Necrosis Disease. (a) and (b) Necrosis of hematopoietic cells in the kidney (c) Spleen (d) Gills and other nuclei are enlarged with marinated chromatin (arrow).

marginated chromatin (Fig. 3) and focal patches of necrosis in gill lamellae (Jung & Miyazaki 1995; Jeffery *et al.* 2007). Karyopyknosis, karyorrhexis (Jung & Miyazaki 1995) with pale basophilic to eosinophilic centres and peripherally displaced chromatin were observed in diffuse necrotic areas of kidney haematopoietic tissue, along with moderate oedema, mild fibrin exudation and cellular debris (Goodwin *et al.* 2006a; Fichi *et al.* 2013; Adamek *et al.* 2017). Lesions in spleen were characterized by mild to severe, multifocal to diffuse degeneration (Groff *et al.* 1998), coagulative necrosis of lymphoid tissue (Jung & Miyazaki 1995; Boitard *et al.* 2016; Adamek *et al.* 2017), hypertrophied nuclei with marginated chromatin (Sahoo *et al.* 2016) and deposition of high number of melanomacrophages (Adamek *et al.* 2017). CyHV-2 causes diffuse hypertrophy and hyperplasia of branchial secondary lamellar epithelium of gills resulting in focally extensive fusion of adjacent lamellae, massive necrosis and sloughing of epithelia in infected fish (Groff *et al.* 1998; Stephens *et al.* 2004; Adamek *et al.* 2017). No histopathological changes were observed in other organs, including muscle, heart and brain, either in fish from the natural outbreak group or those from the experimental infection group by Jung and Miyazaki (1995). Whereas Sahoo *et al.* (2016) reported severe necrosis of gill lamellae, kidney and spleen of naturally infected goldfish samples and also hypertrophied nuclei with margination of chromatin material in spleen. In contrast, Goodwin *et al.* (2006a) found granuloma in brain and mesentery tissue of naturally infected fish. Hypertrophied nuclei of cardiac muscle cells containing marginated chromatin were reported in another study (Lu *et al.* 2016). Similarly, multiple focal necroses and necrotic area with enlarged nuclei and prominent chromatin margination has been reported in heart, small intestine, pancreas and skin by Chang *et al.* (1999). Histological lesions in cultured gibel carp (*C. auratus gibelio*) included acute hepatocellular necrosis, splenic necrosis, kidney necrosis along with oedema in renal glomerulus, hyperplasia of secondary lamellae with focal necrosis in gills, acute necrotic myocarditis, oedema of myocardial cells, accumulation of granulocytes within cardiac lumen, necrosis and oedema in submucosa and mucosa epithelium of intestinal tract and oedema of neurons (Nanjo *et al.* 2016).

Electron microscopy of splenocytes, hematopoietic tissue cells of kidney, epithelial cells of gill and brain cells of infected fish generally reveals the presence of numerous typical enveloped spherical or hexagonal nucleocapsids either in nucleus or in cytoplasm (Jung & Miyazaki 1995; Stephens *et al.* 2004; Hine *et al.* 2006; Jeffery *et al.* 2007). Occasionally extracellular virions can be demonstrated in-between processes of virus-infected cells (Fig. 4). Major nuclear changes in infected host cells include hypertrophy, inclusions, central clearing of nucleoplasm and margination of chromatin (Jeffery *et al.* 2007). In cytoplasm, swelling of organelles and membranes destruction can be noted. Goodwin *et al.* (2006a) demonstrated hexagonal virions of 95 × 106 nm

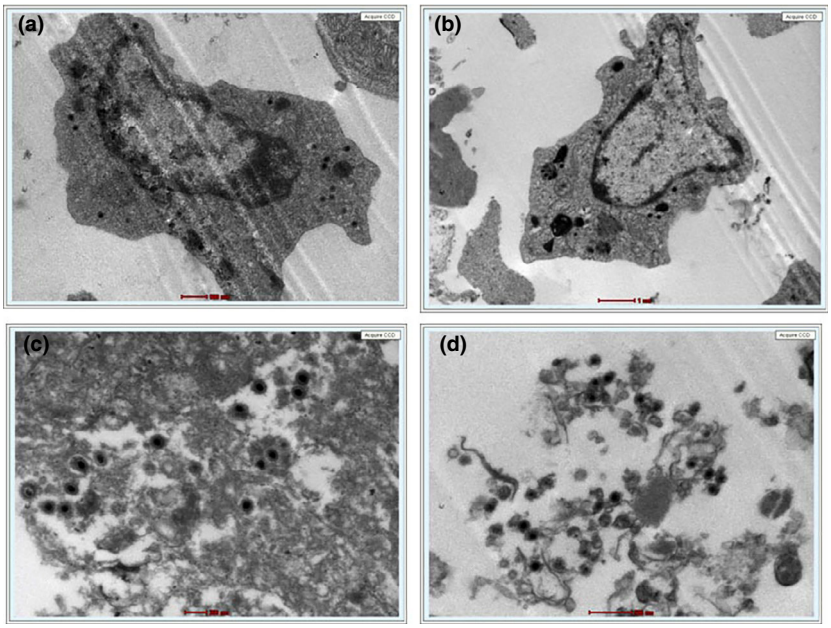

Figure 4 Transmission electron micrograph of infected tissues in infected goldfish. (a) Enlarged nucleus with marginalized chromatin in liver cells; (b) Enlarged nucleus with marginalized chromatin in kidney cells; (c) Fully formed virions had an outer membrane and electron dense core in liver cells of infected goldfish; (d) Fully formed virions had an outer membrane and electron dense core in kidney cells of infected goldfish.

within the cytoplasm of infected tissues. Lovy and Friend (2014) demonstrated different stages of viral morphogenesis viz., empty capsids, capsids with an inner linear concentric density, capsids with an electron-dense core and mature capsids containing an envelope in the tissues fixed in 10% neutral buffered formalin. In infected Prussian carp, Luo *et al.* (2013) reported hexagonal, enveloped virus particles of 170–220 nm in diameter in cytoplasm and extracellular spaces of affected kidney tissue. Wu *et al.* (2013) detailed different stages of CyHV-2 assembly in infected gill, spleen and kidney of Prussian carp such as entire particles with electron-dense cores and incomplete virus-containing empty cores. They further described the maturing process of CyHV-2 through Golgi apparatus, resulting in an enveloped virion within a vesicle inside cytoplasm. The viral nucleocapsids in nuclei and enveloped viral particles in the cytoplasm were approximately 95–110 nm and 170–200 nm in size, respectively. Fichi *et al.* (2013) demonstrated herpesvirus-like particles in gills of infected crucian carp. In diseased gibel carp, ultrastructural lesions of virus-infected cells included enlarged nuclei and margination of chromatin (Xu *et al.* 2013). The authors also measured virion assembly within nuclei of haematopoietic cells as nucleocapsid and mature enveloped virus of 90–120 nm and 170–200 nm in diameter, respectively. Further, they described that the negatively stained purified virions as hexagonal in shape having a diameter of 110–120 nm.

Immune response inside host

Understanding the host immune response in viral infections can provide useful clues for diagnosis, control and prevention. However, such studies pertaining to CyHV-2 infections in fish are very limited. It is proven that initial load of viral dose entering the host plays a significant role in host–viral interactions and thus determines the outcome of CyHV-2 infection (Xu *et al.* 2014). In general, innate immunity as well as adaptive immunity efficiently suppresses the disease progress at lower viral load infections (Xu *et al.* 2014). Whereas, Nanjo *et al.* (2017) investigated the humoral immune response in a passive immunization in native goldfish with the sera of the surviving goldfish. It is also reported that many surviving goldfish after CyHV-2 infection can acquire resistance to the disease after severe infection (Nanjo *et al.* 2016). Water temperature is another factor that plays a significant role in host-CyHV-2 interactions (Nanjo *et al.* 2017). High water temperature treatments are reported to elicit immunity to CyHV-2 infections in survivor fish (Nanjo *et al.* 2017) even though the underlying mechanism has not been resolved. The proposed mechanism may be due to innate immune system attacking and excluding virus-infected cells more effectively at higher water temperatures, reducing mortality. Although virus reactivation was observed in some cases when temperature is back to optimal viral temperature, the reactivated

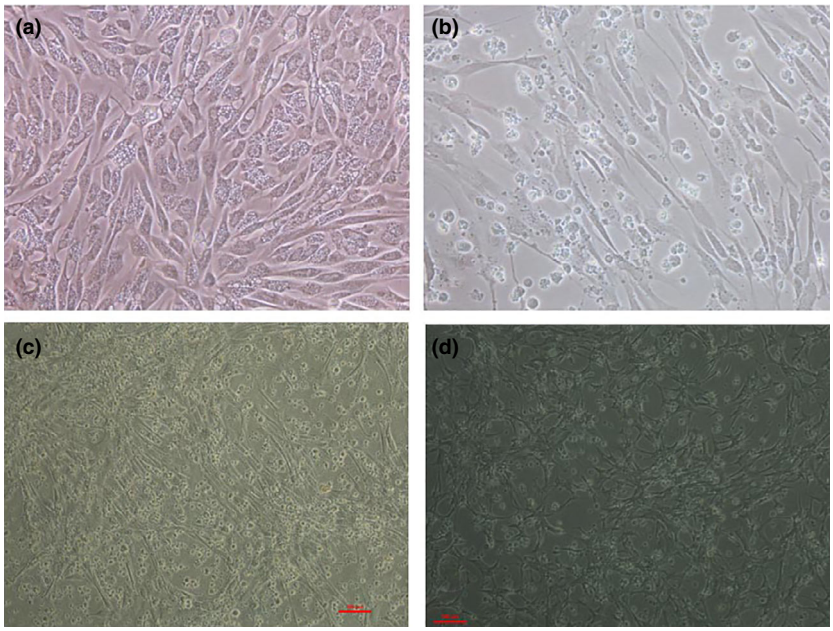

Figure 5 Cytopathic effects of the different goldfish cell line and cell lines from other fish species infected with CyHV-2 in different passages at different days post-inoculations (dpi). (a) Fantail goldfish fin (FtGF) cell line at passage 5 at 7 dpi; (b) Fantail goldfish gill (FtGG) cell line at passage 10 at 5 dpi; (c) Fantail goldfish liver (FtGL) cell line at passage 8 at 6 dpi; (d) Fantail goldfish brain (FtGB) cell line at passage 3 at 10 dp.

virus may continuously stimulate the immune system and contribute to strong adaptive immunity. Thus, shifting the fish rearing water temperature to non-permissive temperature can be a promising strategy for the control of CyHV-2 infection (Nanjo *et al.* 2017). However, further detailed immunological studies are required to define the host responses responsible for reducing mortality during high water temperature treatment.

Immunological analysis of CyHV-2 infections is mainly limited by the lack of immunological tools available for the goldfish, such as antibodies specific to goldfish T-cell subsets. Clonal ginbuna *C. auratus langsdorfii* has been used in many fish immunology studies and was found to be a promising model species for the study of CyHV-2 infection and immunity (Nanjo *et al.* 2017). Elucidating the differences in immune gene expression profiles between dead and surviving fish populations would also provide vital information about viral pathogenicity and shed light to develop antiviral strategies. Hence, Xu *et al.* (2014) attempted to study the differential gene expressions in moribund and surviving crucian carp to CyHV-2 infection thorugh suppression subtractive hybridization (SSH) followed by the sequencing and analyses of ESTs. The authors noted large differences existing in the differential gene expression profiles between the moribund and survivor fish group. Further characterization of keratin8, MPO and dusp1 genes by Podok *et al.* (2014) and NF-Kb inhibitor, Rab GTpase (Rab21), small GTP binding protein (Rac2) genes by Xia *et al.* (2016) confirmed the over-expression of these genes in CyHV-2 infections, pointing out their potential as marker genes in disease investigations. Another study carried out on the expression profiling of kidney tissue of silver crucian carp using digital gene expression tag profiling (DGE) from both control and moribund fish revealed that around 2912 genes were differentially modulated (Lu *et al.* 2017). Out of these 2912 modulated genes, 1422 were up-regulated and 1490 were down-regulated. GKEGG enrichment analysis showed that genes involved in proteasome, neuro-active ligand-receptor interaction, calcium signalling pathway and peroxisome proliferator-activated receptors (PPAR) signalling pathways were enriched in infected fish. Further, quantitative RT-PCR confirmed that three genes namely, major histocompatibility complex-I (MHC-I), interferon regulatory factor 3 (IRF3) and mitogen-Activated Protein Kinase 7 (MAPK7) genes were up-regulated during CyHV-2 infection in silver crucian carp (Lu *et al.* 2017). All these findings might pave the way for future analysis of immune-related genes involved in antiviral immunity of CyHV-2 infection, ultimately helping to design novel diagnostic and antiviral strategies. Recently, Lu *et al.* (2018b) reported that host miRNAs are involved in CyHV-2 infection in gibel carps and participate in the regulation of apoptosis and immune-related genes. Xia *et al.* (2018) identified and characterized crucian carp IFNc

(ccIFNc) ccIFNc as belonging to the type I interferon family with a potential role in countering CyHV-2 infection in crucian carp. Lu *et al.* (2019) reported that CyHV-2 miR-C12 suppresses virus-induced apoptosis and promotes virus replication by targeting caspase 8 and over-expression of miR-C12 reduces the expression of caspase 8 and inhibits CyHV-2 induced apoptosis. Fan *et al.* (2020) cloned and sequenced the complement C3 gene, designated CagC3, from Gibel carp and proved that CagC3 was involved in the innate immune response of Gibel carp to CyHV-2 infection. Briefly, both innate and acquired immunity plays a crucial role in providing protection in goldfish against CyHV-2 diseases and high water temperatures are reported to elicit better immune responses against CyHV-2.

Latency inside host

Considering the lengthy incubation period of the disease and the fact that disease is often precipitated by variations in water temperature or by predisposing factors like stress, CyHV-2 has been generally considered as a latent virus (Goodwin *et al.* 2009). Many reports have also demonstrated that *Cyprinivirus*, mainly CyHV-2 and CyHV-3, can lead to latent infection in infected fish (Reed *et al.* 2014; Wei *et al.* 2019). Virus density of CyHV-2 in an apparently healthy goldfish was found in the range of 10^3–10^5 which may occasionally go as high as $10^{7-}10^9$ (Goodwin *et al.* 2009). A very recent study by Chai *et al.* (2020) confirmed that CyHV-2 established latency in fish following the primary infection and the latency could be reactivated by temperature stress *in vivo*. They also showed that a novel cell line derived from the brain of gibel carp (GCBLat1) supports the CyHV-2 latency, which offers an *in vitro* model to investigate the mechanism of latency and reactivation for CyHV-2. The exact mechanism of latency in CyHV-2 is not established yet, however, production of virus-encoded microRNAs that facilitate viral invasion by exploiting various intracellular signalling pathways of host was demonstrated in these viral infections (Donohoe *et al.* 2015; Lu *et al.* 2017). Establishing the molecular mechanism employed by CyHV-2 in latency may lead to novel antiviral strategies. An interesting study by Lu *et al.* (2017) identified 17 viral miRNAs from CyHV-2-infected crucian carp kidney that are involved in innate immune signalling pathways of host. They have shown that three host genes namely, PIN1, IRF3 and RBMX involved in RIG-I-like immune pathway are the major targets of CyHV-2-encoded miRNAs. The identified miRNAs were found to be distributed across the viral genome with major clusters at ORF42 and ORF114. Application of quantitative PCR and northern blotting techniques revealed that miR-C5 and miR-C4 were the most abundant among these 17 viral miRNAs (Lu *et al.* 2017). Subsequently, it was reported that host miRNAs are also involved in CyHV-2 infection and participate in the regulation of apoptosis and immune-related genes (Lu *et al.* 2018a). Of the total 888 detected miRNAs of this study, 840 were known and rest 48 were novel miRNAs (Lu *et al.* 2018a). Very recently, the same team found that, out of the 17 viral miRNAs, CyHV-2 miR-C12 is the important suppressor of CyHV-2-induced apoptosis of host cells by down regulating caspase 8 expression, promoting viral latency and propagation (Lu *et al.* 2019). In brief, evidences suggest that CyHV-2, may become latent and/or persistent in surviving fish after acute infection. Various stress including change in water temperature, maturation of animal, transportation, injury and secondary infection, may reactivate such latent CyHV-2, leading to the shedding of virus and spreading of infection to other fish in pond.

Diagnosis of CyHV-2 Infection

As in any disease, the first challenge in handling the outbreaks of CyHV-2 infections is to establish the diagnosis. A presumptive diagnosis is usually made based on the history especially, import from enzootic areas, clinical signs, gross and histological lesions which have been detailed earlier. Serum biochemical analyses of diseased fish may be an adjunct to the diagnosis and Lu *et al.* (2018b) showed that there were significant increases in alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and lactate dehydrogenase activities, and significant decreases in total protein, globulin, total bilirubin, creatinine and urea levels in CyHV-2 infection. A more precise presumptive diagnosis is based on electron microscopy findings (described under the section electron microscopy), *in vitro* viral isolation and by specific molecular and immunological methods.

Virus isolation and propagation

In vitro isolation of CyHV-2 has been attempted since the very beginning of 1992. During these earlier studies, CyHV-2 could not be propagated on different cell lines continuously beyond 4-5 passages, which was a major obstacle to gathering information on viral pathogenesis. The first attempt on *in vitro* isolation of CyHV-2 was described by Jung and Miyazaki (1995), where a series of cell lines such as fathead minnow (FHM), epithelioma papulosum cyprini (EPC), eel kidney (EK-1), chinook salmon embryo (CHSE-214), rainbow trout gonad (RTG-2) and tilapia ovary (TO-2) were used. However, CPE (cytopathic effects) were observed only in FHM, EPC and TO-2 cells. Later on different cell lines like koi fin (KF-1), bluegill fibroblast 2 (BF-2), goldfish (GF-1), common carp brain (CCB), standard Ryukin Takafumi (SRTF), Ryukin fin (RKF), *Cyprinus carpio* koi fin (CCKF), Goldfish fin (GFF),

Table 2 Details of isolation of CyHV-2 using different cell lines by various researchers during 1995–2020

S.No	Cell lines	Medium	Incubation	Fish and tissue homogenate	Start of CPE	CPE	Viral Titre	Passage of virus	References
1.	FHM	EMEM with 10% FBS + antibiotics	20°C	Goldfish, *C. auratus*, Spleen and kidney	NM	Vacuolation, pyknosis, lysis, aggregation of rounded, degenerate cells	$10^{6.9}TCID_{50}$ mL^{-1} (14th day culture of 2nd passage)	4th passage	Jung and Miyazaki (1995)
	EPC				NM		NM	NM	
	TO-2	L-15 medium with 10% FBS + antibiotics			NM	Cells exhibited numbers of rounded cells with no further development of the CPE	NM	NM	
	EK-1				–	No CPE	–	–	
	CHSE-214	EMEM with 10% FBS	15°C		–	No CPE	–	–	
	RTG-2				–	No CPE	–	–	
2.	CHSE-214	NM	20°C	Goldfish, *C. auratus*, Kidney	–	No CPE	–	–	Groff *et al.* (1998)
	EPC				–	No CPE	–	–	
	FHM				–		–	–	
3.	KF-1	NM	NM	Goldfish, *C. auratus*, Spleen and gills	–	No CPE	–	–	Goodwin *et al.* (2006a)
	FHM				–	No CPE	–	–	
	EPC				–	No CPE	–	–	
4.	EPC	GMEM with 10% NBCS antibiotic + antimycotic	15°C & 20°C	Goldfish, *C. auratus*, Spleen, kidney and brain tissues	–	No CPE	–	–	Jeffery *et al.* (2007)
	BF-2		15°C		–	No CPE	–	–	
	KF-1	EMEM with 10% foetal bovine + antibiotics	20°C	Gill	10 dpi	Focal areas of granulation, cell vacuolization and detachment of cells	NM	2nd passage	
5.	GF-1	NM	NM	CyHV-2 was provided by H. Fukuda	–	–	–	–	Hedrick *et al.* (2006)
6.	BF-2	EMEM with 10% foetal bovine	15°C and 23°C	Prussian Carp, *C. auratus gibelio,* Gill, Spleen, kidney	NM	CPE in all 4 cell lines at 23°C and in BF-2, FHM and RTG 2 cell lines at 15°C	NM	1st passage	Danek *et al.* (2012)
	EPC								
	RTG-2								
	FHM								

Table 2 (continued)

S.No	Cell lines	Medium	Incubation	Fish and tissue homogenate	Start of CPE	CPE	Viral Titre	Passage of virus	References
7.	Koi Fin	EMEM with 10% foetal bovine	25°C	Gibel Carp, *C. auratus gibelio,* Kidney and spleen	10 dpi	Pyknosis of cells, cells became rounded and cytolysis, vacuolation and the cells detaching from the monolayer and lysing.	NM	5th passage	Xu *et al*. (2013)
8.	BF-2	EMEM with 10% FBS + antibiotics	20°C	Crucian Carp, *C. carassius,* Gills, spleen, brain and kidney	–	No CPE	–	–	Fichi *et al*. (2013)
	EPC				–	No CPE	–	–	
	CCB		25°C		–	No CPE	–	–	
9.	GFF	MEM with 10% FBS + antibiotics	25°C	CyHV-2 Saitama-1 isolate	7 dpi	Granulation, cytoplasmic vacuolization, syncytium formation and the cell detachment	$10^{2.1}$TCID$_{50}$ mL^{-1}	12th passage	Ito *et al*. (2013)
	SRTF				7 dpi		NM	NM	
	RKF				–	No CPE	–	–	
10.	EPC	NM	20°C	Goldfish, *C. auratus,* Spleen, kidney and gill	–	No CPE	–	–	Lovy and Friend (2014)
11.	GiCB	Medium 199 with 10% FBS	25°C	Gibel Carp, *C. auratus gibelio* Kidney and spleen	4 dpi	Shrinkage, rounding and cell fusion with cytoplasmic vacuolization and cell death	$10^{7.5 \pm 0.37}$ TCID$_{50}$ mL^{-1}	50 passages	Ma *et al*. (2015)
	GFF				NM		$10^{3.0 \pm 0.26}$ TCID$_{50}$ mL^{-1}	3rd passage	
	Koi Fin				NM		$10^{2.3 \pm 0.30}$ TCID$_{50}$ mL^{-1}	3rd passage	
	CCB				NM	CPE detected	NM	1st passage	
	CIK				–	No CPE	–	–	
	EPC				NM	CPE detected	NM	1st passage	
12.	CCKF	L- 15 medium + 10% FBS + antibiotics	28°C	Goldfish, *C. auratus* Pools of gills, kidney and spleen	5 dpi	Pyknosis in the cytoplasm of cells, vacuolation, rounding of cells and destruction of whole monolayer	NM	4th passage	Sahoo *et al*. (2016)

Table 2 (continued)

S.No	Cell lines	Medium	Incubation	Fish and tissue homogenate	Start of CPE	CPE	Viral Titre	Passage of virus	References
13.	BF-2	EMEM with 10% FBS + antibiotics	20°C	Crucian Carp, *C. Carassius* Gill, brain, liver, spleen and kidney	After 3 blind passage	–	NM	NM	Fichi *et al*. (2016)
	EPC								
	CCB		25°C						
14.	GFKf	M199 with 15–20% FBS	25°C	Kidney					Jing *et al*. (2016)
15.	GH	in M199 with 10–20% FBS	25°C	Heart					Jing *et al*. (2017)
16.	GiCF	M199 medium supplemented with 10–15% FBS	25°C	Caudal fin of *C. auratus gibelio*	7th day post-infection	Shrinkage, rounding, detachment and cell fusion with cytoplasmic vacuolization	$10^{4.9 \pm 0.22}$ $TCID_{50}$ mL^{-1}	20th passage	Lu *et al*. (2018a)
17.	CrCB	M199 medium with 10% FBS	25°C	Brain tissue of crucian carp *C. carassius*	3rd day post-infection	NM	NM	NM	Xu *et al*. (2019)
18.	GFB		25°C	Brain tissue of goldfish *C. auratus*	7th day post-infection	NM	NM	NM	Xu *et al*. (2019)
19.	FtGF	L- 15 medium + 10% FBS + antibiotics	28°C	Goldfish, *C. auratus* Caudal fin	4th day post-infection	Cell elongation, rounding and cell fusion with cytoplasmic vacuolation, cell death and complete detachment	$10^{7.8 \pm 0.26}$ $TCID_{50}$ mL^{-1}	20th passage	Dharmaratnam *et al*. (2020)

Gibel Carp Brain (GiCB) were also attempted for CyHV-2 isolation (Table 2). Except Jung and Miyazaki (1995), other authors have found that EPC cell line is not successful in CyHV-2 isolation (Groff *et al.* 1998; Goodwin *et al.* 2006a; Jeffery *et al.* 2007; Fichi *et al.* 2013; Lovy & Friend 2014). KF-1 cell line was capable for isolation of CyHV-2 till the fifth passage (Xu *et al.* 2013). Ito *et al.* (2013) propagated CyHV-2 up to 12 passages in GFF and SRTF cell lines. However, the yields of virus from these cells were very low, achieving a maximum titre of $10^{3.0 \pm 0.26}$–$10^{2.3 \pm 0.30}$ $TCID_{50}$ mL^{-1}. Thus, isolation and continuous propagation of CyHV-2 has been very challenging due to the lack of permissive cell lines. In 2015, a cell line was developed from Gibel Carp Brain which gave a lead by propagating CyHV-2 up to 50 passages (Ma *et al.* 2015). The highest viral titre of CyHV-2 was obtained from this GiCB cell line which was about $10^{7.5 \pm 0.37}$ $TCID_{50}$ mL^{-1}. Lu *et al.* (2018b) developed a novel cell line, GiCF cell line (*C. auratus gibelio* caudal fin cell line) for establishing CyHV-2 replication in which the virus induced apoptosis. The virus titre reached $10^{4.9 \pm 0.22}$ $TCID_{50}$ mL^{-1}, and infectious CyHV-2 was produced from the GiCF cells over 30 subcultures. Xu *et al.* (2019) demonstrated CPE in two newly developed brain cell lines CrCB and GFB from 3d after infection with CyHV-2 in silver crucian carp and goldfish, respectively. The cell line GiCF may serve as an ideal infection platform to characterize the apoptosis effect of CyHV-2. Chai *et al.* (2020) established a novel cell line derived from the brain of gibel carp (GCBLat1) which supports the CyHV-2 latency, and the same can be used to investigate the mechanism of latency and reactivation. Recently, we developed a highly sensitive cell lines, Fantail Goldfish Fin (FtGF), Fantail Goldfish Gill (FtGG), Fantail Goldfish liver (FtGL) and Fantail Goldfish brain (FtGB) for continuous propagation of CyHV-2 and compared their permeability in the propagation of CyHV-2; through which we could serially passage the virus in FtGF over 20 times achieving a high titre of $10^{7.8 \pm 0.26}$ $TCID_{50}$ mL^{-1} in the early passage levels (unpublished data).

The major cytopathic affects (CPE) induced by CyHV-2 in all these cell lines include pyknosis, granulation, cytoplasmic vacuolization, syncytium formation in focal areas, appearance of rounded bright cells and lysis in the early stages, which were also observed in our lab (Fig. 5). In later stages the infected cells became rounded, leading to the formation of holes and finally the destruction of monolayer (Jung & Miyazaki 1995; Jeffery *et al.* 2007; Danek *et al.* 2012; Xu *et al.* 2013; Ito *et al.* 2013; Ma *et al.* 2015; Sahoo *et al.* 2016). Hine *et al.* (2006) detailed the measurement of different stages during the assembly of viral particles in infected cell lines *viz.*, capsids, nucleocapsid and virions as 112 ± 4 nm, 103 ± 6 nm and 170 ± 12 nm, respectively. Ma *et al.* (2015) observed complete replication of CyHV-2 in GiCB cells infected with CyHV-2.

Immunological techniques

In virology research, monoclonal antibodies (mAbs) are known as efficient tools for the screening, diagnosis, assessment of viral pathogenesis and immunotherapy of disease (Shi *et al.* 2003; Fofana *et al.* 2013; Wang *et al.* 2013). Ding *et al.* (2014) developed a more specific and stable fluorescence in situ hybridization (FISH) probes labelled with 6-Carboxyfluorescein (6-FAM) for the detection of CyHV-2 polymerase gene sequences in tissue samples. CyHV-2 infection was confirmed in the peripheral blood cells of silver crucian carp, *C. auratus gibelio* (Bloch) using *in situ* hybridization (Wang *et al.* 2016). Monoclonal antibody-based indirect fluorescent antibody method was used to detect CyHV-2 infection in goldfish (Nanjo *et al.* 2016), which produced strong staining signal in infected and dead fish. In another study Kong *et al.* (2017) prepared monoclonal antibodies against recombinant ORF72 protein (pORF72) of CyHV-2 and then established an immunohistochemical protocol and a blood smear method to detect CyHV-2 in carps using the same monoclonal antibody. Monoclonal antibody mAb-1B7 was produced against pORF92 and used for the detection of CyHV-2-infected crucian carp by western blotting and indirect immunofluorescence assays (IFA) methods (Shen *et al.* 2018).

Molecular techniques

The major challenge in diagnosis of CyHV-2 is posed by the latency of virus in apparently healthy fish, because latently infected animals may carry the viral genome without producing infectious particles so that false-negative reactions are possible in cell culture or immunology-based diagnostic methods. Even though quantification of antiviral antibodies that would indicate prior virus exposure can detect latent infections, duration of detectable titres against specific pathogens is quite variable in fish (Bricknell *et al.* 1997). More importantly, fish carrying the virus without developing acute disease will not have antibodies (Goodwin *et al.* 2006b). These limitations in cell culture and immunological methods for detection of latent herpesviral infections make molecular methods which rely on detection of viral genome, indispensable in diagnosis of CyHV-2 infections (Gray *et al.* 2002). Accordingly, a number of conventional and RT-PCR (real time PCR) protocols have been developed for molecular detection of CyHV-2 from natural infections, experimental infections and from cell culture (Table 3). Among the conventional PCR methods, it was Waltzek *et al.* (2005) who first amplified three different genes of CyHV-2 namely, helicase (867 bp), DNA

Table 3 List of primer used for detection of CyHV-2 infection in natural disease outbreak and experimental challenge studies

SI No	Gene	Sequence		Product Size	References
1.	Helicase	*F*	CTGATCATCGACGAGTACGG	867	Waltzek *et al*. (2005)
		R	CACACGCGTGCACACNACRTA		
2.	Intracapsomeric triplex protein	*F*	CACTCTGGCGACGCNTTYATG	259	
		R	CATCACAGAGTTCTTGACNGC		
3.	DNA Polymerase	*F*	CGGAATTCTAGAYTTYGCNWSNYTNTAYCC	497	
		R	CCCGAATTCAGATCTCNGTRTCNCCRTA		
4.	DNA polymerase	*F*	TCGGTTGGACTCGGTTTGTG	170 bp	Goodwin *et al*. (2006a)
		R	CTCGGTCTTGATGCGTTTCTTG		
		P	FAM-CCGCTTCCAGTCTGGGCCACTACC-BHQ1		
5.	DNA Polymerase	*F*	CCAGCAACATGTGCGACGG	362	Jeffery *et al*. (2007)
		R	CCGTARTGAGAGTTGGCGCA		
		F	CGACGGVGGYATCAGCCC	339	
		R	GAGTTGGCGCAYACYTTCATC		
6.	Helicase	*F*	GGACTTGCGAAGAGTTTGATTTCTAC	366	Waltzek *et al*. (2009)
		R	CCATAGTCACCATCGTCTCATC		
7.	Helicase	*F*	GAACACCGCTGCTCATCATC	357	Xu *et al*. (2013)
		R	ACTCTTCGCAAGTCCTCACC		
8.	DNA Polymerase	*F*	CCCAGCAACATGTGCGACGS	362	Boitard *et al*. (2016)
		R	CCGTARTGMGAGTTGGCGCA		
9.	Marker A (mA)	*F*	CCACTTAGAGTAACCACTTAGAG	432	
		R	GCGTTGACTCATTTGCGGTTTG		
10.	Marker B (mB)	*F*	ATCATGGAAGATGTTCTGGCCAG	475	
		R	CAGCAGCAACTGAGCGTCATG		

polymerase (497 bp) and intra capsomeric triplex protein (259 bp) for detection of CyHV-2. Subsequently, another set of primers based on the conserved polymerase gene sequences between the three fish herpesviruses (CyHV-1, CyHV-2 and CyHV-3) was used by Jeffery *et al*. (2007) which successfully amplified 362 bp product from CyHV-2-infected fish. Afterwards, the same primer set was used in numerous studies for confirmation of CyHV-2 infection in both natural disease outbreak and experimental challenge studies (Wang *et al*. 2012; Danek *et al*. 2012; Fichi *et al*. 2013; Boitard *et al*. 2016). Subsequently, Waltzek *et al*. (2009) developed another PCR reaction based on helicase gene of CyHV-2. Xu *et al*. (2013) designed primers that can amplify partial segment of helicase gene (357 bp), for the diagnosis of HVHND in *C. auratus gibelio*. The detection limits for CyHV-2 and spring viraemia of carp virus (SVCV) detection were reported as approximately 88 copies of the cloned viral genomic fragments for both, and there was no cross reaction with other infectious agents and host DNA. Later, two set of primers, one targeting marker A (mA) located in the intergenic region between CDS2 and CDS3 within the terminal direct repeat (TR), and another targeting marker B (mB) located at the 3' end of ORF117 were used for molecular detection of CyHV-2 by Boitard *et al*. (2016). Of these genetic marker, mB was found to be identical in different isolates, suggesting suitability for molecular diagnosis of CyHV2 infections, and unsuitability to be used in molecular tracing studies. On other hand, as the length of mA region among different CyHV-2 strains is different, molecular diagnosis using mA-based primers have to be verified by sequencing to avoid incorrect diagnosis. At the same time, such variability in mA region can be explored for further research on the rate of mutation of the different motifs in this region of CyHV-2 to study the genetic variation and rapid genotyping (Boitard *et al*. 2016; Ito *et al*. 2017).

However, the conventional PCR is very useful for finding out the presence and absence of viral genomes in both diseased fish and cell culture nucleic acid. It may not differentiate between healthy fish and fish with latent infections. Thus, detection of CyHV-2 in conventional PCR may not be a proper diagnostic tool for identifying active disease cases, so that RT-PCR results are more preferred in diagnosis. Further, RT-PCR using specific fluorescent probes has smaller chance of false positives caused by contamination. Accordingly, Goodwin *et al*. (2006b) developed a highly specific real time 5'-nuclease PCR method (TaqMan) targeting DNA polymerase gene of CyHV-2 with a linear response over 8 logs of target concentration. The reported sensitivity was 1 target molecule per reaction. The assay did not cross-react with other similar fish herpesviruses, including CyHV-1 and CyHV-3 and differentiated clinical and latent cases of CyHV-2 infection. Apart from conventional and RT-PCR, molecular methods like *in-situ* hybridization, loop-mediated isothermal amplification

(LAMP) assay and isothermal RPA (Recombinase polymerase amplification) have been applied in CyHV-2 diagnosis. Ding *et al.* (2014) and Xu *et al.* (2014) applied *in-situ* hybridization and fluorescence in situ hybridization (FISH) for the localization of specific probes against nucleic acid for confirmation of cellular changes caused by CyHV-2 in focal necrotic lesions of kidney, spleen, liver and gill tissue. LAMP assay was developed for field level diagnosis of CyHV-2 with limited resources by He *et al.* (2013) and Liang *et al.* (2014) that could detect 10 copies/ μL and 1.09×10^{-4} μg μL^{-1} DNA of CyHV-2, respectively. Zhang *et al.* (2014) also developed a LAMP-PCR assay for the rapid detection of CyHV-2 in Gibel Carp (*C. auratus gibelio*). However, higher chance of false-positive reactions due to cross-contamination should be considered during the application of LAMP-PCR assay in diagnosis. Recently, a rapid and more convenient detection assay based on isothermal RPA (recombinase polymerase amplification) and lateral flow dipstick (LFD) was developed by Wang *et al.* (2018) for detecting CyHV-2 under field conditions. For this, the highly conserved ORF72 of CyHV-2 was targeted by specific and sensitive primers and probes. The optimized RPA assay takes about 45 min (15 min at 38°C using water bath for reaction and 30 min for analysis of products by 2% agarose gel electrophoresis). A simple lateral flow strip based on the unique probe in reaction buffer was developed for visualization. The entire RPA-LFD assay takes 50 min less than the routine PCR method, was 100 times more sensitive and displayed no cross reaction with other aquatic viruses. Recently, we have developed new LAMP and PCR assays for detection of CyHV-2 infection that were highly specific and sensitive and capable of detecting 10 copies of the plasmid construct containing 942 bp fragment of MCP gene of CyHV-2.

Experimental challenge studies

Experimental challenge trials are often required in aquatic animals to study disease pathogenesis, host-pathogen interactions within different environments as well as for the comparison of performance between various existing and novel diagnostic tests, treatments and vaccine efficacy. Experimental challenges of CyHV-2 have been carried out mostly by intraperitoneal (i/p) injections of the homogenates from infected tissues or cell culture fluid isolated from virus-infected flasks (Jung & Miyazaki 1995; Hedrick *et al.* 2006; Ito *et al.* 2013; Xu *et al.* 2013; Zhou *et al.* 2015; Sahoo *et al.* 2016). In the first report of CyHV-2, healthy goldfish inoculated with 0.1 ml of $10^{3.0}$, $10^{3.9}$ and $10^{5.5}$ $TCID_{50}$ of 1st and 2nd passage virus began to die within 3–6 days post-inoculation (dpi) and cumulative mortalities of 100% and 60% occurred within 13 days, among the groups challenged with the highest and lowest viral titre, respectively (Jung & Miyazaki 1995). However, no disease was observed among koi carp after i/p injection of CyHV-2 (Jung & Miyazaki 1995; Hedrick *et al.* 2006; Sahoo *et al.* 2016). Xu *et al.* (2013) carried out experimental infections in healthy *C. auratus gibelio* (gibel carp) by i/p injection of filtrated tissue homogenate of diseased fish, where mortality began at 6 dpi and the mortality reached 100% at 12 dpi. Ito *et al.* (2013) examined the influence of water temperature on the development of disease in goldfish following i/p challenge with CyHV-2 and found that temperature of 20–25°C is highly suitable for disease development, whereas at 13-15°C fish acquired resistance and acted as carriers. Nanjo *et al.* (2016) showed that exposure to higher temperature (34°C for 6 days) reduced the mortalities in experimentally challenged CyHV-2 fish (with a dose of $10^{2.5}$ $TCID_{50}$/ fish i/p) demonstrating initiation of immunity at higher temperature. Apart from these i/p infection studies, intramuscular (i/m) and immersion routes have also been attempted. Wang *et al.* (2012) induced CyHV-2 infection in healthy Prussian carp by i/m injection of the infected tissue filtrate, where 100% mortality was observed. Ito *et al.* (2013) conducted experimental infections in 2 varieties of goldfish (Ryukin and Edonishiki) with CyHV-2 virus passaged 7 times in GFF cells by either i/p or immersion method. The cumulative mortality was 30% and 100% in Ryukin variety following the immersion and i/p challenge at 20°C. There was 90% cumulative mortality in Edonishiki variety in both i/p challenge at 20°C and immersion challenge at 25°C. Through experimental studies either by i/p injection or immersion of CyHV-2-infected GFF cell culture supernatant, Ito and Maeno (2014) showed that susceptibility of Japanese indigenous *Cyprininae* fish viz., ginbuna, *C. auratus langsdorfii*, nagabuna, *C. auratus buergeri*, nigorobuna, *C. auratus grandoculis* and common carp (*C. carpio*) to CyHV-2 is much lower than that of goldfish. Thus, comparison between the experimental challenge studies through i/p and immersion method showed that both methods are effective in causing the infection and disease diagnosis (Ito *et al.* 2017); however, i/p-injected fish showed symptoms of CyHV-2 earlier than the fish infected by immersion method (Sahoo *et al.* 2016). In both routes, the clinical signs produced after challenge studies were same as that of naturally infected fish.

Prophylaxis and control

Owing to serious outbreaks of CyHV-2 infections in many countries and resulting enormous financial losses to aquaculture industry as reported by Xu *et al.* (2013), it has become vital to design suitable preventive and control measures. It was reported that CyHV-2 has spread to almost all main areas of cultured gibel carp in Jiangsu province, China during 2011–2012 (Xu *et al.* 2013). As there are no effective drugs or vaccines currently available for CyHV-2,

control strategies are mainly based on the prevention of this disease rather than to cure. In general, several aquatic animal disease management strategies have to be followed such as health certification and quarantine of imported stocks as well as strict biosecurity measures at farm level, *etc*. Many studies have confirmed that the spread of CyHV-2 is influenced to great extent by global trade of apparently healthy infected goldfish (Adamek *et al*. 2017; Ito *et al*. 2017; Panicz *et al*. 2019). Thus, it is of utmost importance to screen imported goldfish varieties for CyHV-2 in quarantine facilities using sensitive diagnostic methods before introducing fish on local markets. It is also necessary that the female brooder must be inspected for CyHV-2 infections before supplying their fry (Zhu *et al*. 2018).

Studies on the physical and biochemical properties of CyHV-2 will help in designing potential specific mechanisms to interfere the viral pathogenicity. More precisely, the range of 20–25°C and lower temperatures (16.1–20.5°C) are considered as the optimal temperature for CyHV-2 infection in goldfish (Ito & Maeno 2014) and gibel carp (Danek *et al*. 2012), respectively. These observations suggested that CyHV-2 infection can be controlled by maintaining the water temperature at 33–35°C for goldfish (Ito & Maeno 2014) and 32°C for Prussian carp (Liang *et al*. 2015) which have been successfully used by different workers. Shibata *et al*. (2015) also documented that the temperatures of 34°C are non-permissive to CyHV-2. However, as per Panicz *et al*. (2019), stressogenic effect due to fluctuations in water temperature leading to a significant reduction in fish immunity is more important than the absolute value of temperature. Therefore, preventing a broad fluctuation in rearing water is also important in controlling the outbreaks. Results of pH and salinity experiments indicated that CyHV-2 had a high tolerance to pH and salinity; which suggests that disease outbreaks cannot be controlled by adjusting salinity or pH (Liang *et al*. 2015). Among the chemical agents, CyHV-2 was found to be sensitive to IUdR (5-iodo-2-deoxyuridine), acid (pH 3), ether and chloroform (Jung & Miyazaki 1995; Liang *et al*. 2015), which all might be tried in decontamination/inactivation purposes.

Vaccination and use of immunostimulants in aquaculture have emerged as one of the most effective disease preventive strategies against infectious agents (Dadar *et al*. 2017). Experimental studies on CyHV-2 vaccines have been initiated since 2013 which are detailed here. It was Ito and Ototake (2013) who first attempted a formalin-inactivated vaccine against HVHN in goldfish. They found that the vaccine developed from the formalin-inactivated cell culture supernatant of CyHV-2-infected GFF cells provided a relative percentage survival (RPS) value of 57% in the challenged goldfish. After that, Ito and Maeno (2014) found that the protective efficacy period of this vaccine was at least 8 weeks after i/p vaccination. They also demonstrated that a booster vaccination at 4 weeks of initial dose could enhance the protection (RPS value around 64%). Zhang *et al*. (2016) used β-propiolactone to prepare inactivated CyHV-2 vaccine. They could establish that there was an induction of both innate and specific antiviral immune response against this vaccine in cultured gibel carp through various immune assays (phagocytic activity, lysozyme and superoxide dismutase activity, blood cell counting, immune gene expression analysis, neutralizing antibody titration). More importantly, the challenge test demonstrated that the immunized gibel carp had a RPS value of 71.4%. Apart from inactivated vaccines, subunit vaccination studies have also been initiated in CyHV-2 research. Zhou *et al*. (2015) explored yeast expression system (*Pichia pastoris*) for the production of three recombinant truncated proteins of CyHV-2 viz., tORF25, tORF25C and tORF25D and showed that these recombinant truncated proteins could be potential candidate vaccines against CyHV-2 infections in gibel carp through experimental challenge studies. Another study analysing amino acid sequence homology of core ORFs from the alloherpesviruses family showed the 4 core ORFs (ORF33, ORF79, ORF92 and ORF107) are more conserved in this family and can be used as antigen candidates of genetic vaccine against *Alloherpesviridae* (Liu *et al*. 2018), however, *in vivo* validation of this finding has not been conducted till date. Similarly, very recently, Gao *et al*. (2020) identified eight major immunogenic proteins of CyHV-2 in mice, namely pORF92, pORF115, pORF25, pORF57, pORF66, pORF72, pORF131 and pORF132, which are also to be targeted in future immunization studies in fish. Recently, a live vector vaccine against CyHV-2 based on recombinant baculovirus BacCarassius-D4ORFs containing a fused codon-optimized sequence D4ORFs was developed and proved as a potential candidate vaccine against CyHV-2 infection in gibel carp in China (Li *et al*. 2019). Yan *et al*. (2020) proved the efficiency of β-propiolactone-inactivated Cyprinid herpesvirus-2, mixed with β-glucan or anisodamine as immersion immune adjuvant and also provided reference for improving the efficiency of immersion immunity. A DNA vaccine, pEGFP-N1-ORF25 based on the ORF25 gene of CyHV-2 was constructed by Yuan *et al*. (2020) which have a strong immune protection effect against CyHV-2 in Prussian carp. In short, as there are no effective drugs or vaccines available for CyHV-2, control strategies focusing on strict biosecurity measures and strict health certification and quarantine of imported stocks are strongly recommended in the present scenario. Further, research focusing on developing effective vaccines and immunostimulants are urgently required for the real containment of the disease.

Current challenges and future perspectives

Currently, CyHV-2 is widespread in mainly *Carassius* sp. in different countries and outbreaks apparently occur when healthy carriers are exposed to different stressors. The disease is recognized to cause huge economic losses and social impacts on both ornamental and food fish aquaculture industries (Zhu *et al.* 2018). Huge economic losses due to the CyHV-2 infection among cultured gibel carp in China, during 2011–2012 (Xu *et al.* 2013), mass mortality in crucian carp during 2012 in Italy (Fichi *et al.* 2013), 95% mortality in goldfish during 2014 in France (Boitard *et al.* 2016), 85% mortality in goldfish during 2016 in Poland (Panicz *et al.* 2019) had been reported. Identification of the major research gaps, and questions requiring more investigations in CyHV-2 research are outlined here.

We have listed certain important challenges and future perspectives including understanding the complete host range for CyHV-2, establishing the modes and routes of transmission of CyHV-2 in relation to severity of disease, host immune response against CyHV-2, mechanism of latency and reactivation of CyHV-2 and development of specific disease management strategies such as selective fish breeding for disease resistance and efficacious vaccines in mitigating the diseases outbreaks due to CyHV-2 in fish worldwide.

The host range and transmission of CyHV-2 infection is still not well defined. The success of prophylactic efforts cannot be guaranteed until the host range and route of transmission is defined. Even though the disease was reported as a specific disease of goldfish, it is now known that the CyHV-2 can infect a much wider range of cyprinid species (Hedrick *et al.* 2006; Bergmann *et al.* 2010; Fichi *et al.* 2013; Zhu *et al.* 2018). As discussed earlier, severe natural CyHV-2 infections are now reported from crucian carp, Prussian carp and allogynogenetic crucian carp. Further, the recent findings of Zhu *et al.* (2018) indicated that CyHV-2 can cross-infect among different species of fish. In contrast, a specific host recognition factor was suggested in previous reports (Puck 2013; Liang *et al.* 2015). Thus, understanding the host range is an important research area in CyHV-2 research, impacting virus acquisition and dissemination, and may lend itself to adoption of novel preventive and control strategies. Also, the role of common carp in CyHV-2 infection is not defined till date. Common carp reared in neighbouring cages of goldfish did not exhibit any clinical signs as per Boitard *et al.* (2016) who described HVHND mortality outbreaks in France. Additionally, Hedrick *et al.* (2006), and Ito and Maeno (2014) did not report mortalities of common carp immersed in or injected with CyHV-2 suspension. However, Panicz *et al.* (2019) identified CyHV-2 DNA in gill and blood samples of asymptomatic common carp. Thus, further studies are required to provide data on probable role of common carp as vector and other fish species in CyHV-2 infections, routes of penetration of virus into these vectors, clearance rate and the possible replication of CyHV-2, and research on these lines will also throw new insights on control strategies (Panicz *et al.* 2019).

Establishing the modes and routes of transmission in relation to severity of disease will be another important future direction. Emerging evidence suggests vertical transmission is possible for CyHV-2 (Goodwin *et al.* 2009; Zhu *et al.* 2018); even though it is not confirmed and, if possible whether transmission is inside or on outside of the gametes have to be targeted in future studies (Zhu *et al.* 2018). Similarly, even though several potential vectors including fish droppings (Dishon *et al.* 2005), aquatic invertebrates (Kielpinski *et al.* 2010), plankton (Minamoto *et al.* 2011), mechanical transmission of sick fish by piscivorous birds (Ilouze *et al.* 2011) are reported for CyHV-3, such vectors based on transmission studies have not been carried out for CyHV-2 disease outbreaks elsewhere. Understanding the interactions between the virus and their different host/ vectors can underpin the approaches to protect fish from infection by interfering with virus uptake and transmission.

Host immune response against CyHV-2 infection can be another interesting arena to be investigated further, for designing novel diagnostic, therapeutic and prophylactic measures. Identification of the factors contributing to the outcome of CyHV-2 infection (either lethality, sterile immunity or persistent/ latent infection) other than the initial dose and water temperature will help design more effective control measures. Further, elucidating the molecular basis of differential host response in varied dose or water temperature is urgently needed. Many recent studies have demonstrated differential expression of various genes after CyHV-2 infection (Xu *et al.* 2014; Lu *et al.* 2017), which warrant future investigation to determine individual roles of these recognized genes (proviral or antiviral functions) which will index potential marker genes in disease investigations.

Ability of CyHV-2 to cause latent infections is confirmed very recently by Chai *et al.* (2020). However, mechanism of latency and reactivation is not fully established, and deciphering the pathways of latency remains challenging as well. Production of virus- and host-encoded miRNAs to promote CyHV-2 latency has been demonstrated by recent studies in cell lines (Donohoe *et al.* 2015; Lu *et al.* 2017; Lu *et al.* 2018b; Lu *et al.* 2019). These studies have also provided unique insights into the canonical and non-canonical roles of miRNAs in the regulation of host and viral gene expression. This emerging interface between viruses and miRNAs may uncover novel pathways in viral pathogenesis and this knowledge will also help to provide new biomarkers for diagnosis and to guide specific drug design.

However, the existence and role of these miRNAs during CyHV-2 latency *in-vivo* remains to be confirmed. Further, research targeted at ablating expression of miRNAs will ultimately help in designing specific therapeutic interventions to get rid of complete viral infection by forcing the virus to lytic cycle. Anti-miRNA oligonucleotides (AMOs), chemically modified synthetic oligonucleotides complementary to their target sequence, are one of options for ablating the expression of miRNAs (Moen *et al.* 2009). Different classes of AMO have been shown to be efficient in silencing miRNA and may be useful therapeutic tools in latent infections (Krutzfeldt *et al.* 2007; Mattes *et al.* 2007; Esau 2008; Bruscella *et al.* 2017). Such studies have not been initiated in CyHV-2 research and are warranted in future. Further, research on understanding the detailed mechanism of CyHV-2 latency and reactivation would contribute to the prevention and control of CyHV-2 in the aquaculture industry (Chai *et al.* 2020).

Development of specific disease management strategies such as selective fish breeding for disease resistance, and development of safe and efficacious vaccines are another desperately needed area in CyHV-2 to be thoroughly investigated further (Ito & Ototake 2013). Selective breeding programmes in animals have been mostly undertaken with a goal to increase the productivity (Gjedrem & Thodesen 2005), although disease resistance also remains a major aim as mortality caused by diseases is a major threat to aquaculture (Gjedrem 2015). Quantitative trait locus (QTLs) for resistance to viral diseases in Salmonids include QTLs for infectious hemopoetic necrosis virus (IHNV) resistance (Miller *et al.* 2004; Rodriguez *et al.* 2004), infectious salmon anaemia virus (ISAV) resistance (Moen *et al.* 2007), viral haemorraghic septicaemia virus (VHSV) resistance (Verrier *et al.* 2013), salmonid alphavirus (SAV) resistance (Gonen *et al.* 2015) and nervous necrosis virus (NNV) (Liu *et al.* 2016). Likewise, the identification of relevant QTL and the application of molecular markers for marker-assisted selection (MAS) like infectious pancreatic necrosis virus (IPNV) resistant Atlantic salmon, *Salmo salar* in both Norwegian (Moen *et al.* 2009) and Scottish populations (Houston *et al.* 2010), or the direct use of genotype data to perform GS like IPNV resistance (Houston *et al.* 2012). NNV resistance (Tine *et al.* 2014; Palaiokostas *et al.* 2015) are also useful in raising disease resistance fish population. However, no such selective breeding programmes or QTL analysis has been tried to identify the CyHV-2 resistant goldfish stains, which are essential in future research. The current status of experimental studies on CyHV-2 vaccines has been detailed above. Novel approaches for fish vaccines can be expensive to develop, but the limited success of traditional approaches (inactivated cellular preparations) especially in terms of duration of protective efficacy demands further exploration of advanced approaches. Simultaneously, enhancement of efficacy of traditional vaccines using adjuvant and immunostimulants (both conventional and new generation adjuvant like ligands for toll receptors or different cytokines) are also warranted. With the advancement of sequencing technologies, full genomes of 4 different strains of CyHV-2 are available now. Future studies underpinned by these CyHV-2 genome sequences emphasizing pathogenesis, epidemiological, diagnostic and therapeutic innovations will be critical and are necessary to control this pathogen.

In conclusion, despite multiple approaches, CyHV-2 remains a serious epidemic threat causing huge economic losses and social impacts in both ornamental and food fish aquaculture industry. Furthermore, increasing reports of CyHV-2 from various countries illustrate the global spread of this virus through uncontrolled and often unregulated fish trade. Several steps have been recommended to control further spread of CyHV-2 and associated economic losses. However, a robust translational research strategy on different aspects of CyHV-2 infections to enable a more specific and effective therapeutic, preventive and control strategies are urgently required to limit the losses in aquaculture and natural environment associated with this viral disease.

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