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In vitro characteristics of cyprinid herpesvirus 2: effect of kidney extract supplementation on growth

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ABSTRACT: Herpesviral hematopoietic necrosis caused by goldfish hematopoietic necrosis virus (now identified as cyprinid herpesvirus 2, CyHV-2) has contributed to economic losses in goldfish *Carassius auratus* culture and is becoming a major obstacle in Prussian carp *C. gibelio* aquaculture in China. Several reports have described difficulties in culturing the virus, with the total loss of infectivity within several passages in cell culture. We succeeded in propagating CyHV-2 with a high infectious titer in a RyuF-2 cell line newly derived from the fin of the Ryukin goldfish variety using culture medium supplemented with 0.2% healthy goldfish kidney extract. The addition of kidney extract to the medium enabled rapid virus growth, resulting in the completion of cytopathic effect (CPE) within 4 to 6 d at 25°C. The extract also enabled reproducible virus culture with a titer of 10^{5-6} $TCID_{50}$ ml^{-1}. The virus cultured using this protocol showed pathogenicity in goldfish after intraperitoneal injection. The virus grew in RyuF-2 cells at 15, 20, 25, 30, and 32°C but not at 34°C or higher. Higher incubation temperatures allowed earlier development of CPE, but culture at 30 and 32°C yielded a lower virus titer than that obtained at other temperatures because of heat inactivation of the propagated virus during cultivation. Cell lines derived from goldfish and ginbuna *C. langsdorfii* showed high susceptibility to the virus; cell lines from carp were susceptible to the virus using a medium containing goldfish kidney extract, but EPC, FHM, and BF-2 cell lines did not produce any CPE, even in the presence of the extract.

KEY WORDS: Goldfish hematopoietic necrosis virus · GFHNV · Cyprinid herpesvirus 2 · Culture · Kidney extract · Growth temperature · Cell susceptibility · *Carassius*

INTRODUCTION

Herpesviral hematopoietic necrosis (HVHN), resulting from infection with goldfish hematopoietic necrosis virus (GFHNV), was first reported among cultured goldfish *Carassius auratus* in Japan in 1995 (Jung & Miyazaki 1995). GFHNV is currently designated as cyprinid herpesvirus 2 (CyHV-2) and classified in the genus *Cyprinivirus*, family *Alloherpesviridae*. CyHV-2 has spread to most of the goldfish production areas in Japan and has caused significant economic losses in the industry. Subsequently, infection by this virus was found in goldfish in the USA, Taiwan, Australia, New Zealand, and the UK (Chang et al. 1999, Stephens et al. 2004, Goodwin et al. 2006, Hine et al. 2006, Jeffery et al. 2007, Lovy & Friend 2014), as well as in the crucian carp *C. carassius* in Italy (Fichi et al. 2013). More recently, the virus caused mortality in the Prussian carp *C. gibelio*, which is thought to be an ancestral species of gold-

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fish, in the Czech Republic (Daněk et al. 2012) and has caused significant losses in Prussian carp aquaculture in China since 2009 (Wang et al. 2012, Xu et al. 2013). The virus causes acute disease in goldfish of all ages, and mortality following infection with the virus can reach almost 100% at temperatures between 15 and 25°C (Jung & Miyazaki 1995). Diseased fish show no remarkable external signs except pale gills, which result from severe destruction of the hematopoietic tissues (Jung & Miyazaki 1995). Shifting the water temperature to 33–35°C reduces mortality in infected fish, and the surviving fish can acquire immunity against subsequent infection by the virus (Tanaka 2005). Other control methods have been studied, including a formalin-killed vaccine (Ito & Ototake 2013) and selective breeding to establish resistant strains of goldfish (Tanaka 2013).

Some previous reports have pointed out the difficulties in cultivating CyHV-2. Jung & Miyazaki (1995) observed quite extensive cytopathic effect (CPE) in cultures of fathead minnow (FHM) and epithelioma papulosum cyprini (EPC) cell lines used for its initial isolation, yet total infectivity was subsequently lost at the fifth passage in these cells. The same loss of infectivity during virus propagation was reported by Goodwin et al. (2006) using koi fin-1 (KF-1), FHM, and EPC cells and by Jeffery et al. (2007) using KF-1 cells. Successful cultivation was reported by Li & Fukuda (2003) and Ito et al. (2013) using the goldfish fin (GFF) cell line. Ito et al. (2013) successfully conducted infection trials with virus propagated in GFF cells. However, continuous cultivation of the virus remains difficult, even using goldfish-derived cells, and sometimes virus passage requires subcultivation of the virus-infected cells with freshly prepared cells. The infectivity of the virus propagated according to the method of Ito et al. (2013) was only to the level of 10^3 $TCID_{50}$ ml^{-1}. Because analyses based on infectivity are essential to define the characteristics of the virus, the low infectivity of CyHV-2 is a major obstacle in the analysis. As a result, there have been no reports about the *in vitro* characteristics of the virus, such as the growth curve and permissive temperature range, until now.

In this study, we attempted to obtain virus cultures with higher infectious titer as an initial step in the characterization of this pathogen. We asked why the initial isolation of the virus caused extensive CPE on the cells used in some previous studies. We paid attention to the tissues, the kidney homogenate (extract) in particular, included in the inoculum used during the isolation. We therefore added healthy goldfish kidney extract to the culture medium during virus propagation and subsequently succeeded in obtaining a virus stock with high infectious titer after a few passages in a GFF cell line. Using this virus culture, we then studied the *in vitro* characteristics of CyHV-2 such as the virus growth curve, growth temperature, stability at some temperatures, and infectivity in several other fish cell lines.

MATERIALS AND METHODS

Virus and cells

CyHV-2 SaT-1 isolated from diseased goldfish in 1999 in Japan (Ito et al. 2013) was used in this study. The RyuF-2 cell line, newly developed from the caudal fin of the Ryukin variety of goldfish, was used to propagate the virus. These cells were cultured using Eagle's minimum essential medium (Hank's buffered; Sigma) or Medium 199 (HEPES buffered; Sigma) supplemented with 5% fetal bovine serum (FBS; Gibco, Life Technologies), penicillin, and streptomycin (Sigma), which were abbreviated to MEM-5 and M199-5, respectively. Harvested virus cultures were filtered through a 0.45 µm filter unit (HV, Merck Millipore) and stored at –80°C until use.

Kidney extract from healthy goldfish

Goldfish kidney extracts were prepared using 3 lots of body kidney pooled from healthy goldfish: 2 individuals of the Azumanishiki variety (body length: 22 and 24 cm; lot 1), 2 individuals of the Azumanishiki variety (body length: 12.5 and 12.3 cm; lot 2), and 2 individuals of the Wakin variety (body weight: 80 and 86 g; lot 3). These fish were reared using groundwater in the Yoshida Station at the Tokyo University of Marine Science and Technology, where HVHN has never been experienced among goldfish. The kidney pool was homogenized with a 10-fold volume of MEM-5 and then subjected to centrifugation at 1900 × *g* (15 min at 4°C). The supernatant was filtered through 0.45, 0.22, and 0.1 µm pore size filter units (HV, GV, and VV, Merck Millipore), in turn, and stored at –80°C prior to use.

Obtaining virus cultures with high infectious titers

Virus passages 1–7 of the SaT-1 isolate after cultivation by Ito et al. (2013), in RyuF-2 cells seeded in 25 cm^2 flasks (Corning) were performed with

MEM 5 at 25°C. For passages 8–10, the virus was propagated using MEM-5 supplemented with 0.2% (weight of tissue per volume of medium) goldfish kidney extract (lot 1). From passage 11, virus culture was continued using M199-5 supplemented with the extract (lot 1). At passage 23, the virus was cloned in RyuF-2 cells by the limiting dilution method: a 2-fold dilution series of virus was established in a 96-well culture plate using standard procedures. One CPE-positive well of the 96 well-plate at the highest 2-fold dilution of the virus was selected.

Incubation period until completion of CPE

The Sat-1 isolate obtained from passage 14 ($10^{4.8}$ $TCID_{50}$ ml^{-1}) was used to inoculate one 25 cm^2 flask culture of RyuF-2 cells with either MEM-5 or M199-5 supplemented with or without goldfish kidney extract (lot 1) at a concentration of $10^{2.5}$ $TCID_{50}$ ml^{-1}. Cultures were incubated at 25°C until complete formation of CPE, when most of the cells detached from the bottom. To estimate the infectious titer of the resulting virus culture, it was titrated using MEM-5 with 0.2% kidney extract on RyuF-2 cells seeded in a 96-well plate at 25°C.

Stability of the virus during storage

To examine the stability of the virus during storage, a flask containing RyuF-2 cells cultured with MEM-5 or M199-5 supplemented with or without goldfish kidney extract (lot 1) was inoculated with the Sat-1 isolate passage 18 and then incubated at 25°C until CPE formation was complete. Each virus culture (passage 19) was filtered with a 0.45 µm filter unit (HV), dispensed into tubes, and then stored at 4°C, –25°C, or –80°C. A tube was taken out on Days 2 and 7 after storage and titrated on RyuF-2 at 25°C.

Pathogenicity of the cloned virus in goldfish

Because the Sat-1 isolate was propagated more than 20 times in cell culture before cloning the virus, we confirmed the pathogenicity of the clone in goldfish. Ten goldfish of the Ryukin variety (average body weight: 2.6 g) were intraperitoneally injected with clone 1, passage 1 (C1-P1) at a concentration of $10^{2.5}$ $TCID_{50}$ $fish^{-1}$. Control fish were injected with M199-5. The fish of each group were reared in an aquarium at 25°C (24.9 ± 0.1°C). DNA was extracted from the kidneys of dead and surviving fish at the end of the experiment using DNAzol reagent (Life Technologies), according to the protocol for koi herpesvirus described by the OIE (2014). These samples were tested using PCR primers specific to CyHV-2 (Waltzek et al. 2009).

Virus growth curve

RyuF-2 cells seeded in 35 mm culture dishes (Iwaki) were inoculated with the Sat-1 clone C1-P2 at a multiplicity of infection of 0.3. The virus was left to adsorb for 1 h. After rinsing the monolayers 3 times with Dulbecoo's phosphate-buffered saline (PBS; Ca^{2+}, Mg^{2+} free; Nissui Pharmaceutical), MEM-5 or M199-5 supplemented with or without the extract (lot 2) was added to the cells and incubated at 25°C. Single dishes were sampled at 24 h intervals, and both the cells, scraped off with a cell-scraper, and the culture medium were harvested in a tube. The cells and supernatant were then separated by centrifugation at 1000 × *g* for 5 min. The cell pellet was homogenized with a pestle in fresh medium and centrifuged (1000 × *g*, 5 min) to remove cell debris. The supernatants and cell homogenates were titrated on RyuF-2 at 25°C.

Effect of temperature on virus growth

RyuF-2 cells in 25 cm^2 flasks cultured in M199-5 supplemented with kidney extract (lot 3) were inoculated with the isolate Sat-1, C1-P2, at a concentration of $10^{3.8}$ $TCID_{50}$ $flask^{-1}$ and incubated at 15, 20, 25, 30, and 35°C until the formation of CPE was complete. A small aliquot of culture medium was withdrawn daily from each flask until completion of CPE for estimation of the infectious virus titer by titration on RyuF-2 cells at 25°C. Furthermore, to define the non-permissive temperature, flasks containing RyuF-2 cells were inoculated with the virus (Sat-1, C1-P2) in the same way and then incubated at 32 and 34°C.

Virus stability in culture medium at various incubation temperatures

Sat-1, C1-P2, diluted one-third with MEM-5, was dispensed into tubes, which were then placed at 15, 20, 25, 30, and 35°C. A small aliquot was sampled at 1, 3, 5, 7, and 14 d after incubation, and titrated on RyuF-2 at 25°C to estimate residual infectious virus.

Cell line susceptibility to the virus

Sat-1, C1-P2 was titrated on 9 fish cell lines seeded in 96-well plates using MEM-5 supplemented with or without kidney extract (lot 3) at 25°C. The cell lines were RyuF-2 and GFF (Li & Fukuda 2003), derived from goldfish; CFS (Hasegawa et al. 1997) and GTS9 (Katakura et al. 2009), derived from ginbuna *C. langsdorfii*; CCB (Neukirch et al. 1999) and KF-1 (Hedrick et al. 2000), derived from common carp *Cyprinus carpio*; EPC (Fijan et al. 1983, Winton et al. 2010) and FHM (Gravell & Malsberger1965), derived from the fathead minnow *Pimephales promelas*; and BF-2 (Wolf et al. 1966), derived from the bluegill *Lepomis macrochirus*. For CFS and GTS9 cells, Leibovitz's L-15 (Gibco, Life Technologies) supplemented with 5% FBS was used instead of MEM-5. CCB and KF-1 were kindly provided by O. Haenen of the Central Veterinary Institute, The Netherlands, with the permission of Dr. M. Neukirch, and by Dr. R. P. Hedrick of the University of California, Davis, USA, respectively.

RESULTS

Virus culture with high infectious titer

During passage 1–7 of virus culture, in MEM-5 with no kidney extract, a CPE appeared as a few focal plaques on the cell monolayer. These plaques expanded quite slowly, requiring incubation for 2 to 3 wk for complete CPE formation. These cultures showed poor infectivity, at around $10^{2.8}$ $TCID_{50}$ ml^{-1}. Because this virus titer was so low, the majority of the infectivity was sometimes lost when these cultures were stored at –80°C. Upon propagation of the virus in RyuF-2 cells (passage 8–10) in MEM-5 supplemented with 0.2% healthy goldfish kidney extract, the appearance and expansion of CPE occurred earlier than those with MEM-5 without kidney extract. From passage 11, the culture medium was changed to M199-5 supplemented with kidney extract. At passage 12, CPE occurred more rapidly and was completed by the fourth day after inoculation. This virus culture had a higher infectious titer of $10^{4.8}$ $TCID_{50}$ ml^{-1}. Subsequently, we continuously and reproducibly propagated the virus in RyuF-2 cells using M199-5 supplemented with goldfish kidney extract and harvested cultures with high titer ($10^{5–6}$ $TCID_{50}$ ml^{-1}) within approximately 5 d of incubation at 25°C. At passage 23, the virus was cloned using the limiting dilution method.

Incubation period until completion of CPE

The incubation period of virus passage 14 required for complete CPE formation, as well as the infectious titer of the culture, are summarized in Table 1. The incubation period was shorter in cultures with kidney extract than in those without the extract. Cultures using M199-5 had higher viral titers than those using MEM-5, but the virus titer was not different between cultures with and without the extract.

Stability of the virus during storage

The stability of the virus during storage at 4, –25, and –80°C using MEM-5 or M199-5 supplemented with or without goldfish kidney extract is shown in Table 2. The infectivity of the cultures was relatively

Table 1. Incubation periods and infectious titers of cyprinid herpesvirus 2 (passage 14) obtained from RyuF-2 cells cultured using medium supplemented with or without a healthy goldfish kidney extract until cytopathic effect (CPE) formation was complete

Culture medium used	Incubation period until CPE completion (d)	Infectious titer of virus culture (log $TCID_{50}$ ml^{-1})
MEM-5		
With extract	7	4.8
Without	4	4.8
M199-5		
With extract	6	5.3
Without	4	5.6

Table 2. Stability of cyprinid herpesvirus 2 cultured in the medium supplemented with or without a healthy goldfish kidney extract when stored at 4°C, –25°C, and –80°C. Infectious titers are given as the loss of titer during storage for 2 and 7 d

Medium used for virus propagation	Initial titer of culture (log $TCID_{50}$ ml^{-1})	Storage period (d)	Loss of virus titer (log $TCID_{50}$ ml^{-1}) –80°C	–25°C	4°C
MEM-5					
Without extract	4.6	2	0.3	2.5	0.3
		7	1.0	>3.8	0.5
With extract	5.3	2	0.8	3.5	0.5
		7	0.8	>4.5	1.0
M199-5					
Without extract	6.1	2	0	2.0	0.5
		7	0.8	1.8	0.3
With extract	5.8	2	0.3	1.0	0.3
		7	0.3	1.0	1.0

stable at 4°C and –80°C for 7 d. In contrast, it drastically dropped at –25°C, especially when using MEM-5.

Pathogenicity of the cloned virus in goldfish

Mortality in the infected fish group began 2 d after inoculation, and the cumulative mortality reached 100% at 5 d after inoculation. The uninfected control group had no mortality. Ten dead individuals from the infected group were all positive in a PCR-based test for CyHV-2, and fish in the uninfected control group were negative in the test (data not shown).

Virus growth curve

Virus growth curves obtained using RyuF-2 cells cultured in MEM-5 or M199-5 supplemented with or without goldfish kidney extract are shown in Fig. 1.

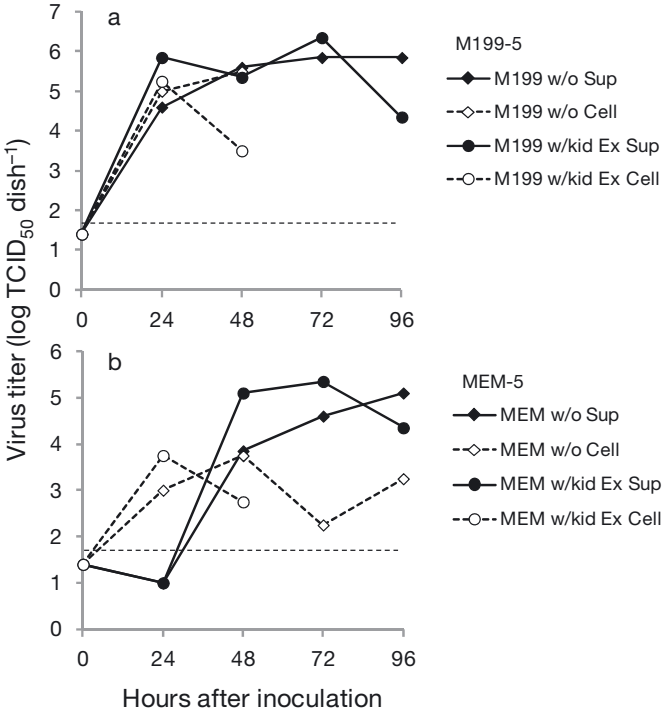

Fig. 1. Growth curves of cyprinid herpesvirus 2 in RyuF-2 cells cultured in (a) Medium 199 (M199) and (b) Eagle's minimum essential medium (MEM) supplemented with (w/kid Ex) or without 0.2% healthy goldfish kidney extract (w/o) at 25°C after inoculation with the virus at a high multiplicity of infection. The culture supernatant (Sup) and cells (Cell) were separated by centrifugation and titrated on RyuF-2 cells at 25°C. Their infectivity is calculated as the total titer of a culture flask. The horizontal dotted line shows the detection limit

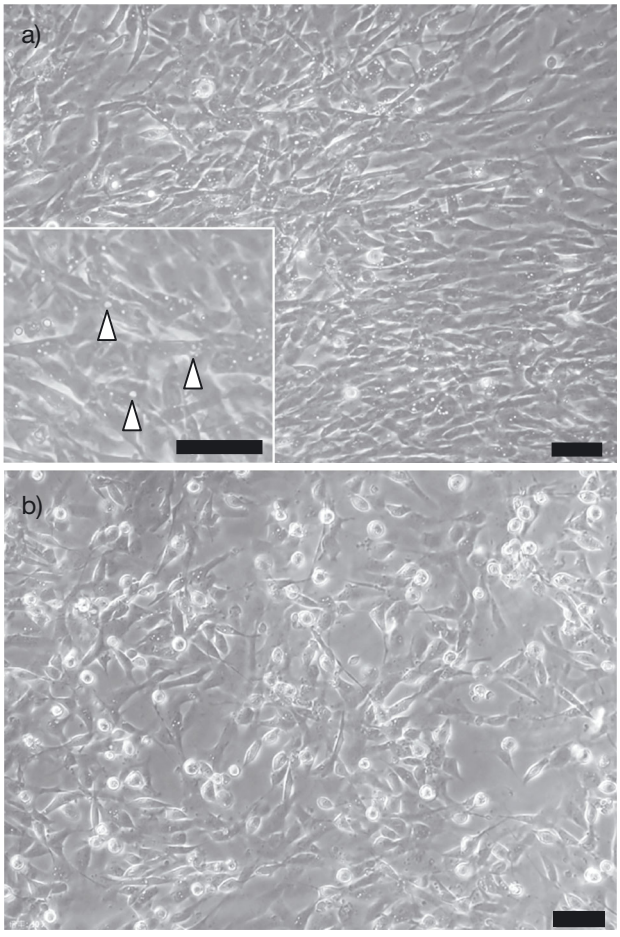

Fig. 2. Cytopathic effect (CPE) in RyuF-2 cells cultured in Medium 199 and inoculated with cyprinid herpesvirus 2. (a) Cytoplasmic vacuolation (arrowheads) appeared in the cells after 24 h (inset shows higher magnification) and (b) rounded and detached cells appeared after 48 h at 25°C. Scale bar = 50 µm

In cultures obtained using M199-5, extensive CPE characterized by cytoplasmic vacuolation appeared in the cells after 24 h incubation (Fig. 2a). The virus infectious titer also increased rapidly (Fig. 1a). The titer of the supernatant was almost the same as that in cells, revealing that the virus particles were quickly released from the infected cells. After 48 h, the cells rounded (Fig. 2b) and subsequently detached from the bottom of the culture dish. The highest titers of the culture supernatant with M199-5 were around 10^6 $TCID_{50}$ ml^{-1}, which is about 10-fold higher than those with MEM-5. In cultures using MEM-5, virus titers of the cell-associated fraction increased 24 h after inoculation, preceding the cell-free supernatants, where the titer was less than the detection limit (0.8 $TCID_{50}$ ml^{-1}; Fig. 1b). At 48 h, the virus titer in the supernatant, especially in media

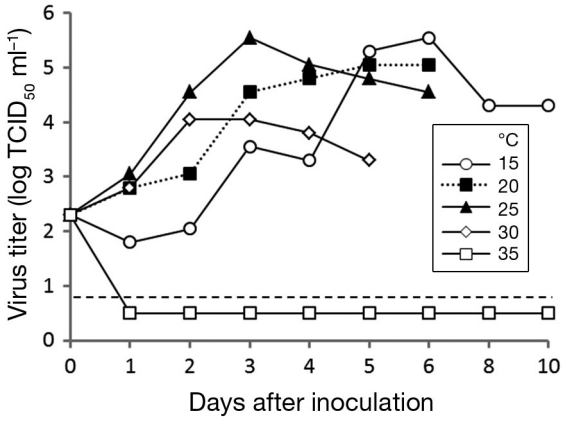

Fig. 3. Effect of incubation temperature on cyprinid herpesvirus 2 growth in RyuF-2 cells. The horizontal dotted line shows the detection limit

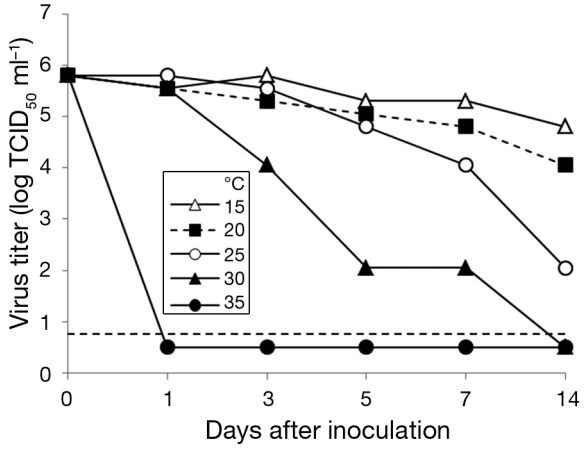

Fig. 4. Stability of cyprinid herpesvirus 2 in Eagle's minimum essential medium supplemented with 5% fetal bovine serum at various temperatures. The horizontal dotted line shows the detection limit

supplemented with kidney extract, quickly elevated. Thus, the results show that goldfish kidney extract likely enhanced virus release from the cells.

Effect of temperature on virus growth

The virus could grow in RyuF-2 cells at 15, 20, 25, and 30°C but not at 35°C (Fig. 3). A higher incubation temperature resulted in earlier CPE development and completion, but cultures at 30°C yielded lower virus titers than incubation at other permissive temperatures. To define the non-permissive temperature, virus growth in cells was evaluated at 32 and 34°C. CPE occurred extensively at 32°C and was completed 3 d after incubation; the virus titer at the time was $10^{2.3}$ $TCID_{50}$ ml^{-1}. In contrast, cells incubated at 34°C showed no CPE, and the virus titer was less than the detection limit ($10^{0.8}$ $TCID_{50}$ ml^{-1}), revealing that the non-permissive temperature of CyHV-2 *in vitro* is 34°C and higher.

Virus stability in culture medium at various incubation temperatures

The stability of the virus decreased at higher temperature, especially at 30 and 35°C (Fig. 4). Ninety-nine percent of the infectivity was lost after 3 d of incubation at 30°C, and total infectivity was lost after incubation at 35°C for 1 d. The virus was comparatively stable at 15 and 20°C; at 25°C, 99% of the infectivity was lost by 7 d.

Cell line susceptibility to the virus

The infectious titer of a virus culture, estimated using 9 fish cell lines, is shown in Table 3. Cell lines derived from goldfish (RyuF-2, GFF) and ginbuna (CFS) were highly susceptible to the virus (Table 3). CPE in CFS cells derived from the ginbuna S3N strain was characterized by cytoplasmic vacuolation and extensive large syncytium formation (data not shown). Cell lines GTS9 from the ginbuna OB-1 strain and KF-1 and CCB from carp were susceptible using a medium containing goldfish kidney extract, but EPC, FHM, and BF-2 did not produce any CPE, even in the presence of the extract.

Table 3. Cell line susceptibility to cyprinid herpesvirus 2 at 25°C using media supplemented with or without a goldfish kidney extract. Values represent the infectious titer of a virus solution determined using each cell line. ND: no CPE detected at the detection limit noted in parentheses

Cell line	Log $TCID_{50}$ ml^{-1}	
	With extract	Without extract
RyuF-2	6.1	5.1
GFF	5.6	4.8
CFS	5.3	5.3
GTS9	3.1	ND (≤1.8)
CCB	2.8	ND (≤1.8)
KF-1	3.3	ND (≤1.8)
EPC	ND (≤0.8)	ND (≤1.8)
FHM	ND (≤0.8)	ND (≤1.8)
BF-2	ND (≤0.8)	ND (≤1.8)

DISCUSSION

Before beginning this investigation of CyHV-2 propagation *in vitro*, we asked the question: Why did the initial isolation of CyHV-2 show extensive CPE even when using non-*Carassius* cells? Our preliminary study revealed that the titer of an inoculum prepared by the usual protocol (homogenization of the kidney of infected goldfish with a 50-fold volume of medium) was $10^{3.8}$ $TCID_{50}$ ml^{-1}, which is not very different from that obtained from cell culture; we therefore assumed that another factor is involved, other than the amount of virus in the inoculum during virus isolation. In this study, we focused on the inclusion of a kidney homogenate (extract) in the inoculum and attempted virus propagation using a culture medium supplemented with healthy goldfish kidney extract. The concentration of the extract was adjusted to 0.2% because routine virus isolation is achieved by the inoculation of the medium in a 25 cm^2 flask with a filtered homogenate at a final concentration of 1/500 (0.2%). Supplementation with 3 preparations of pooled kidney extract at this concentration caused no toxic effect on RyuF-2 cells and a slight promotion of cell growth. Because the final concentration of the extract (0.2%) in the medium was sufficient to enhance virus production (data not shown), 3 lots of the extract produced similar enhancement of CPE development on RyuF-2 cells due to CyHV-2.

In the earlier passages of the CyHV-2 Sat-1 isolate, CPE appeared as small plaques on the cell monolayer and expanded slowly in a concentric fashion. As a result, the completion of CPE required 2 to 3 wk of incubation. It seemed that the virus infection occurred in an almost cell-to-cell manner. Beginning with passage 8, addition of kidney extract to the culture medium resulted in CPE that was initially observed as plaques similar to the earlier passages, but subsequently spread rapidly over the entire cell monolayer, producing multiple new plaques in the monolayer. The virus released into the medium from infected cells appeared to infect separate cells. This observation was confirmed with the finding that goldfish kidney extract can enhance virus release from the infected cell, as shown in Fig. 1b. Through multiple virus passages using a medium containing the kidney extract, the process of CPE development on the cell monolayer started earlier and progressed more rapidly. Thus, we succeeded in obtaining a virus culture with a high infectious titer ($10^{4.8}$ $TCID_{50}$ ml^{-1}) after 6 passages in cells using a medium supplemented with 0.2% goldfish kidney extract.

RyuF-2 cells are routinely cultured in M199-5, which supports cell growth very well, but they can also be maintained in MEM-5. Therefore, we compared MEM-5 and M199-5 supplemented with the kidney extract for the propagation of CyHV-2 in cells. We also compared the virus stability during storage in these media to determine an efficient culture protocol. Virus culture using M199-5 usually resulted in greater virus production than with MEM-5 (Tables 1 & 2). These results were supported by the results of the virus growth curve experiment (Fig. 1). Regarding virus growth, results demonstrated that supplementation with the kidney extract can shorten the incubation period required to complete CPE formation. Although addition of the extract did not seem to contribute as much to the infectious titer of the culture using M199-5 (see Table 2), the effect of supplementation with the goldfish kidney extract appeared to be more evident when virus from an earlier passage number was cultured. This envelope-bearing virus particle of CyHV-2 is not stable at higher temperatures (Fig. 3), so that a shorter incubation period is favorable to obtain a culture with high infectivity. Conversely, virus cultures at earlier passage numbers required long incubation periods before the formation of CPE was complete, and it is likely that the longer incubation time causes a substantial reduction in infectivity of the propagated virus. Furthermore, the results showed that the infectivity was stable at 4°C and −80°C, but decreased drastically at −25°C, especially when cultured using MEM-5 (Table 2). Taken together, the results concerning the incubation period, virus growth, and stability upon freezing lead us to conclude that M199-5 supplemented with 0.2% goldfish kidney extract is suitable for CyHV-2 propagation *in vitro*. Finally, by culturing CyHV-2 using this medium at 25°C, we have succeeded in consistently obtaining virus cultures with high infectious titers of around 10^{5-6} $TCID_{50}$ ml^{-1}. However, when titrating CyHV-2 in 96-well plates, M199-5 medium can result in overgrowth of RyuF-2 cells. This can be avoided by using MEM-5 supplemented with kidney extract for the titrations.

We further studied the *in vitro* characteristics of CyHV-2 using a cloned virus isolate. However, passing a virus more than 23 times in culture generally influences virus pathogenicity. For example, koi herpesvirus (= CyHV-3), which is related to CyHV-2, was attenuated by 20 serial passages in a carp cell line (Ronen et al. 2003). Therefore, we conducted an experimental infection trial using goldfish. The results of this trial showed that the cloned virus maintains pathogenicity in goldfish, as evidenced by the

acute nature of the infection in goldfish, at 25°C. Thus, the cloned virus could be used in experiments to define the basic *in vitro* characteristics of CyHV-2.

CyHV-2 grew rapidly in RyuF-2 cells cultured in M199-5 and achieved high virus titer by 24 h (Fig. 1a). At 24 h after inoculation, cells only showed cytoplasmic vacuolation; they did not become round and detach from the bottom of the flask (Fig. 2a). Virus release from the cells occurred rapidly. This manner of growth *in vitro* is consistent with the acute disease nature of CyHV-2 infection. Thus, virus harvest can be achieved by merely collecting the supernatant from the culture before CPE formation is complete. Moreover, supplementation with the kidney extract likely enhanced the release of virus from the infected cells.

In this study, CyHV-2 growth in RyuF-2 cells occurred between 15 and 32°C, which is slightly higher than the temperature used to culture CyHV-3 (Gilad et al. 2003). Higher incubation temperatures result in earlier CPE development, but culture at 30 and 32°C yielded a lower virus titer. Incubation of the virus for 3 d at 30°C gave 10^4 $TCID_{50}$ ml^{-1}, and heat inactivation of the virus in culture medium at 30°C caused the loss of 10^2 $TCID_{50}$ for 3 d (Fig. 4). This result may simply indicate that a total virus production of 10^6 $TCID_{50}$ ml^{-1} occurred in the cells, which is not very different from virus production at the other permissive temperatures. During infection experiments, fish death appeared earlier at 30 or 31°C than it did at 25°C, but the cumulative mortality rate was lower than that at 25°C (Tanaka 2005, Ito & Maeno 2014b). These observations may reflect the growth characteristics of the virus. In contrast, fish mortality rate at 15°C was quite low (Ito & Maeno 2014b), although our study showed that the infectious titer of the virus culture at 15°C approached the same level as it did at 25°C. Further investigations are required to assess the relationship between host damage due to virus propagation and host defense capability at the relevant temperatures. Furthermore, this study showed that 34°C (and above) is the non-permissive temperature for CyHV-2. Shifting the water temperature to 33–35°C can decrease mortality among infected goldfish (Tanaka 2005), and this treatment has been applied in some aquaculture farms in Japan. This treatment temperature is consistent with the non-permissive temperature demonstrated in the present study.

Among the cell lines tested, cells from goldfish showed high CyHV-2 susceptibility, as did 1 cell line (CFS) derived from the fin of an isogeneic strain (S3N) of ginbuna, which is related to goldfish and Prussian carp. Another cell line (GTS9) from the thymus of the isogeneic ginbuna strain OB-1 was less sensitive to the virus. Experimental infection with CyHV-2 caused 20% mortality in the ginbuna OB-1 strain, which demonstrates low susceptibility to the virus (Ito & Maeno 2014a). Since there are many indigenous, geographically isolated strains of ginbuna in Japan, further infection experiments using some of the ginbuna strains, including S3N, are needed to predict the risk of HVHN in wild ginbuna populations.

Carp cell lines showed some degree of susceptibility in the presence of goldfish kidney extract. Thus, enhancing cell susceptibility by supplementation with the extract was also seen in cyprinid cell lines other than *Carassius* cell lines. This phenomenon may explain the fact that the initial isolate of CyHV-2 from diseased goldfish showed extensive CPE on the carp cell line KF-1 (Goodwin et al. 2006, Jeffery et al. 2007, Xu et al. 2013). In the present study, no CPE was observed on EPC cells. Some previous studies reported that this cell line was permissive to the virus (Goodwin et al. 2006, Daněk et al. 2011), while other reports found no CPE in these cells (Groff et al. 1998, Jeffery et al. 2007). This discrepancy may result from differences in the amount of virus in the inoculum as well as differences in the susceptibility of the various cell lineages which have been maintained in various laboratories over the long term.

Our study suggests that goldfish kidney extract has some ability to enhance CPE development in cells, and may also enhance virus release from cells. We are currently interested in the substance(s) responsible for these activities. Arginine is essential for the replication of herpes simplex virus and to promote virus growth *in vitro* (Tankersley 1964). Since we used culture media supplemented with 5% FBS in the present study, deficiencies of nutrients essential for replication were unlikely to occur. Therefore, there is little possibility that this is a simple nutritional effect. As another possibility, the kidney extract may contain some specific enzymes that activate the virus, as seen in the cleavage of the virus surface protein HA_0 of influenza virus (Klenk et al. 1975). A preliminary experiment in which CyHV-2 was incubated with goldfish kidney extract for 1 h prior to inoculation showed no promotion of CPE development. Although it is currently unclear, we deduce that kidney extract might act mostly on the infected cell. Thus, identification of the substance(s) and mechanism(s) responsible for the effects of goldfish kidney extract should be clarified in future stud-

ies, as should the activities that may be present in other goldfish organs. These studies will lead to the development of more efficient ways to propagate CyHV-2 *in vitro* and to better understand the pathogenicity of the virus in fish, consequently helping to establish more effective vaccines and improved selection of CyHV-2-resistant strains of fish. In addition, it may be worthwhile to investigate whether supplementation of culture medium with tissue extracts can enhance the growth of other poorly culturable viruses.

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