Vaccination against cyprinid herpesvirus 2 (CyHV-2) infection in goldfish Carassius auratus

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Abstract

Cyprinid herpesvirus 2 (CyHV-2) is known as the causative agent of herpesviral haematopoietic necrosis (HVHN) of goldfish (Carassius auratus). However, in recent years, the virus has also been detected from Prussian carp (Carassius gibelio) and crucian carp (Carassius carassius) from several countries. In this study, the efficacy of a vaccination against HVHN in goldfish was evaluated. The cell culture supernatant of CyHV-2-infected goldfish fin (GFF) cells was inactivated with formalin (0.1% v/v) for 2 days at 4°C. Goldfish of the variety Edonishiki were intraperitoneally injected with the vaccine and the fish were injected with a booster vaccine 9 days after the first vaccination. The fish were then CyHV-2-challenged by immersion route (10 TCID50). 21 days after the initial vaccination. Statistical analysis showed significantly higher survival rates in the vaccinated fish compared to the control and the un-vaccinated groups. The relative percentage survival (RPS) value of the fish injected with the inactivated vaccine was 57% and onset of mortality occurred later in the vaccinated group. Although viral DNA was detected from all surviving fish within the vaccinated group, amplicons/polymerase chain reaction products appeared with decreased intensity indicating that lower amounts were present compared to the deceased fish. Moreover the virus was not re-isolated from the surviving fish of the vaccinated group. The present study clearly shows the potential efficacy of vaccines derived from formalin inactivated cell culture supernatant of CyHV-2 infected GFF cells against HVHN of goldfish.

Introduction

Herpesviral haematopoietic necrosis (HVHN) has caused great economic damage to goldfish (Carassius auratus) aquaculture in Japan since it was initially reported in 1992 (Aichi Prefecture, 2008; Jung and Miyazaki, 1995). The disease is now recognized as a major pathogen of goldfish not only in Japan but also in the USA (Goodwin et al., 1999), Australia (Stephens et al., 2004), New Zealand (Hine et al., 2006) and the United Kingdom (Jeffery et al., 2007). The causative agent of the disease was called goldfish haematopoietic necrosis virus (GFHNV) in the first report (Jung and Miyazaki, 1995), and as this virus is the second herpesvirus isolated from a cyprinid species, it was formally designated

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as the species *Cyprinid herpesvirus 2* (CyHV-2) following the rules for nomenclature of the International Committee on the Taxonomy of Viruses. Although it was initially thought that this virus only demonstrated virulence towards goldfish, Wang et al. (2012) reported that mass mortality caused by CyHV-2 infection occurred in farmed Prussian carp (*Carassius gibelio*) in China. Moreover CyHV-2 was also detected from domestic cultured Prussian carp in China (Luo et al., in press; Wu et al., 2013) and Daněk et al. (2012) detected this virus from the same species in the Czech Republic. Furthermore, Fichi et al. (2013) detected this virus from the crucian carp (*Carassius carassius*) in Italy. These reports suggest that this virus may potentially be spread by movement of diseased or carrier fish and also CyHV-2 has the potential to infect other host species of the genus *Carassius*. It is anticipated that a vaccine can be effective for the prevention of this viral disease, but there is to our knowledge no reported vaccination for HVHN since isolation from diseased fish and the subsequent continuous propagation of this virus in cell culture has been limited (Jung and Miyazaki, 1995; Li and Fukuda, 2003). However, recently, we reported a method for sustainable propagation of CyHV-2 using goldfish fin (GFF) cells as well as results of a successful experimental infection of goldfish by intraperitoneal injection and immersion using an *in vitro* propagated virus isolate (Ito et al., 2013). In this report, we describe the potential efficacy of vaccination against HVHN of goldfish.

**Materials and methods**

**Cell line**

The goldfish fin (GFF) cell line (Li and Fukuda, 2003; Ito et al., 2013) from the fin of the Ryukin variety of goldfish, was used in this study. Cultures of the cell line were maintained in minimum essential medium (MEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio) and antibiotics (100 U penicillin ml⁻¹ and 100 mg streptomycin ml⁻¹) at 25°C.

**Virus and vaccine preparation**

The virus used in the present study was the CyHV-2 Saitama-1 (SaT-1) isolate which was isolated in GFF cells from diseased Calico variety of goldfish in Saitama Prefecture, Japan in 1999 (Ito et al., 2013). This isolate was propagated five times in GFF cells in 75cm² flasks (Greiner Bio-one) at 25°C. Aliquots of the cell culture supernatant containing CyHV-2 were placed in 3.6 ml cryo tubes (Nunc®, Thermo Fisher Scientific) and stored at -85°C until use. One aliquot was thawed and a TCID₅₀ titration of the viral stock used in the subsequent experiments was performed by the method of Reed and Muench (1938).

To prepare a vaccine from the viral supernatant of CyHV-2 infected GFF cells, the culture medium was centrifuged at 400 x g for 10 min at 4°C. Formalin (0.1% v/v) was then added to the supernatant, which contained 10¹⁰ TCID₅₀ ml⁻¹ of the virus, and the preparation was incubated for 48 h at 4°C to inactivate the virus.

**Vaccination procedure**

The goldfish variety, Edonishiki, were used in the challenge trials. Edonishiki were bred from CyHV-2 free broodstock at the Tamaki Laboratory, National Research Institute of Aquaculture. To evaluate the efficacy of the vaccine, 60 Edonishiki goldfish were divided into 4 groups. Fifteen fish were intraperitoneally inoculated with 0.1 ml of vaccine solution made from the cell culture supernatant of CyHV-2-infected GFF cells (vaccinated group).
Nine days after the first vaccination, all the fish of the vaccinated group were given a booster shot which was a repeat of the first vaccination procedure as described above. Fifteen fish were intraperitoneally inoculated with 0.1 ml of the supernatant of non-infected GFF cell cultures (control group). All injected fish were anaesthetized by 2-phenoxyethanol diluted at 1:1000 with well water. Thirty fish constituting the other two control groups received no injection. All experimental groups were kept in 60 l tanks with a continuous supply of flow-through freshwater (0.6 l min⁻¹) at 24.5°C (24.0-25.0°C). They were fed a commercial diet (Saki-Hikari®, Kyorin) once a day and were observed over a 21 day period to ensure that no detrimental effects from the vaccination procedure occurred.

**Challenge test**
An immersion challenge using the CyHV-2 Saitama-1 (SaT-1) isolate was conducted utilizing the aforementioned vaccinated (n=15) and control groups (n=15) as well as fifteen fish from a non-injection group (un-vaccinated group). The fish were immersed for 1 h in diluted supernatant of virus culture (10 TCID₃₀ l⁻¹), the same batch used for the production of the vaccine. The fourth group of fish (n=15; un-treated group) were immersed in diluted uninfected cell culture medium using the same protocol. All 4 groups were kept in 60 l tanks as described previously and were observed over a 21 day period. The experimental design and fish size of each group is summarized in Table 1.

**Detection of viral DNA from fish by polymerase chain reaction (PCR)**
For the specific detection of CyHV-2, the PCR described by Waltzek et al. (2009) was employed. The DNA template for positive control was extracted from the supernatant of virus-infected cell cultures using InstraGene™Matrix (Bio-Rad Laboratories). For samples of experimentally infected fish, total DNA was extracted from the kidney of all experimental fish using Gentra Puregene Tissue Kit (Qiagen). The PCR reaction mixture used was TaKaRa Ex Taq® Hot Start Version (TaKaRa) according to the manufacturer’s protocol. The thermocycling profile was performed according to published protocols (Waltzek et al. 2009).

**Virus re-isolation from experimental fish**
Kidney (40-50 mg per fish) homogenate sampled from all the experimental fish was prepared using approximately 50-times volume of MEM and passed through a 0.45 µm filter. Twenty-four h after the preparation of GFF cells in 25cm² flasks (Greiner Bio-one), the culture medium was removed from the prepared flask and 500 µl of the inoculum was added and absorbed at 25°C for 1 h. Fresh medium supplemented with 10% FBS was added to the flask and incubated at 25°C. The cells were observed daily for cytopathic effect (CPE) for 14 days.

**Statistical analysis**
The statistical difference between the vaccinated group and the respective control groups were determined by Fisher’s exact test, P<0.01 values were considered significant. The relative percentage survival (RPS) was also determined by the formula RPS = (1-% loss of immunization fish/ % loss of control) x 100.

**Results**
One fish from the vaccinated group died 9 days after the booster was administered due to an injection accident. CyHV-2 DNA was not detected by PCR and virus was not re-isolated from the
showed a normal appetite and similar activity to healthy fish.

The survival rate of the 4 groups after challenge are shown in Figure 1. The survival rates in the vaccinated group, RPS value of 57 %, were significantly higher than those in the control and un-vaccinated groups P=0.0007. Mortality within the respective negative control groups occurred between 3 and 11 days post-viral challenge. In contrast, the beginning of mortality in the vaccinated group was delayed, occurring between 10 and 14 days post-viral challenge. A behavioral change (lethargy) was observed, occurring just a few days prior to mortality among the vaccinated, control and non-vaccinated groups. There was no mortality in the non-challenged control group during the experimental period.

<table>
<thead>
<tr>
<th>Group</th>
<th>Challenge</th>
<th>No. of samples</th>
<th>Total length (cm)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated</td>
<td>Yes</td>
<td>15</td>
<td>8.09 ± 0.82</td>
<td>23.69 ± 4.34</td>
</tr>
<tr>
<td>Control</td>
<td>Yes</td>
<td>15</td>
<td>7.70 ± 0.46</td>
<td>22.61 ± 2.93</td>
</tr>
<tr>
<td>Un-vaccinated</td>
<td>Yes</td>
<td>15</td>
<td>7.87 ± 0.84</td>
<td>23.81 ± 5.89</td>
</tr>
<tr>
<td>Un-treated</td>
<td>No</td>
<td>15</td>
<td>7.45 ± 1.21</td>
<td>21.57 ± 6.69</td>
</tr>
</tbody>
</table>

Table 1. Experimental design, and mean ± SD of total length and body weight of fish used in this study.

Figure 1. Cumulative survival curves of vaccinated goldfish challenged with CyHV-2: (▲) Edonishiki were infected with CyHV-2 following vaccination, (●) Edonishiki were infected with CyHV-2 following injection with cell culture supernatant of non-infected GFF cells, (■) Edonishiki were infected by bath immersion with CyHV-2 without receiving vaccination, (◆) Edonishiki of non-challenged control. Asterisk: the survival rate at the end of the experiment was significantly different from the group of non-vaccinated (P<0.01 by Fisher’s exact test) goldfish.
The survival rate at the end of the challenge and results of CyHV-2 DNA detection by PCR and re-isolation of the virus from the experimental fish are shown in Table 2. CyHV-2 DNA was detected from kidney samples collected from all deceased fish in the infected groups after the viral challenge. Although viral DNA was detected from all surviving fish in the vaccinated group, the amplicons from these surviving fish were less intense than from the kidney material sampled from the vaccinated fish that died during the experiment (Figure 2). Viral DNA was not detected from the non-challenged control fish. The virus was re-isolated from the kidneys of 5/6 dead fish in the vaccinated group after viral challenge. In contrast, no CPE was observed in cultures inoculated with samples from surviving fish in the vaccinated group. The virus was re-isolated from the kidneys of 29 of 30 dead fish in the control and un-vaccinated groups after viral challenge.

**Discussion**
The present study clearly demonstrates the potential efficacy of vaccines developed from formalin inactivated CyHV-2 cell culture viral supernatant against HVHN in goldfish.

No previous report of vaccination against CyHV-2 infection have been published as it has been difficult to achieve sustainable propagation of this virus due to the lack of a suitable cell line for viral culture. However, a method describing the sustainable propagation of CyHV-2 using GFF cell cultures was recently published (Ito et al., 2013). From the vaccinated group only one fish died prior to the challenge trial as a result of an injection accident when administering the booster vaccine. The RPS value of the inactivated vaccine was determined to be 57% and the onset of mortality within the vaccinated group was delayed compared to the control and un-vaccinated groups. Therefore, the inactivated vaccine appears to have the potential to be effective against HVHN of goldfish.

Naturally diseased fish resulting from CyHV-2 infection do not show any specific external signs (Jung and Miyazaki, 1995). While in our previous study there was a lack of pathognomonic

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**Figure 2.** Detection of CyHV-2 DNA from kidney samples obtained from the vaccinated group of Edonishiki infected by immersion treatment. A PCR using specific primers targetting the helicase gene of CyHV-2 (Waltzek et al. 2009) was applied. The arrow indicates the position of the 366-bp amplicon. M, Molecular weight makers (Bioline, HyperLadder II); P, positive control; N, negative control.
clinical signs due to CyHV-2 experimental infection, we did observe in the immersion infection experiment a behavioral change (lethargy) which occurred just a few days before death (Ito et al., 2013). These indistinct clinical signs of CyHV-2 infection may be the reason that goldfish farmers, wholesalers and importers have difficulty in detecting infected fish. In fact, it is likely that farmers may overlook infected, sub-clinical carriers resulting in transport not only to domestic but also foreign markets.

We demonstrated the efficacy of a vaccine derived from formalin inactivated cell culture supernatant of goldfish GFF cells infected with CyHV-2. However, titer values of CyHV-2 in cell culture are low compared with other CyHVs such as CyHV-1 or CyHV-3 although the sustainable propagation of the virus has succeeded. The development of a method to increase the in vitro viral titer is one of next themes that must be investigated. Furthermore, trialing the vaccine on a larger scale to establish the validity of the vaccine and the possibility that vaccinated surviving fish become virus carriers are themes of future studies.

Acknowledgement
This study was funded by the Fisheries Research Agency in Japan. We thank Y. Maeno of National Research Institute of Aquaculture, Fisheries Research Agency for his comments on an earlier version of the manuscript.

References


Table 2. Results of survival rate at 21 days post-viral challenge, detection of CyHV-2 DNA and re-isolation of the virus from the kidneys of experimental fish. Values are given as the number of positive fish / the number of fish tested. NT: not tested.

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival rate post-challenge (%)</th>
<th>Dead fish</th>
<th>Surviving fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Surviving /Challenged)</td>
<td>PCR</td>
<td>Virus re-isolation</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>57 (8/14)</td>
<td>6/6</td>
<td>5/6</td>
</tr>
<tr>
<td>Control</td>
<td>0 (0/15)</td>
<td>15/15</td>
<td>15/15</td>
</tr>
<tr>
<td>Un-vaccinated</td>
<td>0 (0/15)</td>
<td>15/15</td>
<td>14/15</td>
</tr>
<tr>
<td>Un-treated</td>
<td>100 (15/15)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
carassius (L.), in Italy. *Journal of Fish Diseases* 36, 823-830.


