Recent studies on koi herpesvirus (KHV) for diagnosis and prevention in Japan

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Contents of this presentation

1. Historic events on KHV disease
2. Characteristics of KHV
3. Control measures to prevent spreading KHV in Japan
4. Present studies in our laboratory.
   1) Susceptibility of goldfish to KHV
   2) Evaluation of cohabitation test
   3) Attenuated vaccine for KHV
## Historic events on KHV disease

Table 1. Events on KHV (from initial outbreak to listing to the OIE manual)

<table>
<thead>
<tr>
<th>Years</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>~1997</td>
<td>Mortality in koi carp in European countries</td>
</tr>
<tr>
<td>1998</td>
<td>Mass mortality in common carp farm in Israel and koi carp in USA</td>
</tr>
<tr>
<td>1999/2001</td>
<td>the 9th and 10th International Conference of EAFP</td>
</tr>
<tr>
<td>2002</td>
<td>Identification of causative agent, Development of diagnostic method using PCR</td>
</tr>
<tr>
<td>2002</td>
<td>Mass mortality in common carp farm in Indonesia</td>
</tr>
<tr>
<td>2003</td>
<td>Regulated as ‘specific disease’ in the Japanese law.</td>
</tr>
<tr>
<td>2003</td>
<td>Mass mortality in carp farm in Japan</td>
</tr>
<tr>
<td>2004</td>
<td>Mass mortalities in Thailand and Taiwan</td>
</tr>
<tr>
<td>2005</td>
<td>the 6th symposium on diseases in Asian Aquaculture</td>
</tr>
<tr>
<td>2006</td>
<td>Listing disease by the OIE (Reference lab.: CEFAS and NRIA)</td>
</tr>
<tr>
<td>Criteria for listing</td>
<td>KHV</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----</td>
</tr>
<tr>
<td>A. Consequences</td>
<td></td>
</tr>
</tbody>
</table>
| The disease has been shown to **cause significant production losses** at a national or multinarinal (zonal or regional) level.  
  or  
The disease has been shown to scientific evidence indicates that it is likely to cause significant morbidity or mortality in wild aquatic animal populations.  
  or  
The agent is of public health concern.  
  and  
| KHV                |     |
| B. Spread           |     |
| Infectious aetiology of the disease is proven.  
  or  
  An infectious agent is strongly associated with the disease, but the aetiology is not yet known.  
  and  
  Likelihood of international spread, including via live animals, their products or fomites.  
  and  
  Several countries or **countries with zones may be declared free** of the disease based on the general surveillance principles  
  and  
| C. Diagnosis        |     |
| A repeatable and robust means of detection/diagnosis exists. |
Characteristics of KHV

- Herpesvirus (cyprinid herpesvirus 3, commonly koi herpesvirus)
- high virulence (Mortality in carp reaches 100 % after exposure to virus)
- systemic infection
- Indistinct clinical signs
- limited host range (only Cyprinus carpio)
- temperature-dependent
- international spreading
Phylogram depicting relationships among fish and amphibian herpesviruses based on amino acid sequences of the DNA polymerase and terminase genes (Waltzek et al., 2009)
Cumulative mortalities of two types of common carp after virus exposures.
Detection of KHV genome by PCR by organ of infected fish kept at 28, 23 or 18°C.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time</th>
<th>Mucus</th>
<th>Gill</th>
<th>Liver</th>
<th>Gut</th>
<th>Spleen</th>
<th>Kidney</th>
<th>brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td>8d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>23°C</td>
<td>8d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>18°C</td>
<td>10d</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Clinical signs of affected common carp with natural infection

Swelling of the gills
Patch on the gills
Gill rot
Sunken eyes
Skin lesion
Irregular coloration of the skin due to hemorrhage, congestion of fins (d3-d7)

Formation of white patches on the skin due separation of epidermal cells (d5-d10)

Sunken eyes (d10-)
Abnormal swimming (d10-)

Clinical signs of affected common carp with experimental infection
# Susceptibility of carp and other fish species to KHV

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Scientific name</th>
<th>mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>carp, koi</td>
<td><em>Cyprinus carpio</em></td>
<td>30-100 %</td>
</tr>
<tr>
<td>goldfish</td>
<td><em>Carassius auratus auratus</em></td>
<td>0 %</td>
</tr>
<tr>
<td>gin-buna</td>
<td><em>Carassius auratus langsdorfii</em></td>
<td>0 %</td>
</tr>
<tr>
<td>ayu</td>
<td><em>Plecoglossus altivelis</em></td>
<td>0 %</td>
</tr>
</tbody>
</table>
Number of diagnoses for KHV disease at each month in 2004 - 2006

(Data from the NRIA)
### KHV disease outbreaks at 3 different types of culture sites for common carp in Indonesia

<table>
<thead>
<tr>
<th>Types of culture</th>
<th>Outbreaks of KHV</th>
<th>Water temperature (daily range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Floating cage at lake</td>
<td>occur</td>
<td>24—25 °C</td>
</tr>
<tr>
<td>Tank with running water</td>
<td>occur</td>
<td>23—26 °C</td>
</tr>
<tr>
<td>Rice field (for nursery)</td>
<td>Not occur</td>
<td>24—29 °C</td>
</tr>
</tbody>
</table>

Floating cages in Cirata Lake (West Java prov.)
Concrete tanks with running water from channel in a private farm (South Smatra Prov.)
Rice field (South Smatra Prov.)
Occurrences of KHV disease in the world

Asia
- Korea
- Indonesia

USA, Israel
- USA
- Israel

Europe
- Germany
- Belgium
- Netherlands
- Denmark
- Austria
- France

Years:
- 1996
- 1997
- 1998
- 1999
- 2000
- 2001
- 2002
- 2003
KHV positive: 30 countries


KHV suspected: 3 countries

India*, Guatemala*, Russia*

(* = in closed system; # = in wild carp)

Results of global hoi herpesvirus questionnaire 2009 (Haenen et al.)
### Genetic types of KHV based on the sequence variations of 9/5, Sph-5 and TK regions

<table>
<thead>
<tr>
<th>Samples</th>
<th>9/5 region</th>
<th>Sphl-5 region</th>
<th>TK region</th>
<th>type</th>
</tr>
</thead>
<tbody>
<tr>
<td>USA  F98–50</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Taiwan 1</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Taiwan 2</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Philippines (China)</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C deletion</td>
<td>C deletion AA</td>
</tr>
<tr>
<td>Indonesia 1</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Indonesia 2</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Indonesia 3</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Indonesia 4</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Indonesia 5</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Indonesia 6</td>
<td>T × 4</td>
<td>A × 6</td>
<td>C deletion</td>
<td>C AAC C G AT</td>
</tr>
<tr>
<td>Japan  34 samples</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 1</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 2</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 3</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 4</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 5</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 6</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 7</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT AA</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 8</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT AA</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 9</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 10</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 11</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 9</td>
</tr>
<tr>
<td>Netherlands 12</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td></td>
</tr>
<tr>
<td>Netherlands 13</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td></td>
</tr>
<tr>
<td>UK (Malaysia)</td>
<td>T × 4</td>
<td>A × 7</td>
<td>C AAC C G AT deletion</td>
<td>T × 8</td>
</tr>
</tbody>
</table>

Kurita et al (Fish Pathol.2009)
Distribution of KHV and control measures against spreading KHV in Japan
Inspection chart for KHVD in the guideline established by Japanese government

Epidemiological findings

Clinical observation

- Water temperature
- Spreading

gross observation

PCR: Improved Sph method (Gray et al. (2002), improved by Yuasa et al. (2005))
or LAMP: (Yoshino et al. 2006).

+ or ±

Virus isolation

PCR: Improved Sph method and
9/5 method (Gilad et al (2002))

Samples for diagnosis
- Frozen gill
- Maximally 3 in a case

Judgment

Prefectural Fisheries
Experimental Station

the NRIA of FRA
Impact of KHVD outbreak in culture common carp at Kasumigaura-lake

Total production of common carp in Japan a year (2002) : 9,949 tons
In Kasumigaura-lake (2002) : 5,138 tons (more than half of the total)

↓ Outbreak of KHVD in Oct. 2003

Loss of 1,190 tons (about 20% of annual production) in 2003
Alive infected carp produced in Lake Kasumigaura had been transferred to aquaculture farms, wholesalers, restaurants and game fishing facilities throughout Japan during Oct 2003 when KHVD occurred.

The NRIA confirmed KHVD on 2nd Nov 2003.

The transfer of carp in Lake Kasumigaura was regulated by the government.
A start of tragedy for carp farms in Japan

KHV infected carp were found in 23 prefectures by the end of 2003.

During Nov to Dec 2003, a half of prefectures were contaminated with KHV.

No mortality was observed during Jan to Mar 2004.

Some Japanese believed that KHV epidemic had been stamped out.

However, - - -.
April, 2004
Diagnosed by NRIA

4 pref. positive
May, 2004

24 prefectures positive
June, 2004

30 prefectures positive
July, 2004

Carp in Kasumigaura had been transferred to Hokkaido in Oct 2003.

29 prefectures positive
August, 2004

14 pref. positive
September, 2004

20 pref. positive
October, 2004

16 pref. positive
November, 2004

8 pref. positive
Cumulative in 2004

39 pref. positive
38 out of 64 cases occurred in a prefecture, where a farm was contaminated with KHV after a flood with heavy rain. Then KHV was spread to retail and hobbyist in the prefecture by transferring infected carp.
Japanese regulation at present

- Once natural river or lake was contaminated with KHV, KHV can survive with carp in the area for a long period (more than 10 years).

- More than a half of natural river or lake has been contaminated with KHV.

Japanese government continuously regulates on the items as follows:

1. Disposal of the whole population in the tank or pond where KHV has been confirmed by the NRIA diagnosis.
2. Ban on transferring KHV-infected carp to KHV-free area.
3. Ban on transferring naïve carp to KHV-contaminated area.
4. Koi exporting farm’s obligation to certify free of KHV twice a year.

Keep low level of contamination
The fact is that outbreak in Kasumigaura was not a first case of KHV infection in Japan.

Mortality occurred in wild carp in an western prefecture in May 2003, but the prefectoral experimental station could not diagnose the case. Later (after outbreaks in Kasumigaura), KHV was detected from frozen samples.

<Actual event>
An western prefecture (May)
Kasumigaura lake (Sep to Oct)
23 or 39 prefectures

<Ideal event (dream)>
An western prefecture (May)
Kasumigaura lake
other prefectures

If local staff could have diagnosed KHV and stopped transferring carp,
Our role to achieve an ideal condition

Who can first perform the diagnosis of emerging disease?

Only local staff who is in charge of diagnosis in the field can do it.

An important role of us is to **train** the local staff to perform rapid and accurate diagnosis of emerging diseases.

Local staff’s role to achieve an ideal condition

Who can first get in contact with the emerging disease?

Farmer or retailer or hobbyist can do it.

An important role of local staff is to **educate** farmer, retailer and hobbyist to understand the disease and to report its occurrence to local staff quickly.
Recent studies in our laboratory

1. Susceptibility of goldfish to KHV
2. Evaluation of cohabitation
3. Attenuated vaccine for KHV
1. Susceptibility of goldfish to KHV

Recently it was reported that KHV genome can be detected from goldfish cohabited with infected carp. (ML-Matbouli et al. 2007; Sadler et al. 2008)

Further, Naïve carp cohabited with KHV-exposed goldfish died of KHV infection. (ML-Matbouli and Soliman 2011)

Is goldfish carrier of the virus or only mechanical vector?

1) Koi carp and common carp exposed to KHV died of KHV infection.
2) Hybrid may become carrier of virus.
3) Goldfish cannot be infected by KHV and not be carrier of virus.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Mortality (Number of dead fish/number of fish injected)</th>
<th>Virus detection</th>
<th>Survivors (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koi carp</td>
<td>19/20</td>
<td>18/19</td>
<td>1/1</td>
</tr>
<tr>
<td>Common carp</td>
<td>2/12</td>
<td>2/2</td>
<td>7/10</td>
</tr>
<tr>
<td>Hybrid</td>
<td>1/20</td>
<td>1/1</td>
<td>4/10</td>
</tr>
<tr>
<td>Goldfish</td>
<td>0/20</td>
<td>0/20</td>
<td>0/10</td>
</tr>
</tbody>
</table>

*a* All or up to 10 fish surviving infection were examined for the presence of viral DNA in the tissues.
Susceptible species: Means a species of aquatic animal in which infection has been demonstrated by natural cases or by experimental exposures to the pathogenic agent that mimics the natural pathways for infection.

Infection: Means the presence of a multiplying or otherwise developing or latent pathogenic agent in a host. This term is understood to include infestation where the pathogenic agent is a parasite in or on a host.

Presence of multiplying pathogen in a host should be demonstrated by experimental exposure to pathogen.

Experimental infection (exposure to KHV)

1. KHV DNA and mRNA were detected from goldfish.

   **Our question:**
   - Was goldfish really infected by KHV? (Gill and intestine were exposed to environment)
   - Is RT-PCR specific to mRNA?

2. Naïve carp cohabited with the goldfish died of KHV infection.

   **Our question:**
   - Source of infection was the virus on the surface of goldfish?

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**Fig. 1.** Agarose gel electrophoresis (1.5%) demonstrates amplification of the TK gene of CyHV-3 (409 bp) by PCR (A) and RT-PCR (B) from different organs of goldfish. **Lane Mar** = 100 bp DNA ladder; lane **Gill** = DNA extracted from Gill; lane **Liver** = DNA extracted from liver; lane **Int** = DNA extracted from intestine; lane **Kidn** = DNA extracted from kidney; lane **Brain** = DNA extracted from brain; lane **Sple** = DNA extracted from spleen; lane **+veco** = positive control; lane **-veeco** = negative extraction control; lane **-veco** = no-template control.
Development of RT-PCR for KHV-replicating stage (Yuasa et al: DAO, 2012)

<Genomic DNA>

84245* 83445* 83365* 83162* 56513* 55128*

Exon 1 Exon 2 Exon 3

Intron 1
<splicing>

Intron 2
<splicing>

<mRNA>

Exon 1 Exon 2 Exon 3

KHVRT F3 → KHVRT R1

KHVRT F1 → KHVRT R1

Primer set A (219 bp)

Primer set B (224 bp)

Template 1 and 2: DNA and mRNA

Template 3: DNA only
Detection of KHV replicating stage with the developed RT-PCR

160 goldfish 160 carp

1 hour exposure to KHV

0h, 12h, 24h, 36h, 48h, 3d, 7d, 14d

Sample 20 fish: gills, fin, kidney

mRNA detection with developed RT-PCR
KHV mRNA detection from 3 organs of goldfish and carp

<table>
<thead>
<tr>
<th></th>
<th>0h</th>
<th>12h</th>
<th>24h</th>
<th>36h</th>
<th>48h</th>
<th>3d</th>
<th>7d</th>
<th>14d</th>
</tr>
</thead>
<tbody>
<tr>
<td>goldfish</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>kidney</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>carp</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>gills</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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</tr>
<tr>
<td>kidney</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Can goldfish exposed to KHV transmit the virus to naïve carp?

20 goldfish and 10 koi carp were exposed to KHV.

Each 10 fish post viral exposure were cohabited with 5 naïve carp.

10 goldfish + 5 naïve carp

Cohabit with 0-1 dpe goldfish (1 day cohabitation)

10 goldfish + 5 naïve carp

Cohabit with 2-24 dpe goldfish (22 days cohabitation)

10 koi carp + 5 naïve carp

Cohabit with 2-24 dpe koi carp (22 days cohabitation)
Mortality of wild type carp cohabited with koi carp or goldfish

<table>
<thead>
<tr>
<th>Aquarium</th>
<th>Period of cohabitation</th>
<th>Motarity of wild type carp</th>
</tr>
</thead>
<tbody>
<tr>
<td>goldfish</td>
<td>A</td>
<td>at 0-1 dpe</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>at 2-24 dpe</td>
</tr>
<tr>
<td>koi carp</td>
<td>C</td>
<td>at 2-24 dpe</td>
</tr>
</tbody>
</table>

*All dead fish were KHV-positive

Goldfish exposed to KHV can be vector of virus, but not be carrier or host.

Yuasa et al (Fish Pathology, 48, 52-55)
2. Evaluation of cohabitation

In Japan, PCR is adopted as diagnostic method for the purpose of zoning koi farm for KHV-free area as well as certifying KHV-free in fish for exportation.

- Periodically twice a year
- Just before exportation

Cohabitation is generally useful to detect KHV infection, but not described in OIE manual as standard diagnostic method.
Present examination to certify KHV-free in fish for exportation

Rearing koi and cheap carp in a pond through the culture period

- Present examination
- Cohabitation

Rearing only koi in pond

- Examine 30 cheap carp with PCR
- Cohabit koi with several naive carp at 20-24°C for a few weeks in a tank
- Examine cheap carp with PCR

PCR - → dispose
PCR + → export

PCR - → Export
PCR + → Examine cheap carp with PCR
Experiment for evaluating cohabitation -1

10 carp + KHV (1 hour)

Carp exposed to KHV (low titer) (n=10)

Naïve carp (n=3 x 10)

- - - - 10 aquariums

3 weeks rearing at 23°C

Sampling a part of fins at 3 days, 1, 2 and 3 weeks after cohabitation

Real time PCR
## Amount of KHV genome in the fins of carp (KHV cope: 1-10^2 +, 10^2-10^3 ++, 10^3-10^4 +++ , 10^4-10^5 ++++, 10^5-10^6+++++)

<table>
<thead>
<tr>
<th>Cohabitation period</th>
<th>No. 1</th>
<th>No. 2</th>
<th>No. 3</th>
<th>No. 4</th>
<th>No. 5</th>
<th>No. 6</th>
<th>No. 7</th>
<th>No. 8</th>
<th>No. 9</th>
<th>No. 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-4 days (3 days)</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Carp exposed to KHV (4d)</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>++++</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Recipient carp-1</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Recipient carp-2</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Recipient carp-3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>1-8 days (1 week)</td>
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<td></td>
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<tr>
<td>Carp exposed to KHV (8d)</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Recipient carp-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>++++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-2</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>1-15 days (2 weeks)</td>
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<td></td>
</tr>
<tr>
<td>Carp exposed to KHV (15d)</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-1</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-2</td>
<td>++++</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-3</td>
<td>++++</td>
<td>+</td>
<td>-</td>
<td>++++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>1-22 days (3 weeks)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carp exposed to KHV (22d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Recipient carp-1</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>Recipient carp-2</td>
<td>++++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Recipient carp-3</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++++</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Detection limit of conventional PCR: ++
Experiment for evaluating cohabitation -2

Carp survived infection (24 days post-viral exposure) + Naïve carp (n=3 x 10)

- - - 10 aquariums

Two weeks rearing at 23°C → real time PCR

Two weeks rearing at fluctuated temperature → real time PCR
1. PCR should be performed at 2 to 3 weeks after cohabitation.
2. To detect fish shedding virus, cohabitation followed by PCR is superior to direct PCR.
3. Cohabitation cannot detect survivor due to its lack of virus shedding.
4. But, survivor may not be a source of infection? (based on our experiments)
Quantitative change of anti-KHV antibody in carp experimentally infected with high concentration of KHV (measured by ELISA)
## Sensitivity and specificity of 4 diagnostic methods for different targets

<table>
<thead>
<tr>
<th>Target</th>
<th>Hazard to spread</th>
<th>Detection method</th>
<th>Sensitivity(^1))</th>
<th>Specificity(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish shedding virus</td>
<td>high</td>
<td>cohabitation</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR</td>
<td>high</td>
<td>low to intermediate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RT-PCR</td>
<td>high</td>
<td>intermediate to high</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ELISA</td>
<td>low</td>
<td>low</td>
</tr>
</tbody>
</table>

| Fish infected by virus      | intermediate to high | cohabitation     | intermediate to high | high               |
|                             |                      | PCR              | high                | intermediate to high|
|                             |                      | RT-PCR           | intermediate to high | high               |
|                             |                      | ELISA            | intermediate        | intermediate        |

| Fish survived KHV infection (Survivor) | low to intermediate | cohabitation     | low                 | -                  |
|                                       |                      | PCR              | low to intermediate | high               |
|                                       |                      | RT-PCR           | low                 | -                  |
|                                       |                      | ELISA            | high                | intermediate\(^3\))|

---

1) Means the proportion of true positive tests given in a diagnostic test.
2) Means probability that absence of infection will be correctly identified by a diagnostic test.
3) Due to cross reaction with fish infected by CyHV-1.
### 3. Attenuated vaccine for KHV

<table>
<thead>
<tr>
<th>Vaccine Type</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attenuated vaccine</td>
<td>higher efficacy</td>
<td>sometimes not high safety</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(some virulence)</td>
</tr>
<tr>
<td>Inactivated vaccine</td>
<td>lower efficacy</td>
<td>high safety (no virulence)</td>
</tr>
</tbody>
</table>

We demonstrated that formalin-killed KHV (inactivated vaccine) has little efficacy to reduce mortality due to KHV.

Is KV3 produced by KoVax (Israeli) effective for preventing Japanese carp from KHV?
Safety of Israeli vaccination (KV3) for koi and wild-type carp

Disadvantage of KV3

Days after vaccination

Cumulative mortality (%)

Koi carp

Wild-type carp

mortality: 5%

mortality: 25%
Advantage of KV3

Koi carp  Wild-type carp

Efficacy of KV3 for reducing mortality in koi and wild-type carp

RPS (Relative percent survival) (%) : 
\[
RPS = \left(1 - \frac{\text{mortality of vaccinated fish}}{\text{mortality of non-vaccinated fish}}\right) \times 100
\]

RPS > 60% : effective

RPS: 87.6%  RPS: 73.3%

Days after challenge with KHV

Cumulative mortality (%)
Thank you for your attention