Isolation and preliminary characterization of several viruses from koi (*Cyprinus carpio*) suffering gill necrosis and mortality

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Abstract
The present communication describes the isolation of seven viruses from diseased koi carp during the last two years in our laboratory. All isolates caused cytopathogenic effects in CCB and CaF-2 cell lines. Six isolates were sensitive to chloroform treatment, four isolates passed 100 nm filters and only one isolate were found to be sensitive against treatment with JUDR. Two isolates were identified as strains of spring viremia of carp virus (SVCV). The results inclusive electron microscopical visualization allow a preliminary classification, but further investigations are necessary for exact taxonomic classification of the pathogens and for the determination of their pathogenicity.

Introduction
Since 1997, mass mortalities of koi (*Cyprinus carpio*) have been observed in Europe, USA, South Africa and Israel (Body et al., 2000; Walster, 2000). Most outbreaks occur at temperatures higher than 20°C (Walster, 1999) characterized by an acute or peracute course combined with epidermal lesions and gill necrosis. The causative agent seems to be highly contagious and virulent and the disease restricted to koi and common carp populations (Ariav et al., 1999; Hoffmann et al., 2000; Walster, 1999). In diseased fish virus like particles could be demonstrated by electron microscopical investigations (Ariav et al., 1999; Bretzinger et al., 1999; Hoffmann et al., 2000) as well as by isolation procedures (Neukirch et al., 1999; Body et al., 2000). Hedrick et al. (2000) described the isolation of a herpesvirus associated with mass mortality of adult koi in two outbreaks in Israel and USA. The present communication deals with the preliminary characterization of several viruses isolated from diseased koi in our laboratory during the last two years.

Material and Methods
Samples
The koi fish derived from garden ponds of hobbyist koi keepers or from koi dealer units. After external inspection and examination of gill and skin scrapings for ectoparasites and external lesions portions of the gill, skin, liver spleen and kidney were collected for virus isolation procedures under greatest sterile conditions possible. Isolate 1551 has already been described in part (Neukirch et al., 1999), and sample No 1617 was sent by R. Hoffmann, Munich.
Virus isolation

Samples from diseased fish were processed by standard virological methods (Mayr et al. 1974). Tissue homogenates were inoculated onto four fish cell lines from common carp: EPC (Fijan et al., 1983), CCB and CCG (Neukirch et al., 1999) and the carp fin (Ca F-2; Neukirch, unpublished), recently developed in our laboratory according to the method described earlier (Neukirch et al., 1999). The cell lines were propagated at 25°C in Eagle’s minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, antibiotics and in the case of CCB cells with glucose (4.5g/l). Tissue culture flasks of 25cm² were inoculated with 1ml of 1:10 and 1:100 dilutions (w/v) of the tissue homogenates. Virus growth medium (Dulbecco’s modified EMEM, supplemented with 5% fetal bovine serum, and additionally glucose for CCB cells) was added after an adsorption period of two hours at 20°C and the culture flasks were further incubated at 20°C. Cell cultures were examined for CPE daily. Infectivity titers were calculated by the method of Kärber (1931) using the 10 fold end point dilution test in microtiter plates.

Neutralization

Neutralization tests were carried out in microtiter plates (Mayr et al., 1974) using polyvalent anti-IPNV serum and anti-SVCV serum. (Antiserum against SVCV was kindly provided by Dr. Enzmann, Tübingen). Isolates 1592 and 1621 were tested against anti- SVCV serum and isolate 1620 against anti-IPNV serum.

Treatment with chloroform and IUDR

The sensitivity of infectious supernatants against lipid solvents (10% chloroform) and IUDR (5-Iodo-2-deoxyuridine, 100µg ml⁻¹) was investigated according to the methods described by Mayr et al. (1974).

Bouyant densities of the viruses

Densities of the virus isolates were determined by isopycnic centrifugation in sucrose or CsCl gradients. Clarified infectious supernatant was either layered on a preformed sucrose gradient (20-60%) or mixed with CsCl (isolate 1620, resulting density:1.33gml⁻¹) and centrifuged for 40 h at 40 000 rpm in a SW41 rotor (Beckman). One-ml fractions were collected by piercing the bottom of the centrifuge tubes. The density of each fraction was calculated from the refractive index and viral infectivity was quantified by the endpoint dilution test after the fractions had been dialysed against phosphate buffered saline.

Electron microscopy

Cover slips with CCB cell monolayers placed in small petri dishes were infected with the isolates. After CPE became obvious CCB cells were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH: 7.4). The monolayers were postfixed with 1% OsO₄ in the same buffer, dehydrated in a series of ascending grades of acetone and embedded in the resin Durcupan (Fluka, Germany). Ultrathin sections (50-70nm) were mounted onto copper grids, stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10 A electron microscope. Preparations of isolates 1592 and 1621 resuspended in 0.1 M Tris buffer were placed on formvar-coated copper grids after the isolates had been concentrated.
Figure 1. Cytopathic effects (CPEs) of Koi isolates in CCB cells. (a) non-infected CCB cells; (b) isolate 1609, 7 days p.i.; (c) isolate 1592, 2 days p.i.; (d) isolate 1551, 6 days p.i.; (e) isolate 1620, 4 days p.i.; (f) isolate 1617, 10 days p.i.; (g) isolate 1621, 2 days p.i.; (h) isolate 1632, 12 days p.i. May-Grünwald-Giemsa stain. Bar represents 80µm.
and purified by sucrose density centrifugation. The grids were negatively stained with neutralized 2% phosphotungstic acid and examined as mentioned above.

**Calculation of particle sizes**

Particle sizes of the different isolates were provisionally examined by titration of virus infectivity after filtration of infectious cell culture supernatants through 450, 200 and 100 nm filters (Millipore). Additionally, particle dimensions were measured on photographic prints after electron microscopical visualization.

**Results**

**Anamnesis and pathological findings**

Most of the koi keepers mentioned the introduction of new fish two to five weeks before outbreak of the disease in the old population. Diseased fish showed unspecific symptoms as lethargy or disorientation, anorexia and frequent ventilation. Mortality was usually high.

The most common pathological findings were disorders of the external epithelia and an irregular coloration of the gills often combined with more or less severe branchial necrosis. Focal losses of the epidermis sometimes with a sandpaper-like structure or hemorrhages and ulcers were also seen.

**Isolation of viruses**

The development of cytopathic effects (CPEs) depended on kind of isolates and cell cultures used. CPEs of isolates 1592, 1620 and 1621 appeared within two days in all cell lines and were characterized first by focal rounding and thereafter by detachment of the cells. A few days later the cell cultures were completely destroyed. Isolate 1617 (organ samples were sent by R. Hoffmann, Munich) caused rounding and detachment of cells as well, but this phenomenon was detected first after 10 days and did not occur in CCG and EPC cell lines. The CPE of the isolates 1551 and 1609 was characterized by forming syncytia in all cell lines used first observed 5 (isolate 1551) or 10 days (isolate 1609) after inoculation. The characteristics of the CPE initiated by isolate 1632 were first a more or less extensive vacuolization (fig 3: in CCB cells) of the cells of all cell lines (fig. 2). In CCB and CaF-2 cells the CPE appeared 8-9 days after inoculation and spread in the cell culture within the next 5 to 6 days. In CCG and EPC cell lines the isolate 1632 caused a few focal but transient CPEs (fig. 2) first observed after 10-11 days. Nearly two weeks later CPEs had vanished. No CPE could be initiated in subsequent passages. Table 1 shows the sensitivity of the four cell lines to the different isolates. In figure 1 various kinds of CPE initiated by the isolates in CCB cells are documented.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Ca F-2</th>
<th>CCB</th>
<th>CCG</th>
<th>EPC</th>
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<tr>
<td>1551(1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1592</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1609</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1617(2)</td>
<td>+</td>
<td>+</td>
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<td>1620</td>
<td>+</td>
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</tr>
<tr>
<td>1632</td>
<td>+</td>
<td>+</td>
<td>?</td>
<td>?</td>
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</table>

Table 1. Sensitivity of carp cell lines used for virus isolation. (1)Isolate partially described by Neukirch et al., 1999. (2)Organ sample sent by R. Hoffmann, Munich. (3) No CPE was observed. (4)CPE transient.
Figure 2. Cytopathic effect (CPE) of Koi isolate 1632 in four different carp cell lines. (a + a’): CCB cells, 12 days p.i.; (b + b’): CaF-2 cells, 10 days p.i.; (c + c’): CCG cells, 14 days p.i.; (d + d’): EPC cells, 22 days p.i. May-Grünwald-Giemsa stain. Bars represent 300µm (a-d) and 45µm (a’-d’).
Treatment with chloroform and IUDR

All viruses isolated except isolate 1620 were found to be sensitive against chloroform treatment.

Supplementation of IUDR in the virus growth medium caused no significant reduction in virus infectivity of the isolates 1551, 1592, 1609, 1617, 1620 and 1621 indicating that these viruses possess a RNA as genome. However, multiplication of isolate 1632 was completely suppressed by IUDR. Formation of CPE were not observed and infectivity did not occur as it is known for DNA viruses.

Buoyant density of the viruses

After isopycnic centrifugation in sucrose gradients the maximum of infectivity was found at a density of 1.16 g ml\(^{-1}\) for isolates 1592 and 1621, and of 1.19 g ml\(^{-1}\) for isolates 1551, 1609 and 1617. The highest yield of infectivity of isolate 1632 was detected at 1.22 g ml\(^{-1}\). Density of isolate 1620 was determined in a CsCl gradient showing maximum infectivity at 1.32 g ml\(^{-1}\) (table 2).

Neutralization

Isolate 1620 was not neutralized by a trivalent anti-IPNV serum. Isolates 1592 and 1621, however, showed a significant reduction of infectivity in the neutralization test against anti-SVCV serum.

Particle sizes:

All isolates passed 450 nm filters without loss of infectivity. After filtration through 200 nm filters only isolate 1609 showed a slight reduc-

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Characteristics of CPE</th>
<th>Chloroform sensitivity</th>
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<th>Density(2) (g cm(^{-2}))</th>
<th>Nucleic acid</th>
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<td>+</td>
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<td>1.19</td>
<td>RNA</td>
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<td>1.16</td>
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</tr>
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<td>&lt;100</td>
<td>1.16</td>
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<td>1.22</td>
<td>DNA</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of viruses isolated from diseased Koi carp *cyprinus carpio*.\(^{(1)}\) Estimated by titration of infectivity after filtration through filters with different pore sizes. \(^{(2)}\) Density was determined in sucrose gradients, density of isolate 1620 in a CsCl gradient. \(^{(3)}\) Isolate 1551 was partially described by Neukirch *et al.*, 1999. \(^{(4)}\) Organ sample was sent by R. Hoffmann, Munich.
Figure 4: Electron micrographs of thin sections showing virus particles of koi isolates grown in CCB cells. (a, a'): isolate 1609; (b, b'): isolate 1617; (c, c'): isolate 1632. Bars represent 1µm (a, b, c), 200nm (a', c'), and 100nm (b').
tion of virus infectivity. The 100 nm filters were passed by the isolates 1592, 1617, 1620 and 1621 but not by 1609 and 1632. The results are summerized in table 2.

**Electron microscopy**

Virions of isolate 1609 have been found in the cytoplasm and vacuoles of infected giant CCB cells and revealed usually spherical morphology (Fig. 4a). Sometimes filamentous forms (Fig. 4a’) are visible. The enveloped particles carry surface projections, the diameters vary between 160 and 190 nm. Similar virus particles represent the isolate 1551, too (not shown).
Micrographs of isolate 1617 exhibit a collection of spherical virus particles in the cytoplasm and cytoplasmic vacuoles (Fig. 4b). The internal structure of some virions seems to be granular, the outer surface of the enveloped particles is covered with projections (Fig. 4b’). Diameters of the particles vary between 70 and 80 nm.

The electron micrographs of Fig. 4c,c’ reveal particles of isolate 1632 in the nuclei of a giant cell formation of the CCB cell line. The virions with diameters between 160 and 180 nm are found as single particles or clusters near the nuclear membranes. Capsids are 100 to 110nm.

Virus particles of isolate 1592 show the typical bullet-shaped structure approximately 100-120nm in length and about 70 nm in diameter (Fig. 5a). Some virions revealed surface projections (Fig. 5a’) representing their G protein. Electron microscopical examinations of isolate 1621 disclosed bullet-shaped virions (not shown) identical with virus particles of isolate 1592.

Isolate 1620 (Fig. 5b) is represented by non-enveloped cytoplasmic virus particles appearing mostly spherical in shape. Some particles show an icosahedral morphology (Fig. 5b’). Virion diameters are between 60 and 70 nm.

Discussion
Mass mortality in koi carp populations is the main symptome of a new disease which seems to be restricted to koi and common carp populations (Ariav et al., 1999; Hoffmann et al., 2000). It is widely accepted that a virus is the causative agent as bacteria, parasites or variable water parameters were excluded as cause of this disease and virus-like particles have been found in diseased fish by electron microscopical investigations (Ariav et al., 1999; Bretzinger et al., 1999) and isolation procedures (Body et al., 2000; Hedrick et al., 2000; Neukirch et al., 1999).

The viruses isolated from diseased fish in our laboratory are of taxonomic diversity. The isolates can be classified to five families due to the formation of different CPEs in the cell culture, the sensitivity against chloroform and IUDR, the neutralization test, the maintenance or loss of infectivity after filtration through filters of different pore sizes, the different bouyant densities after isopycnic centrifugation and the electron microscopical structure of the virus particles. Six of seven isolates were found to be sensitive against treatment with chloroform indicating the presence of an envelope. Results concerning the treatment of the isolates with IUDR revealed RNA as viral genome in six cases. The multiplication of isolate 1632, however, was completely suppressed, indicating that this virus contains DNA as nucleic acid.

Based on the above mentioned investigations and the electron microscopical visualization of the virus particles the isolates can be provisionally classified as paramyxovirus (isolates 1551 and 1609), rhabdovirus (isolates 1592 and 1621, identified as strains of spring viraemia of carp virus, SVCV), reo- or birnavirus (isolate 1620), orthomyxovirus (isolate 1617) and herpesvirus (isolate 1632).

Isolation of viruses from diseased koi suffering mass mortality has already been described (Neukirch et al., 1999; Body et al., 2000; Hedrick et al., 2000). But pathogenic tests with
the isolates obtained by Body et al. (2000) are still in progress and the isolate 1551 (Neukirch et al., 1999) which seems to be very similar to the pathogens isolated by Body et al. (2000) is of weak pathogenicity. However, exposures to a herpesvirus, isolated from adult koi during two outbreaks, resulted in high mortality and reisolation of the virus from tissues of infected fish (Hedrick et al., 2000). Herpesvirus-like particles have also been demonstrated by electron microscopy in tissues of diseased koi (Ariav et al., 1999; Bretzinger et al., 2000; Hedrick et al., 2000). Furthermore, the herpesvirus 1632 has been isolated from koi suffering mass mortality. Therefore, and in spite of missing pathogenic investigations with the above mentioned isolates it is believed that a herpesvirus is responsible for the new koi disease associated with high mortalities.

Another point should briefly be stressed. Hoffmann et al. (2000) reported the first outbreak of the new disease with mass mortality in mirror carp of a German fish farm after some koi carp had been introduced into the farm eight to ten days before. Symptoms resembled those of diseased koi and in tissues of affected carp herpesvirus-like particles were demonstrated by electron microscopy. This outbreak of the new koi disease in common carp bred for human consumption is considered to be most important, as it indicates the risk of the introduction of new diseases in the aquaculture industry by ornamental fish. Pathogenic studies with isolate 1632 and the other virus isolates are clearly necessary and further investigations to give more precise classification of the viruses should also be performed.

Acknowledgement

We would like to thank B. Luckhardt for her skilled technical assistance.

References


