First Report of Spring Viremia of Carp Virus in Feral Common Carp (*Cyprinus carpio)* in North America

AUDREY L. DIKKEBOOM, CRAIG RADI and KATHY TOOHEY- KURTH *

Wisconsin Veterinary Diagnostic Laboratory, University of Wisconsin-Madison, 6101 Mineral Point Road, Madison, Wisconsin 53705-4494 USA

SUSAN MARCQUENSKI

Wisconsin Department of Natural Resources, 101 South Webster Street, Box 7921, Madison, Wisconsin 53707 USA

MARTY ENGEL

Wisconsin Department of Natural Resource, 890 Spruce Street, Baldwin, WI 54002 USA

ANDREW E. GOODWIN

Aquaculture/Fisheries Center, University of Arkansas at Pine Bluff, 1200 North University Drive, Mail Slot 4912, Pine Bluff, Arkansas 71601 USA

KEITH WAY, DAVID M. STONE and CLARE LONGSHAW

Centre for Environment, Fisheries and Aquaculture Science, Weymouth Laboratory, Barrack Road, The Nothe, Weymouth DT4 8UB UK

* to whom correspondence should be addressed

Running head: SVCV in feral common carp

Key words: rhabdovirus, SVCV, North America, common carp, cyprinid, dropsy

Abstract

In spring 2002, an estimated 1,500 common carp(*Cyprinus carpio*) (over 10,000 kg of biomass) died over a six week period (late April through the first week in June) in Cedar Lake which is located in Polk and St. Croix Counties, in northwestern Wisconsin. Three moribund carp were necropsied and had signs consistent with spring viremia of carp (SVC) disease, including petechiae and ecchymotic hemorrhages on the skin, ascites, and edematous kidney and spleen. A virus was isolated on FHM cells and was shown to be a rhabdovirus by electron microscopy. Immunoassay results indicated a close serological relationship with SVC virus and this was confirmed by RT-PCR assay and subsequent sequence analysis.

Immunocytochemistry and serum neutralization tests indicated that the Cedar Lake isolate did not share complete antigenic identity with the European reference SVCV. Also, the isolate showed an inhibition of cytopathic effect after repeated sub-culture in EPC and BF-2 cells when compared to other SVCV isolates. In virus transmission studies the isolate was shown to be of low virulence for juvenile carp. Sequence analysis showed that the Cedar Lake SVCV isolate was more closely related to a recently isolated North Carolina strain of SVCV (98.6% nucleotide identity) and to strains of Asian origin rather than the European reference strain of SVCV. This is the first report of a SVC epizootic in a feral common carp population in North America.

Spring viremia of carp virus (SVCV) causes a rhabdoviral disease of cultured common carp, *Cyprinus carpio* and has been isolated from other cyprinids under natural conditions (Fijan 1999). SVCV is one of only five fish pathogens listed as notifiable in the International Aquatic Animal Health Code of the Office International des Epizooties (OIE) (OIE 2000). SVC disease outbreaks have been reported most often in carp farms in Europe (Fijan 1999, Ahne et al. 2002), but have also been reported, on one occasion, in the Middle East (Fijan 1999). The first report of SVC disease in North America occurred in a private fish farm in North Carolina in the spring of 2002 (Goodwin 2002). Reports of SVC epizootics in wild fish are rare. Marcotegui and Coll (1992) reported SVCV in common carp from wild ponds and lakes in Spain.

SVCV is tentatively assigned to the Vesiculovirus genus of the Rhabdovirus family (Ahne et al. 2002). Pike fry rhabdovirus (PFRV) is a closely related member of this genus and shares 64.6% amino acid sequence homology with the glycoprotein of the SVCV reference strain (Stone et al. 2003). This relatively high degree of amino acid sequence homology has caused some difficulty in assigning isolates when using either the ELISA or virus neutralization tests (Rowley et al. 2001) because of serum cross reactivity. However, problems with virus identification were overcome by comparative analysis of partial G-gene sequences. Further, phylogenetic analysis of partial G-gene sequences identified distinct lineages separated on geographical isolation. Four subgroups of SVCV have been identified to date that correspond to viruses from Asia, Western Europe, Eastern Europe and States of the former USSR (Stone et al. 2003).

Clinical signs of SVC include edema, inflammation of the swim bladder, ascites and petechial hemorrhages in the gill and skin (Fijan et al. 1971). Moribund fish display lethargy, loss of equilibrium, uncoordinated swimming, exopthalmia and low respiration (Jeney and Jeney 1995). Outbreaks of SVC generally occur when water temperatures are between 10 and 17°C; however fry can be affected at temperatures as high as 22-23°C (Ahne et al. 2002).

An extensive common carp kill occurred in Cedar Lake, Wisconsin from ice-out in mid-April through the first week in June, 2002. Cedar Lake is located on the border of Polk and St. Croix Counties in northwestern Wisconsin and is a 448-hectare recreational lake. The lake is a natural water body with an inlet and an outlet stream seasonally controlled by a low head dam (0.61 m). The outlet stream discharges into the Apple River which flows into the St. Croix River which in turn joins the Mississippi River at Prescott, WI. The kill was specific to carp and approximately 1,500 fish or 20 % of the population died. Dead fish ranged in size from 46 to 90 cm and larger fish weighed an average of 6 kg.

This article describes the diagnosis of SVCV as the causative agent of the common carp kill in Cedar Lake and is the first report of SVCV from feral common carp in North America.

Methods

Necropsy. Three moribund common carp were collected on 22 May 2002 by local Wisconsin Department of Natural Resources (WI DNR) fisheries staff, euthanized and transported on ice to the WI DNR fish health lab in Madison, WI and necropsied the next day. At necropsy, bacterial cultures were made from the skin, gills, kidney, liver

and spleen on trypticase soy agar (TSA) and from skin and gills on Ordal's medium and submitted to the Wisconsin State Laboratory of Hygiene for bacteriology. Kidney, spleen, swim bladder and approximately 1 ml of ascites were homogenized, diluted 1:50 with Hanks balanced salt solution (HBSS) and submitted to the Wisconsin Veterinary Diagnostic Laboratory for virology. Tissues for histology were not collected due to the length of time between death and necropsy (30 hours).

Bacteriology. Mixed bacterial cultures were sub-cultured at 21°C to obtain pure colonies and biochemical tests (cytochrome oxidase, KOH, TSI, motility, morphology) were performed to screen for significant bacteria. These bacterial isolates were then identified using API 20E identification strips (bioMerieux Vitek, Inc., Hazelwood, Missouri).

Virus isolation on cell culture. Tissue homogenates for virological examination were clarified by centrifugation at 2000 g for 20 minutes and 0.2 μm membrane filtration. Filtrates were then diluted to 1:100 (w/v) with culture medium (Eagles Minimal Essential Medium (EMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin, 100 μg /ml streptomycin, 30 units/ml nystatin and 0.05 μg/ml gentamycin and inoculated onto epithelioma papulosum cyprini (EPC; Fijan et al. 1983) and fat head minnow (FHM; Gravell & Marlsberger 1965) cell monolayers in 24-well cluster dishes. Monolayers of EPC cells were incubated at 15°C and FHM at 25°C and examined daily for cytopathic effect (CPE). Cell monolayers exhibiting CPE were sub-cultured onto EPC, FHM and Bluegill fibroblast (BF-2; Wolf et al. 1966) cell monolayers in 75cm^2 flasks at serial log^{10} dilutions ranging from 10^2 to 10^8 and incubated at 20°C. In

collaborating laboratories, the SVCV-Cedar Lake, WI (WI02-131) isolate was grown on EPC cell monolayers at 20°C.

Electron Microscopy. Monolayers of BF-2 cells exhibiting 90% CPE at a 10^8 dilution in 75cm^2 flasks were frozen overnight at –70°C. After thawing, the medium containing virus was harvested and centrifuged at 550 x g for 20 minutes on a fixed angle rotor. The supernatant was removed and centrifuged for an additional 2 h at 150,000 x g. The supernatant was discarded and pellet resuspended with filtered water and 0.01% albumin then negatively stained with 4% phosphotungstic acid. The solution was then aspirated onto a 300 mesh copper grid for examination on a Hitachi H-600 transmission electron microscope at an acceleration voltage of 100 kV at 30,000X magnification.

In vivo transmission trials. Monolayers of BF-2 cells were inoculated with SVCV-North Carolina isolate (NC02-46) and SVCV-Cedar Lake, WI (WI02-131) then incubated at 22°C until most of the cell monolayer was destroyed (3-5 d). Supernatants were passed onto EPC cells that were incubated until most of the monolayer was destroyed (3-5 d) then the supernatants were frozen at -80°C.

Twenty common carp 5-8 cm in total length, and 12 koi 7-10 cm in total length from commercial fish farms with a history of negative SVCV inspections, were placed in each of two 35 L aquariums containing dechlorinated municipal water at 20°C. Fish from each aquarium were injected intraperitoneally with 50µl of EPC cell culture supernatant containing WI02-131 or NC02-46 diluted with HBSS so that each fish received 10^6 $TCID_{50}$ of the virus. Estimates of virus titers were determined in EPC by serial dilution at 22°C according to the methods of Reed and Muench (1938). The temperature in the aquariums was then dropped rapidly to 15°C and held at that

temperature with flow-through chilled water for the duration of the experiment. Fish were observed for 45 days for mortalities and clinical signs of SVCV.

Immunocytochemistry. Immunocytochemistry was performed on infected EPC flasks showing CPE using a polyclonal rabbit anti-SVCV antibody (generously supplied by Niels Olesen of the National Veterinary Laboratory in Denmark) derived from a European reference strain of SVCV. Uninfected cells were used as a negative control and a reference SVCV isolate was used as a positive control.

ELISA. Virus was identified by enzyme-linked immunosorbent assay (ELISA) following the procedure recommended for the identification of SVC virus in the OIE Diagnostic Manual for Aquatic Animal Diseases (OIE 2000). Virus antigen was extracted from EPC cell monolayers with phosphate buffer containing 2% Nonidet P-40. The extracted antigen was tested in an amplified sandwich-ELISA using rabbit immunoglobulin (Ig) and biotinylated rabbit IgG specific to SVCV and PFRV and ExtrAvidin horseradish peroxidase (Sigma, St. Louis, MO) as the conjugate. Absorbance was measured at 450nm (A450) and the values given are averages of duplicate wells on a microtiter plate.

Serum neutralization. Serum neutralization tests were carried out following recommended procedures (OIE 2000) using polyclonal rabbit antiserum raised against the European reference strain of SVCV. This antiserum was raised by intra-venous injection of concentrated virus using the multiple inoculation program described by Hill et al. (1981). Supernatants from cell monolayers exhibiting CPE were diluted at 1:5,000, 1:50,000 and 1:500,000 and incubated for 1h with aliquots of antiserum diluted to 1:200, 1:400 and 1:800 and then inoculated onto EPC monolayers in 96-well tissue culture

microplates. The microplates were then incubated at 20°C and observed daily for the appearance of CPE.

RT-PCR. The identity of the virus was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and a semi-nested PCR following the method of Stone et al. (2003). Total RNA was extracted from infected cell culture supernatant using the TRIZOL™ (Invitrogen, Carlsbad, CA) reagent and the RT-PCR was carried out using MMLV reverse transcriptase and the SVCV R2 and SVCV F1 primer set. The semi-nested PCR was carried out with the SVCV R4 and SVCV F1 primer set (Stone et al. 2003).

Sequence analysis. Products generated by RT-PCR were purified using the Geneclean® (Lucernachem, Switzerland) spin system, inserted into the pGEM-T vector (Promega, Madison, WI) according to the manufacturer's recommendations and sequenced using the M13-20 and reverse primers (Stratagene, Cedar Creek, TX) and the ABI PRISM™ (Applied Biosystems, Foster City, CA) cycle sequencing system. Sequence alignments were performed using the Clustal V package within the MEGALIGN software (DNAstar Inc., Madison, WI) and the phylogenetic analysis was performed using Phylip (Seattle, WA) within the phylogenetic inference environment facility at the Human Genome Mapping Project Resource Centre, Hinxton, UK.

Results

Fish kill. During the disease outbreak approximately 1,500 common carp (over 10,000 kg of biomass) died in Cedar Lake from ice-out (April 14) through June 8, 2002. In the fall of 2001, there were anecdotal reports of dead carp in Cedar Lake, but ice formation prevented collecting fish for necropsy at that time. The kill was specific to

carp. Dead fish ranged in size from 46 to 90 cm and the larger fish weighed an average of 6 kg. Carp ceased dying around June 8, when water temperature was consistently above 19°C.

Necropsy. The three moribund carp collected for necropsy were adult females, 80, 81 and 90 cm in length. Gross lesions included abundant petechiae and ecchymotic hemorrhages on the ventral skin, inflamed vent, copious clear, golden ascites and edematous kidney and spleen. No hemorrhages were observed on the swim bladder.

Bacteriology. No bacterial growth was observed on TSA or Ordal's media incubated at 21°C from the kidney and spleen for all three fish. *Aeromonas hydrophila* was cultured from the liver of one fish; *A. hydrophila, Pseudomonas fluorescens* and *Flavobacterium sp.* were isolated from the skin and gills of all three fish.

Virus isolation on cell culture. Tissue homogenates from each of the three carp produced CPE in FHM cells within 3 days at 25°C. CPE was slower to develop in EPC cells, occurring in 5-7 days at 15°C. Subcultures of FHM, BF-2 and EPC supernatants produced CPE in FHM cells in 2-3 days at 25°C, BF-2 cells in 3-5 days at 20°C and in EPC cultures in 2-3 days at 15°C. Complete destruction of the monolayers occurred in 6-7 days. When CPE was present, cells developed vacuoles, rounded up and lifted from the cell sheet. In later sub-cultures of the isolate WI02-131, the CPE developed more slowly and complete CPE was only seen in flasks receiving high dilutions of virus.

Electron Microscopy. Electron microscopy revealed a bullet shaped rhabdovirus with the inner nucleocapsid measuring 32 x 95 nm and the virion measuring 111 –127 nm in length and 80-95 nm in width (Figure 1). Presumably, competition from defective interfering particles prevented formation of completely developed rhabdoviral particles in

flasks inoculated with concentrated virus, which prevented cytopathic effect from occurring. Complete formation of the virions (and resultant CPE) was accomplished by using a 10^8 dilution of the rhabdovirus suspension when inoculating new flasks.

In vivo transmission trials. Mortality began 13 days post injection and peaked at 20 days. Fish died with signs consistent with SVC disease and SVCV was isolated from 2 of the carp. The rate of death declined through day 45. Total mortality after 45 d was 75% in koi and 85% in common carp injected with NC02-46. Over the same time period, mortality was 0% for koi and 10% in common carp injected with WI02-131 (Figure 2).

Immunocytochemistry. SVCV antibodies used in the immunocytochemistry assay did not recognize the Cedar Lake isolate, WI02-131. However, positive controls did react with the antiserum.

ELISA. Harvested supernatants from EPC cells infected with WI02-131 gave high absorbance values at 450nm (A_{450}) in the SVCV ELISA system (Table 1). All of the SVCV isolates tested, including isolate WI02-131, gave lower A_{450} values in the PFRV ELISA system compared to the reference PFRV isolate.

Serum Neutralization. Neutralization of the Cedar Lake isolate WI02-131 occurred at the 1:50,000 virus dilution with the 1:200 and 1:400 antiserum dilutions. In contrast a European SVCV isolate (UK-E117), included as a positive control in the test, was neutralized at a 1:5,000 virus dilution with the 1:800 antiserum dilution. This indicates that WI02-131 was only weakly neutralized by SVCV antiserum.

RT-PCR. The Cedar Lake isolate WI02-131 was confirmed as SVCV by RT-PCR. Using the SVCV specific primers, a product of 606 bp was generated when using

RNA extracted from WI02-131, as well as the SVCV reference positive control. No product was generated with the negative control (results not shown).

Sequence analysis. Two independent extraction and amplifications were performed for sequence analysis to eliminate the effect of errors introduced by the Taq polymerase and to identify the consensus sequence within what was likely to be a complex heterogeneous 'quasi-species'. A 426 bp segment from the glycoprotein (G-gene) sequence (nucleotides 429-855) derived from the 606 bp PCR amplicon was used for phylogenetic analysis. Previously published sequences for representatives of the four SVCV subgroups (Stone et al. 2003) confirmed that the Cedar Lake isolate WI02-131 was SVCV, sharing 87.7% nucleotide identity with the European SVCV reference strain (S30). WI02-131 shared 89.2% nucleotide identity with isolates from the Ukraine (N1-5) and Moldova (2-90), and 99.3% nucleotide identity with 980528, an isolate recovered from Chinese koi imported into the United Kingdom from the Republic of China in 1998 (Figure 3). Comparing partial G-gene sequences of WI02-131 and NC02-46 and NC02-94 from North Carolina SVCV (Goodwin 2002) showed there were 6 nucleotide substitutions or 98.6% nucleotide identity. NC02-46 and NC02-94 shared 100% nucleotide identity. Stone et al. (2003) used Neighbor-Joining and maximum parsimony analysis of the 426 bp partial G-gene sequence and identified 4 SVCV genogroups. Using this method, all three US isolates were assigned to the same SVCV subgroup (Subgroup Ia). Analyses were done on 1000 bootstrapped data sets and values >600 are shown on the trees (Figure 4). The tree shown for the Neighbor-joining method was generated using non-bootstrapped analysis to retain branch length information, and the bootstrapped values from a parallel bootstrapped analysis were placed on the analogous

branches of the tree. A detailed examination of nucleotide sequence analysis of the G-gene of SVCV and additional information on SVCV subgroups may be found in Stone et al. (2003).

Discussion

Bacteriology results did not suggest a bacterial etiology as the cause of the common carp kill in Cedar Lake. Homogenates of kidney, spleen, swim bladder and ascites produced a virus-like CPE in cell culture that developed most rapidly in FHM cells at 25°C. A rhabdovirus was observed in tissue culture supernatant using negative staining electron microscopy and was shown to be of low virulence for juvenile carp in transmission studies. It was serologically similar to SVCV based on ELISA results. However, the immunocytochemistry and serum neutralization results indicated the WI02-131 isolate did not share complete antigenic identity with the European reference SVCV. Analysis of partial G-gene sequences generated by RT-PCR confirmed the Cedar Lake isolate WI02-131 was SVCV and shared 87.7% nucleotide identity with the European reference strain. In rhabdoviruses, neutralization is a function of antibodies directed against the envelope glycoprotein (Kelly 1972, Ahne 2002). Further analysis of the gene that encodes the glycoprotein may elucidate the sequences responsible for the weak neutralization response of WI02-131.

Growth in cell culture suggests that WI02-131 exhibits unique characteristics when compared to other SVCV isolates. During growth of the isolate in BF-2, EPC and FHM cells the CPE developed more slowly after each sub-culture. Development of CPE was improved when WI02-131 was sub-cultured at high dilution suggesting that incomplete or defective virus particles may be produced at low dilution sub-culture of the

virus. Rhabdoviruses, and in particular the vesiculovirus, vesicular stomatitis virus, have been shown to produce defective interfering particles (DIPs) after repeated passage in cell culture resulting in a reduction in virus yield and inhibition of cell cytopathogenicity (Holland 1987). Of the fish rhabdoviruses, DIP production has been most studied in the novirhabdovirus, infectious hematopoietic necrosis virus and there is evidence that DIPs may mediate virus persistence in fish (Kim et al. 1999). It is not clear why the Cedar Lake SVCV isolate, WI02-131, exhibited such low virulence for juvenile carp compared to the North Carolina isolate NC02-46. Both viruses were isolated from clinically diseased carp. The unusual growth characteristics displayed by WI02-131 and possible production of DIPs may be one explanation for the apparent attenuation of the virus and low virulence and warrants further investigation.

Partial G-gene sequence analysis shows a close genetic relationship between the Cedar Lake isolate WI02-131 and the two isolations made in North Carolina NC02-46 and NC02-94 (Goodwin 2002). However, there are 6 base pair substitutions, indicating the isolates are not identical. The Cedar Lake and North Carolina isolates are placed on distinct branches of the neighbor-joining distance tree which is supported by high bootstrap values of >68%. WI01-131 shared greater homology (99.3% nucleotide identity) with 980528, which was cultured from a shipment of Chinese koi in the UK in 1998.

Stone et al. (2003) have established four subgroups of SVCV; based on partial G-gene sequence analysis. These subgroups differentiate SVCV viruses from Asia, Western Europe, Eastern Europe and States of the former USSR. SVCV isolates from Cedar Lake, Wisconsin and North Carolina were placed into SVCV subgroup Ia. This

subgroup also includes SVCV isolates originating from Asian shipments of carp (Stone et al. 2003), suggesting the US isolates may have their origins in Asia.

There are insufficient data to conclude whether these are new SVCV introductions or whether the virus has been present and undetected in the US for some time. There are a number of examples of virus isolations made from healthy asymptomatic fish, where an additional trigger is required to induce the clinical disease. The mortalities in common bream *Abramis brama* reported by Rowley et al. (2001) is a good example of a situation where an apparently benign virus can spontaneously produce high levels of mortality in a wild fish population. The rhabdovirus isolated in the bream was also isolated from brown and rainbow trout before the outbreak and from asymptomatic bream and roach in the years following the outbreak (Adair & McLoughlin 1986). This suggests that the trigger for disease is absent most of the time. Although the trigger has not yet been identified in this particular case, poor water quality was implicated (Rowley et al. 2001).

In conclusion, despite differences in *in vitro* and *in vivo* characteristics compared to other SVCV isolates, the Cedar Lake isolate WI02-131 was confirmed as a rhabdovirus by electron microscopy and subsequently specifically identified as SVCV by ELISA and RT-PCR. Partial G-gene sequence analysis placed WI02-131 in SVCV subgroup Ia, which suggests a possible Asian origin of the virus. This is the first time SVCV has been reported from feral common carp in North America.

Acknowledgements

We thank Marty Collins at the Wisconsin State Laboratory of Hygiene for providing bacteriology results and Dr. Hui-Min Hsu at the Wisconsin Veterinary Diagnostic Laboratory for her consultation and insights on this case.

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Table 1. Absorbance values at 450nm (A_{450}) in the SVCV and PFRV ELISA systems of EPC harvests of the Cedar Lake SVCV isolate (WI02-131) compared with the European reference strain (S30), imports of Chinese koi into the UK in 1998 (980528) and Russia (P4), North Carolina (NC02-46), PFRV reference (F1) isolates and EPC cells. Values are average readings from duplicate wells.

Virus	Dilution	SVCV A_{450}	PFRV A_{450}
WI02-131	1/8	0.552	0.333
WI02-131	1/16	0.481	0.228
WI02-131	1/40	0.349	0.156
S30	1/10	0.468	0.152
980528	1/10	0.541	0.274
P4	1/10	0.518	0.263
NC02-46	1/10	0.524	0.220
PRFV F1	1/10	0.411	0.570
EPC negative control	1/10	0.050	0.067

Figure 1. Electron micrograph of isolate WI02-131 from a BF-2 cell culture negatively stained with phosphotungstic acid. Bar = 100nm.

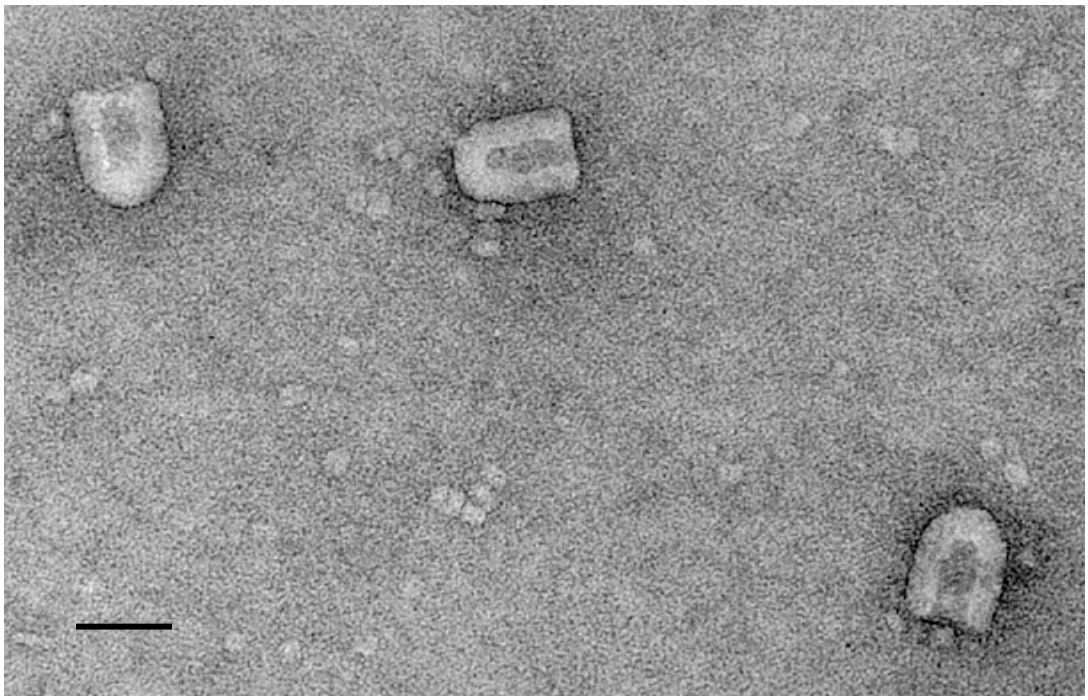
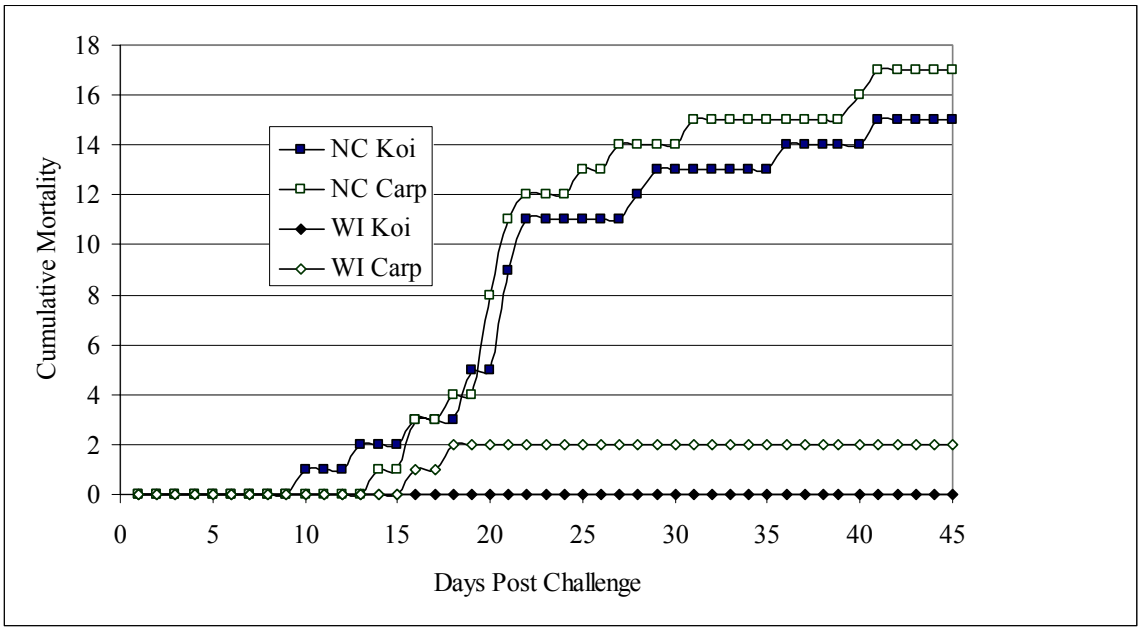
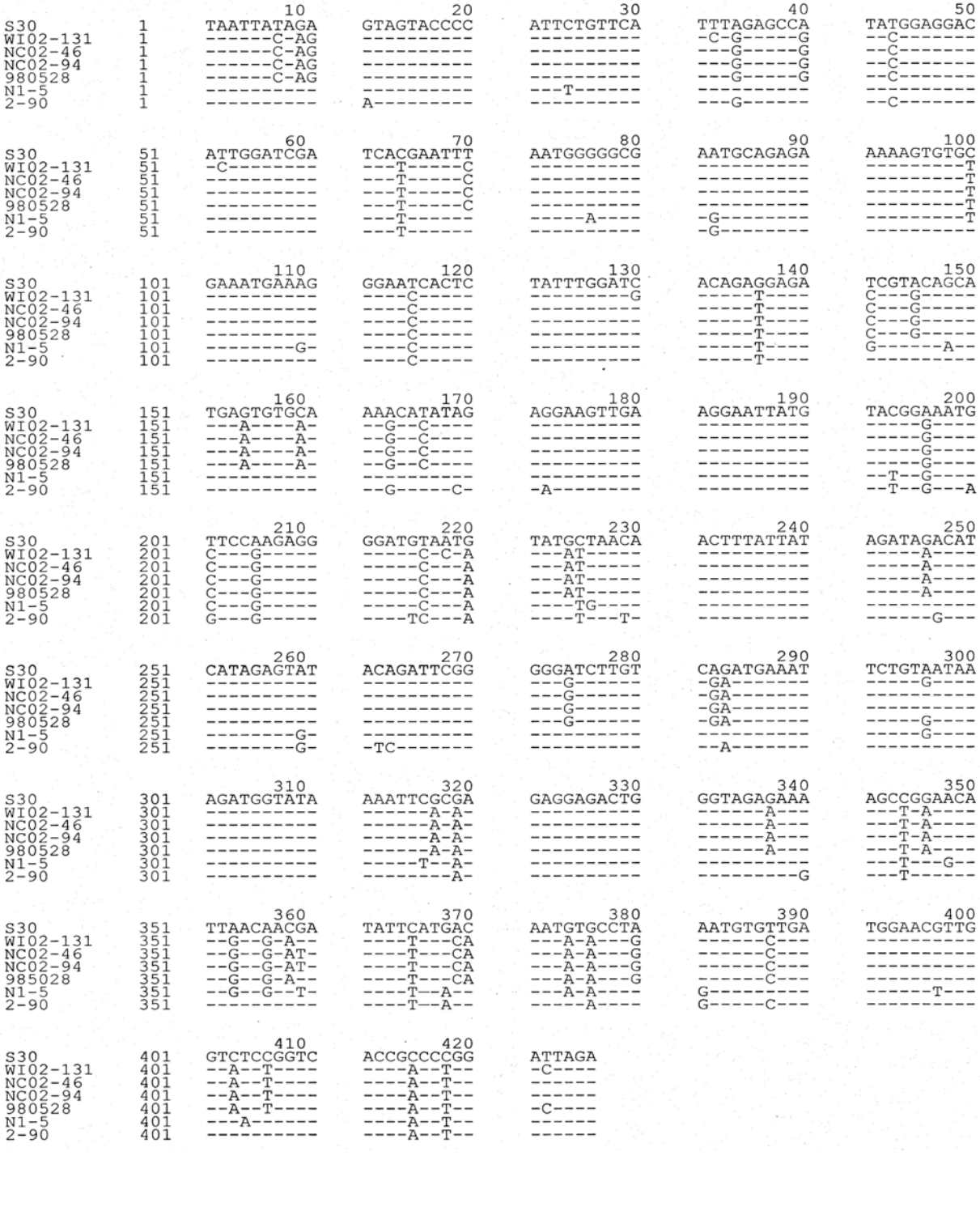


Figure 2. Cumulative mortality of common carp and koi injected with SVCV isolates NC02-46 and WI02-131 at 10^6 $TCID_{50}$/fish.



1 Figure 3. Alignment comparison of the nucleotide sequence of SVCV isolates from the
2 European reference strain (S30), Cedar Lake, Wisconsin (WI02-131), North Carolina
3 (NC02-46 and NC02-94), imports of Chinese koi into the UK in 1998 (980528) and from
4 Ukraine (N1-5) and Moldova (2-90).



		10	20	30	40	50
S30	1	TAATTATAGA	GTAGTACCCC	ATTCTGTTCA	TTTAGAGCCA	TATGGAGGAC
WI02-131	1	------C-AG	----------	----------	-C-G-----G	--C-------
NC02-46	1	------C-AG	----------	----------	---G-----G	--C-------
NC02-94	1	------C-AG	----------	----------	---G-----G	--C-------
980528	1	------C-AG	----------	----------	---G-----G	--C-------
N1-5	1	----------	----------	---T------	----------	----------
2-90	1	----------	A---------	----------	---G------	--C-------

		60	70	80	90	100
S30	51	ATTGGATCGA	TCACGAATTT	AATGGGGGCG	AATGCAGAGA	AAAAGTGTGC
WI02-131	51	-C--------	---T-----C	----------	----------	---------T
NC02-46	51	----------	---T-----C	----------	----------	---------T
NC02-94	51	----------	---T-----C	----------	----------	---------T
980528	51	----------	---T-----C	----------	----------	---------T
N1-5	51	----------	---T------	-----A----	-G--------	---------T
2-90	51	----------	---T------	----------	-G--------	----------

		110	120	130	140	150
S30	101	GAAATGAAAG	GGAATCACTC	TATTTGGATC	ACAGAGGAGA	TCGTACAGCA
WI02-131	101	----------	----C-----	--------G	-----T----	C---G-----
NC02-46	101	----------	----C-----	----------	-----T----	C---G-----
NC02-94	101	----------	----C-----	----------	-----T----	C---G-----
980528	101	----------	----C-----	----------	-----T----	C---G-----
N1-5	101	--------G-	----C-----	----------	-----T----	G------A--
2-90	101	----------	----C-----	----------	-----T----	----------

		160	170	180	190	200
S30	151	TGAGTGTGCA	AAACATATAG	AGGAAGTTGA	AGGAATTATG	TACGGAAATG
WI02-131	151	---A----A-	--G--C----	----------	----------	-----G----
NC02-46	151	---A----A-	--G--C----	----------	----------	-----G----
NC02-94	151	---A----A-	--G--C----	----------	----------	-----G----
980528	151	---A----A-	--G--C----	----------	----------	-----G----
N1-5	151	----------	----------	----------	----------	--T--G----
2-90	151	----------	--G-----C-	-A--------	----------	--T--G---A

		210	220	230	240	250
S30	201	TTCCAAGAGG	GGATGTAATG	TATGCTAACA	ACTTTATTAT	AGATAGACAT
WI02-131	201	C---G-----	-----C-C-A	---AT-----	----------	-----A----
NC02-46	201	C---G-----	-----C---A	---AT-----	----------	-----A----
NC02-94	201	C---G-----	-----C---A	---AT-----	----------	-----A----
980528	201	C---G-----	-----C---A	---AT-----	----------	-----A----
N1-5	201	C---G-----	-----C---A	----TG----	----------	----------
2-90	201	G---G-----	----TC---A	----T---T-	----------	------G---

		260	270	280	290	300
S30	251	CATAGAGTAT	ACAGATTCGG	GGGATCTTGT	CAGATGAAAT	TCTGTAATAA
WI02-131	251	----------	----------	---G------	-GA-------	-----G----
NC02-46	251	----------	----------	---G------	-GA-------	----------
NC02-94	251	----------	----------	---G------	-GA-------	----------
980528	251	----------	----------	---G------	-GA-------	-----G----
N1-5	251	--------G-	----------	----------	----------	-----G----
2-90	251	--------G-	-TC-------	----------	--A-------	----------

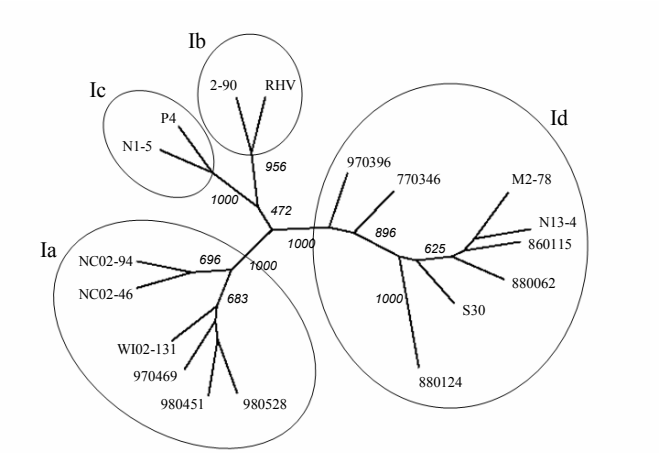
		310	320	330	340	350
S30	301	AGATGGTATA	AAATTCGCGA	GAGGAGACTG	GGTAGAGAAA	AGCCGGAACA
WI02-131	301	----------	------A-A-	----------	------A---	---T-A----
NC02-46	301	----------	------A-A-	----------	------A---	---T-A----
NC02-94	301	----------	------A-A-	----------	------A---	---T-A----
980528	301	----------	------A-A-	----------	------A---	---T-A----
N1-5	301	----------	-----T--A-	----------	----------	---T---G--
2-90	301	----------	--------A-	----------	---------G	---T------

		360	370	380	390	400
S30	351	TTAACAACGA	TATTCATGAC	AATGTGCCTA	AATGTGTTGA	TGGAACGTTG
WI02-131	351	--G--G-A--	----T---CA	---A-A---G	------C---	----------
NC02-46	351	--G--G-AT-	----T---CA	---A-A---G	------C---	----------
NC02-94	351	--G--G-AT-	----T---CA	---A-A---G	------C---	----------
985028	351	--G--G-A--	----T---CA	---A-A---G	------C---	----------
N1-5	351	--G--G--T-	----T--A--	---A-A----	G---------	------T---
2-90	351	----------	----T--A--	-----A----	G-----C---	----------

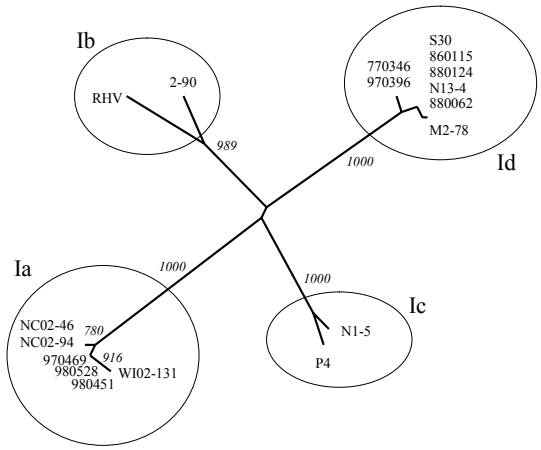
		410	420	
S30	401	GTCTCCGGTC	ACCGCCCCGG	ATTAGA
WI02-131	401	--A--T----	----A--T--	-C----
NC02-46	401	--A--T----	----A--T--	------
NC02-94	401	--A--T----	----A--T--	------
980528	401	--A--T----	----A--T--	-C----
N1-5	401	---A------	----A--T--	------
2-90	401	----------	----A--T--	------

1
2
3

Figure 4. Phylogenetic trees generated by maximum parsimony (A) and Neighbor-Joining (B) analyses of 426 bp partial glycoprotein gene sequences from the North Carolina and Wisconsin isolates and those published previously by Stone et al. (2003). Subgroup Ia contains SVCV cultured from fish originating in Asia (970469, 980528, 980451) as well as Cedar Lake (WI02-131) and North Carolina (NC02-46 and NC02-94) isolates. Subgroup Ib contains isolates from Moldova (RHV and 2-90), Ic isolates from Ukraine (N1-5) and Russia (P4) and Subgroup Id contains isolates from the UK (770346, 970396, M2-78, 880062, N13-4, 880124, 860115) and the European reference (S30).



A.



B.