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First detection of pike fry-like rhabdovirus in barbel and spring viraemia of carp virus in sturgeon and pike in aquaculture in the Czech Republic

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ABSTRACT: Rapid antigen detection enzyme-linked immunosorbent assay (ELISA) testing of cell cultures with organ homogenate from fish, collected from farms with a predominance of common carp or in natural aquaculture in the Czech Republic between 1995 and 2008, identified piscine vesiculovirus in 27 of 178 samples. Using reverse transcription semi-nested PCR, targeting a 550 nucleotide region of the glycoprotein (G) gene, piscine vesiculovirus was confirmed in 23 of the 27 organ samples diagnosed by ELISA as infected. PCR products were amplified and sequenced from 18 isolates from common carp *Cyprinus carpio* (family Cyprinidae), 2 isolates from northern pike *Esox lucius* (family *Esocidae*), and 1 isolate each from Siberian sturgeon *Acipenser baerii* (family *Acipenseridae*), common barbel *Barbus barbus* (family *Cyprinidae*), and koi carp *Cyprinus carpio koi* (family Cyprinidae). The sequences (based on 401 nucleotides) clustered into 2 genogroups. The majority of isolates (n = 22), including those from sturgeon and pike, grouped with the spring viraemia of carp virus (SVCV) Genogroup I and Subgroup Id. The 22 isolates could be further subdivided into 2 groups: Id1 (n = 20) and Id2 (n = 2). A marker (a non-conservative nucleotide substitution) for the Id1 SVCV group was identified. It was specifically found in all sequences of Id1 isolates when testing SVCV originating from different countries. The remaining isolate from barbel, was classified in the pike fry-like rhabdovirus Genogroup IV. This is the first confirmation of natural SVCV infection in sturgeon and pike, and pike fry-like rhabdovirus infection in barbel. In the case of the pike fry-like rhabdovirus, this is also its first identification in the Czech Republic. According to the presence/absence of evident clinical signs of rhabdoviral disease in the 3 infected hosts, only the sturgeon seemed to be susceptible to the monitored rhabdovirus.

KEY WORDS: *Vesiculovirus* · Spring viraemia of carp virus · SVCV · Pike fry-like rhabdovirus · ELISA · RT-PCR · *Cyprinidae* · Barbel · Sturgeon · G gene · Phylogenetic analysis

INTRODUCTION

Piscine rhabdoviruses cause seasonal hemorrhagic infections which result in serious economic losses. Some of the most important piscine rhabdoviruses are classified within the genus *Vesiculovirus* with *Spring viraemia of carp virus* (SVCV) being listed as a type species (http://talk.ictvonline.org/media/p/1214.aspx, accessed 12 February 2010). Members of the genus *Vesiculovirus* have a linear non-segmented single-stranded negative-sense RNA genome consisting of 5 genes in the order 3′ N-P-M-G-L 5′ coding for the viral nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and polymerase, respectively (Wunner & Peters 1991, Coll 1995, Ahne et al. 2002). Phylogenetic analysis of the SVCV-related fish vesiculoviruses reveals 4 distinct genogroups (Stone et al. 2003, Sheppard et al. 2007). Genogroup I comprises all SVCV isolates, Genogroup III comprises a single isolate named pike fry rhabdovirus (PFRV), and Genogroups II and IV include infectious agents originally characterised as pike fry-like rhabdoviruses. Within Genogroup I, the

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isolates have been further classified into 4 subgroups and these subgroups correlate with the geographical origin of isolates. Isolates from Asia cluster into Subgroup Ia, Eastern European isolates are grouped into Subgroups Ib and Ic and Western European isolates mostly form Subgroup Id which also contains the reference strain of spring viraemia of carp S30 (Fijan et al. 1971) from the former Yugoslavia. Further phylogenetic analysis including SVCV isolates from Austria distinguished 3 clusters within the Subgroup Id, designated Id1, Id2 and Id3 (Basic et al. 2009). With respect to the Austrian isolates, the viruses falling within the Id1 cluster were isolated during the period 1994 to 2007, while those found within the Id2 cluster were all detected after 2003. A single Ia isolate was also detected in Austria.

Differentiation of vesiculoviruses is based both on serological and molecular methods. Since piscine vesiculovirus antigenic determinants (e.g. glycoprotein G, the N and M proteins) are closely related, conventional serological methods such as the indirect fluorescent antibody test or ELISA can only detect the virus and do not allow further classification (Jorgensen et al. 1989, Way 1991, Rodak et al. 1993, Ahne et al. 1998, Dixon & Longshaw 2005). For genogroup and subgroup classification, sequencing of 401 to 550 nucleotides (nt) of the G gene is most frequently used (Stone et al. 2003, Garver et al. 2007, Miller et al. 2007, Sheppard et al. 2007, Warg et al. 2007), although an alternative target gene has been tested with similar results (Miller et al. 2007). Molecular differentiation, specifically sequence analysis, of the G gene of aquatic vesiculoviruses is accepted by the World Organization for Animal Health (OIE) (OIE 2010) for the identification of SVCV in cell culture. Genogroup I vesiculoviruses infect mainly common carp *Cyprinus carpio*, and silurid (*Siluridae*) fish in the natural environment, and northern pike *Esox lucius* and roach *Rutilus rutilus* have been infected under experimental conditions (Ahne 1985, Haenen & Davidse 1993). Vesiculoviruses of Genogroups II, III and IV can infect a wide range of cyprinid fish (Ahne 1975, Ahne et al. 1982, Ahne & Thomsen 1986, Haenen & Davidse 1989, Rowley et al. 2001, Way et al. 2003) as well as pike *E. lucius* (Bootsma & van Vorstenbosch 1973, De Kinkelin et al. 1973, Bekesi et al. 1984), the latter being a typical host of PFRV of Genogroup III. The wide range of potentially susceptible fish species indicates that 'non-typical' host species might represent reservoirs of vesiculoviruses in fish aquaculture.

The global trade in fish favours infectious disease transmission. Therefore, the assessment of virus isolate characteristics is important for obtaining better information about current epidemiology and for early adoption of preventive control or eradication measures. As the epidemiological situation in the Czech Republic has not previously been studied by molecular biological means, we classified assumptive piscine vesiculovirus isolates of Czech origin into phylogenetic groups based on genetic characterisation using the glycoprotein G gene, and looked for associations with geographic origin and year.

MATERIALS AND METHODS

Sample collection. From January 1995 to December 2008, 178 samples originating from fish from farms and fisheries in different parts of the Czech Republic were examined for the presence of vesiculovirus using virus isolation in cell cultures (Fig. 1, see Table 1). The infected cell cultures were further analysed by ELISA. Most fish submitted to the laboratory for analysis displayed signs of disease such as haemorrhages, exophthalmia and mortality. Fish species included common carp *Cyprinus carpio,* koi carp *Cyprinus carpio koi*, grass carp *Ctenopharyngodon idella*, common barbel *Barbus barbus,* northern pike *Esox lucius* and Siberian sturgeon *Acipenser baerii*. The samples were pooled organs consisting of kidney, spleen and heart tissue from a maximum of 30 fish or pools of a maximum of 30 whole fish in the case of fry. The organ pools were homogenised in Tris/Eagle's minimum essential medium (MEM), pH 7.6, supplemented with 10% foetal bovine serum (FBS) and 0.1% gentamycin in a tissue to volume ratio of 1:5. After 15 min centrifugation at $1500 \times g$ at 4°C, the supernatant was filtered through a 0.45 µm pore size filter membrane and was used for virus isolation, and for PCR analysis retrospectively.

Virus isolation on cell cultures. For virus propagation, epithelioma papulosum cyprini (EPC) or fathead minnow (FHM) and rainbow trout gonad (RTG) cell lines were inoculated with 1:100, 1:1000 and 1:10000 dilutions of filtered homogenised pools. Two cell lines in 24-well plates were propagated in parallel in Tris MEM supplemented with 10% FBS and standard concentrations of antibiotics for 24 h before infection. Inoculated cell cultures were incubated at 15°C. Cell cultures were collected for virus identification when cytopathic effect (CPE) appeared, usually 4 to 7 d later. The cells were frozen and thawed, the cell debris pelleted and the supernatant used in ELISA. If no CPE was observed after a week, the cultures were frozen, thawed, the cell debris pelleted and the supernatant applied to newly prepared cells. These were cultured for a further 7 d during which development of potential CPE was monitored.

ELISA. To identify potential vesiculovirus inducing CPE in cell cultures, a test kit from TestLine, Brno, based on the sandwich ELISA antigen detection

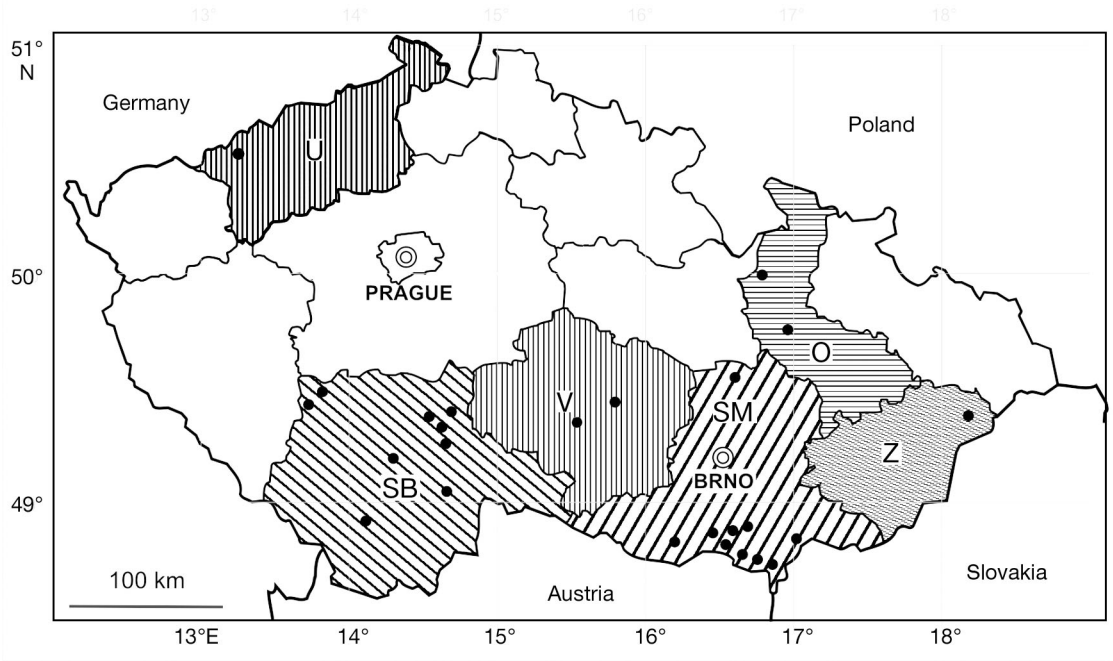


Fig. 1. Localities in the Czech Republic regions with confirmed vesiculovirus isolates (●). O = Olomouc (Id1: 1; nd: 1), SB = South Bohemia (Id1: 8; Id2: 2), SM = South Moravia (Id1: 10; nd: 1), U = Usti (IV: 1), V = Vysocina (Id1: 1), Z = Zlin (nd: 2). The numbers of isolates of characterised genogroups sampled from localities of respective regions are shown in brackets; nd (no data) indicates the number of isolates not classified into genogroups

method (Rodak at al. 1993, Dixon & Longshaw 2005) was applied. Virus identification was based on vesiculovirus specific polyclonal antibodies: swine antibody as the microplate-coating agent and a primary rabbit antibody. To confirm vesiculovirus RNA presence and for subsequent phylogenetic analysis, ELISA-positive cell cultures were further analysed by RT-PCR and PCR products were sequenced.

RNA extraction and polymerase chain reaction. Total RNA used in RT-PCR was obtained from 140 µl of sample (supernatant of homogenised organ material or supernatant of lysed infected cell culture) using RNA-affinity spin columns (QIAamp Viral RNA Kit, Qiagen) according to the manufacturer's instructions. Purified RNA was eluted in 60 µl of RNase-free water containing 0.04 % sodium azide and stored at –20°C. To obtain a primary PCR product, the One Tube RT-PCR System (Roche Diagnostics) was used, according to the manufacturer's instructions, with the primer pair SVCV F1 5′-TCT TGG AGC CAA ATA GCT CAR RTC-3′ and SVCV R2 5′-AGA TGG TAT GGA CCC CAA TAC ATH ACN CAY-3′ (Stone at al. 2003). The reaction mix contained 6 µl of extracted RNA, 20 pmol of each of the primers, 0.2 mM of each of the 4 dNTPs, 5.0 mM of dithiothreitol, 40 U of RNase inhibitor (Promega), 1× RT-PCR buffer (with 1.5 mM $MgCl_2$), 1 µl of enzyme mix (Roche Diagnostics) and RNase-free water up to a reaction volume of 50 µl. Reverse transcription of 30 min at 50°C with an initial denaturation of 2 min 30 s at 94°C was followed by 34 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 68°C, and a final extension step of 7 min at 68°C. To obtain a secondary PCR product for sequencing, semi-nested PCR with the primer pair SVCV F1 and SVCV R4 5′-CTG GGG TTT CCN CCT CAA AGY TGY-3′ was used (Stone at al. 2003). The reaction mix contained 0.5 µl of the primary PCR product, 0.25 µM of each of the primers, 0.25 mM of each of the 4 dNTPs, 1× PCR buffer, 2.5 mM $MgCl_2$, 1.25 U GoTaq DNA Polymerase (Promega), and RNase-free water up to a volume of 50 µl. The reaction was subjected to 25 cycles of 30 s at 94°C, 30 s at 60°C and 30 s at 72°C, followed by a final extension step of 7 min at 72°C. The products were detected by electrophoresis in 2 % agarose gel stained with ethidium bromide. The expected sizes of the primary and secondary PCR products of vesiculovirus were 714 and 606 bp, respectively.

Sequencing and phylogenetic analysis. Secondary amplification products were purified using the QIAquick Purification Kit (Qiagen) and sequenced from both directions using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were aligned using BioEdit version 5.0.9 (Hall 1999) and the alignment proofread and manually edited where the software was considered to have introduced misalignments. The sequences of the 550 bp region of the G gene of Czech isolates were identified as SVCV or pike fry-like rhabdovirus by the Basic Local

Alignment Search Tool (BLAST) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed 22 November 2010). The sequences of 23 isolates (corresponding to nucleotides 3498 to 4047 of GenBank sequence U18101) were deposited in the GenBank with accession numbers FN424053 to FN424075 (see Table 1).

Phylogenetic analysis of the 401 nt sequences (corresponding to nucleotides 3545 to 3945 of GenBank sequence U18101) of newly and previously isolated vesiculoviruses (Björklund et al. 1996, Stone et al. 2003, Garver et al. 2007, Miller et al. 2007, Sheppard et al. 2007, Teng et al. 2007, Warg et al. 2007, Yue et al. 2008, Basic et al. 2009) was done, as this allowed most isolates for which sequence data is available to be included. Phylogenetic analyses were also performed with subsets of the isolates for which 550 nt (nucleotides 3498 to 4047 of GenBank sequence U18101) and 520 nt (nucleotides 3498 to 4017 of GenBank sequence U18101) were available. The analyses were carried out using Clustal X version 2.0 (Thompson et al. 1997) and Mega version 4 (Tamura et al. 2007). The neighbour-joining trees were generated using the p-distance substitution model with a bootstrap test of 1000 replicates. All positions with alignment gaps and missing bases were removed using the pairwise deletion option.

RESULTS

Virus isolation, ELISA identification

Investigations of the 178 fish samples during the study period confirmed virus presence with positive CPE for 24 samples following first passage of the cell culture post inoculation, and for 3 samples after the second passage (data not shown). Piscine vesiculovirus was detected by ELISA in all 27 CPE-positive samples (Table 1).

Sequencing and phylogenetic analysis

Apart from 4 vesiculovirus isolates from the susceptible hosts *Cyprinus carpio* and *Ctenopharyngodon idella*, which were not available for sequence analysis, the remaining 23 piscine vesiculovirus isolates identified by ELISA were characterised molecularly (Table 1).

Based on the 550 nt sequence corresponding to the glycoprotein G gene obtained from each isolate, 22 isolates were identified as SVCV by BLAST analysis, with identities of between 98.0 and 100.0% (0 to 11 nt substitutions over 550 bp) when compared with each other. When each of the 22 SVCV isolates were compared with the sequence of the reference virus SVCV VR-1390 (accession number U18101), 5 to 7 nt substitutions were observed in each isolate, which corresponded to nucleotide identities of between 98.7 and 99.0%. Derived amino acid (AA) sequence identities among the Czech SVCV ranged between 98.9 and 100% (0 to 2 AA substitutions out of 183), and when

Table 1. Piscine vesiculovirus isolates detected in the Czech Republic. Regions in the Czech Republic: O = Olomouc, SB = South Bohemia, SM = South Moravia, U = Usti, V = Vysocina, Z = Zlin. Host species name: *Cyprinus carpio* = common carp, *Cyprinus carpio koi* = koi carp, *Acipenser baerii* = Siberian sturgeon, *Esox lucius* = northern pike, *Barbus barbus* = common barbel, *Ctenopharyngodon idella* = grass carp. Observations: S: symptoms relating to rhabdovirus infection (haemorrhages, exophthalmia); M: mortality; +: infectious haematopoietic necrosis virus; ++: retrovirus-like particles and symptoms of retrovirus infection. nd: no data. Categorisation of isolates into the genogroups was a part of this study

Isolate marker	Year/ region of isolation	Host name	Observations	GenBank accession no.	Genogroup category
V-640	2006/SB	*Cyprinus carpio*		FN424070	Id1
V-637	2006/SB[a]	*Acipenser baerii*	S/M	FN424074	Id2
V-634	2006/SM	*Cyprinus carpio*	S	FN424071	Id1
V-635	2006/SB	*Cyprinus carpio*	M	FN424069	Id1
V-632	2005/SM	*Cyprinus carpio*	S	FN424072	Id1
V-636	2005/SB[a]	*Cyprinus carpio koi*		FN424073	Id2
PI05	2005/SM	*Esox lucius*	+	FN424068	Id1
V-630	2004/U	*Barbus barbus*	++	FN424075	IV
V-612	2002/SM	*Cyprinus carpio*	M	FN424067	Id1
V-609	2001/O	*Cyprinus carpio*	S	FN424057	Id1
PL01	2001/O	*Cyprinus carpio*		nd	nd
V-552	1999/SB	*Cyprinus carpio*		FN424056	Id1
V-555	1999/SB	*Cyprinus carpio*		FN424062	Id1
V-556	1999/SB	*Cyprinus carpio*		FN424064	Id1
V-551	1999/SM[a]	*Cyprinus carpio*	M	FN424061	Id1
V-649	1999/SB	*Cyprinus carpio*		FN424066	Id1
V-613	1999/SB	*Cyprinus carpio*		FN424059	Id1
V-590	1999/SM	*Esox lucius*		FN424058	Id1
V-541	1998/SB	*Cyprinus carpio*		FN424063	Id1
PO98	1998/SM	*Cyprinus carpio*	S/M	nd	nd
V-627	1997/SM	*Cyprinus carpio*	M	FN424055	Id1
V-539	1997/SM[a]	*Cyprinus carpio*	M	FN424060	Id1
VM97	1997/V	*Cyprinus carpio*		FN424065	Id1
V-540	1997/SM	*Cyprinus carpio*	M	FN424054	Id1
V-500	1996/SM	*Cyprinus carpio*	M	FN424053	Id1
VSK95	1995/Z[a]	*Cyprinus carpio*		nd	nd
VSA95	1995/Z[a]	*Ctenopharyngodon idella*		nd	nd

[a]Same locality examined within a particular region

compared with VR-1390, ranged between 98.9 and 100% (0 to 2 AA substitutions out of 183). The latter comparison (with VR-1390) showed the highest and lowest AA identities for the V-636 (V-637) sequence and the V-612 (V-540) sequence, respectively (data not shown). The majority of the 17 different nucleotide substitutions detected in virus isolates from the Czech Republic were in the last two-thirds of the G gene sequence. Only 3 nucleotide changes led to changes in the derived AA sequences, namely in positions 191, 216, and 290 of the reference SVCV VR-1390 G gene sequence. AA changes Val^{191}→Ile and Met^{216}→Ile were present in the isolates V-612 and V-540, respectively. The Val^{191}→Ile AA substitution also occurred in Austrian isolate 122-02 from goldfish, which belongs to Subgroup Id1 (Basic et al. 2009). Of the Subgroup Id isolates, the AA change Met^{216}→Ile was the only substitution unique to a Czech isolate. This substitution was further present in Subgroup a, b, and c member sequences. A Val^{290}→Ile substitution was observed in the AA sequences of 20 of the 22 Czech SVCV isolates studied, with the isolates V-637 and V-636 being the exceptions (data not shown). The Val^{290}→Ile substitution was also seen in all Id1 nucleotide sequences. The nucleotide sequence of isolates V-637 and V-636 was found to be identical to that of Id2 Austrian isolate 38-06 (Basic et al. 2009). The present study did not find any unique nucleotide substitution shared only among the Id Czech vesiculovirus isolates (data not shown).

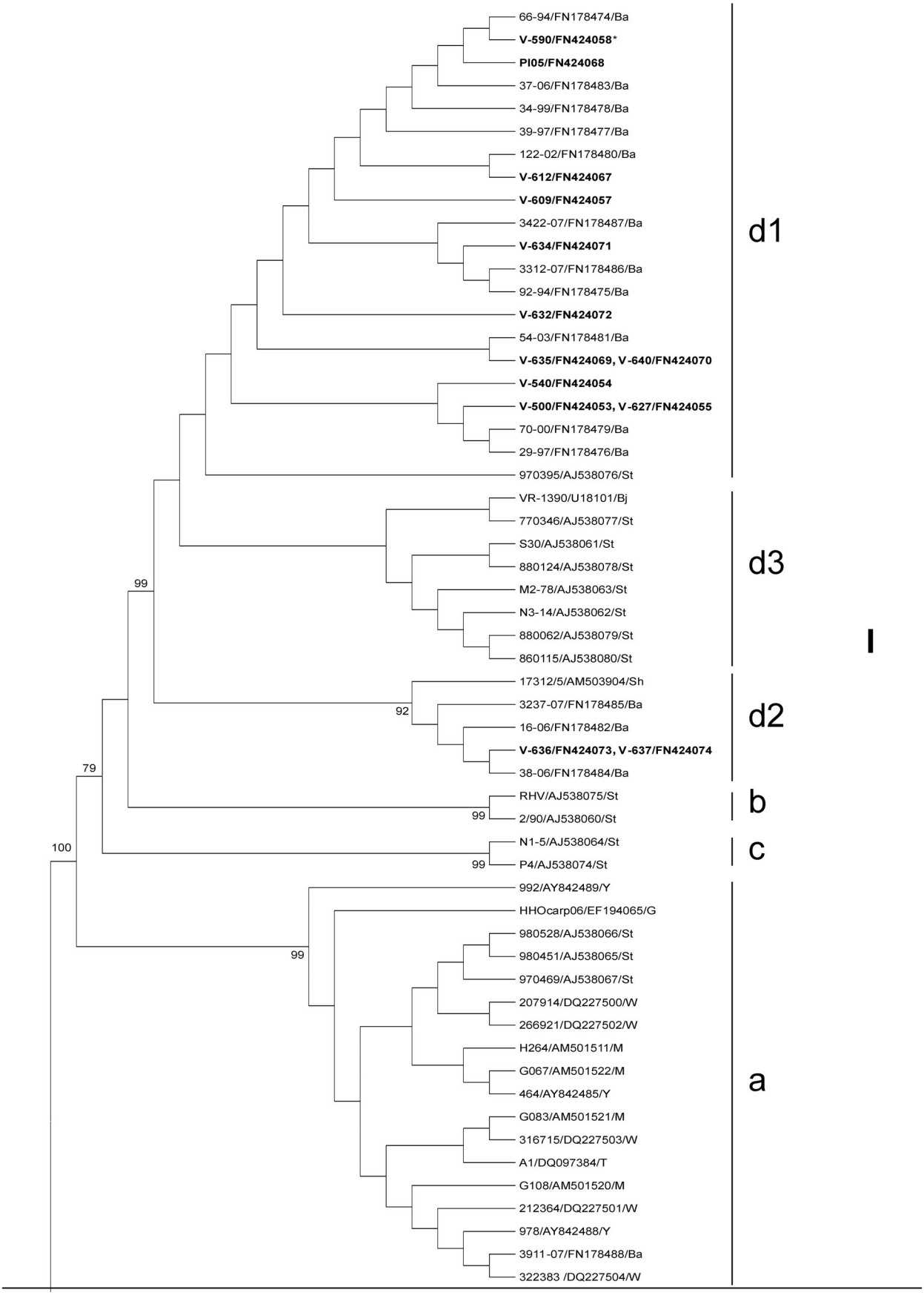
The 550 nt sequence of the G gene of the remaining isolate from barbel (sample V-630) was identified as pike fry-like rhabdovirus and shared nucleotide identities of between 64.1 and 65.0% (193 to 198 nt substitutions in 550 bp) with the sequences of the 22 SVCV isolates, with the highest homology (65.0%) found with isolate V-636 (V-637). Based on a 401 nt sequence comparison, the barbel isolate was determined to be closest to rhabdoviruses: isolates 3605 from grass carp *Ctenopharyngodon idella* (98.7%), 880137 from orfe *Leuciscus idus* and GRV from grass carp *Ctenopharyngodon idella* (98.2%). However, when translated sequences were compared, the barbel isolate was most closely related to rhabdoviruses 98-93 and 994663-2 from brown trout *Salmo trutta* and roach *Rutilus rutilus*, respectively. The barbel rhabdovirus shared AA identities of between 93.9% (isolate 332) and 99.2% (isolates 98-93 and 994663-2) with the Genogroup IV representative isolates, and 78.1 and 90.2% with the Genogroups II (V76) and III (F4) isolates, respectively (data not shown).

Phylogenetic analyses based on the 401 nt, as well as the 550 nt, region of the G gene showed that all Czech vesiculovirus isolates, except for the barbel isolate (V-630), were grouped with the representative SVCV isolates (Björklund et al. 1996, Stone et al. 2003, Garver et al. 2007, Miller et al. 2007, Sheppard et al. 2007, Teng et al. 2007, Warg et al. 2007, Yue et al. 2008, Basic et al. 2009) in Genogroup I (Fig. 2) and therefore should be classified as SVCV. This genogroup was supported by a bootstrap value of 1000. The phylogenetic analyses further clustered the Czech isolates, including the isolate from sturgeon fry (V-637), which was linked with pathological changes and occurrence of mortality in its sturgeon host, into the Subgroup Id, supported by bootstrap values ≥990. The analyses divided the Czech isolates belonging to Subgroup Id, and the representative Id isolates (Björklund et al. 1996, Stone et al. 2003, Sheppard et al. 2007, Basic et al. 2009), into 2 distinct groups: Id1 and Id2 (Fig. 2). Analysis based on the 520 nt region of the G gene supported the Id1 and Id2 groups with bootstrap values of 880 and 990, respectively (data not shown). The remaining isolate V-630 was again assigned to the Genogroup IV based on the 401 nt, as well as the 550 nt, G gene region analyses, supported by bootstrap values ≥990 (Fig. 2). Analyses using the different length nucleotide regions gave the same groupings for those isolates for which the relevant sequence was available.

DISCUSSION

This is the first complete survey of piscine vesiculovirus infection in aquaculture in the Czech Republic based on molecular characterisation of isolates. Regular surveillance testing of asymptomatic fish for vesiculoviruses is not obligatory in the Czech Republic (OIE 2010) and it is not frequently utilised by farmers for preventive health measures. Therefore, fish delivered for laboratory testing are most often those showing clinical signs of a viral disease. Consequently, ELISA (Rodak at al. 1993, Dixon & Longshaw 2005) detected a high portion of positive samples from the overall tested samples (27 of 178) during the study period 1995 to 2008. Virus-infected samples giving a positive result with a polyclonal antibody detecting all genogroups of piscine vesiculoviruses (Rodak at al. 1993, Dixon & Longshaw 2005) were subsequently used for PCR and sequencing of a G gene region, apart from 4 presumed vesiculovirus isolates detected by ELISA which were not further investigated in the present study. Using primers specific to SVCV (Koutná et al. 2003) 1 of these 4 isolates (PL01) was previously detected in a carp host (*Cyprinus carpio*) diagnosed with spring viraemia of carp disease (M. Koutná et al. unpubl. data).

Phylogenetic analysis revealed that 22 of the isolates belonged to SVCV Subgroup Id, which confirmed a common West-Central European origin for the Czech


66-94/FN178474/Ba
V-590/FN424058*
PI05/FN424068
37-06/FN178483/Ba
34-99/FN178478/Ba
39-97/FN178477/Ba
122-02/FN178480/Ba
V-612/FN424067
V-609/FN424057
3422-07/FN178487/Ba
V-634/FN424071
3312-07/FN178486/Ba
92-94/FN178475/Ba
V-632/FN424072
54-03/FN178481/Ba
V-635/FN424069, V-640/FN424070
V-540/FN424054
V-500/FN424053, V-627/FN424055
70-00/FN178479/Ba
29-97/FN178476/Ba
970395/AJ538076/St
VR-1390/U18101/Bj
770346/AJ538077/St
S30/AJ538061/St
880124/AJ538078/St
M2-78/AJ538063/St
N3-14/AJ538062/St
880062/AJ538079/St
860115/AJ538080/St
17312/5/AM503904/Sh
3237-07/FN178485/Ba
16-06/FN178482/Ba
V-636/FN424073, V-637/FN424074
38-06/FN178484/Ba
RHV/AJ538075/St
2/90/AJ538060/St
N1-5/AJ538064/St
P4/AJ538074/St
992/AY842489/Y
HHOcarp06/EF194065/G
980528/AJ538066/St
980451/AJ538065/St
970469/AJ538067/St
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266921/DQ227502/W
H264/AM501511/M
G067/AM501522/M
464/AY842485/Y
G083/AM501521/M
316715/DQ227503/W
A1/DQ097384/T
G108/AM501520/M
212364/DQ227501/W
978/AY842488/Y
3911-07/FN178488/Ba
322383 /DQ227504/W
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I
99
92
79
99
100
99

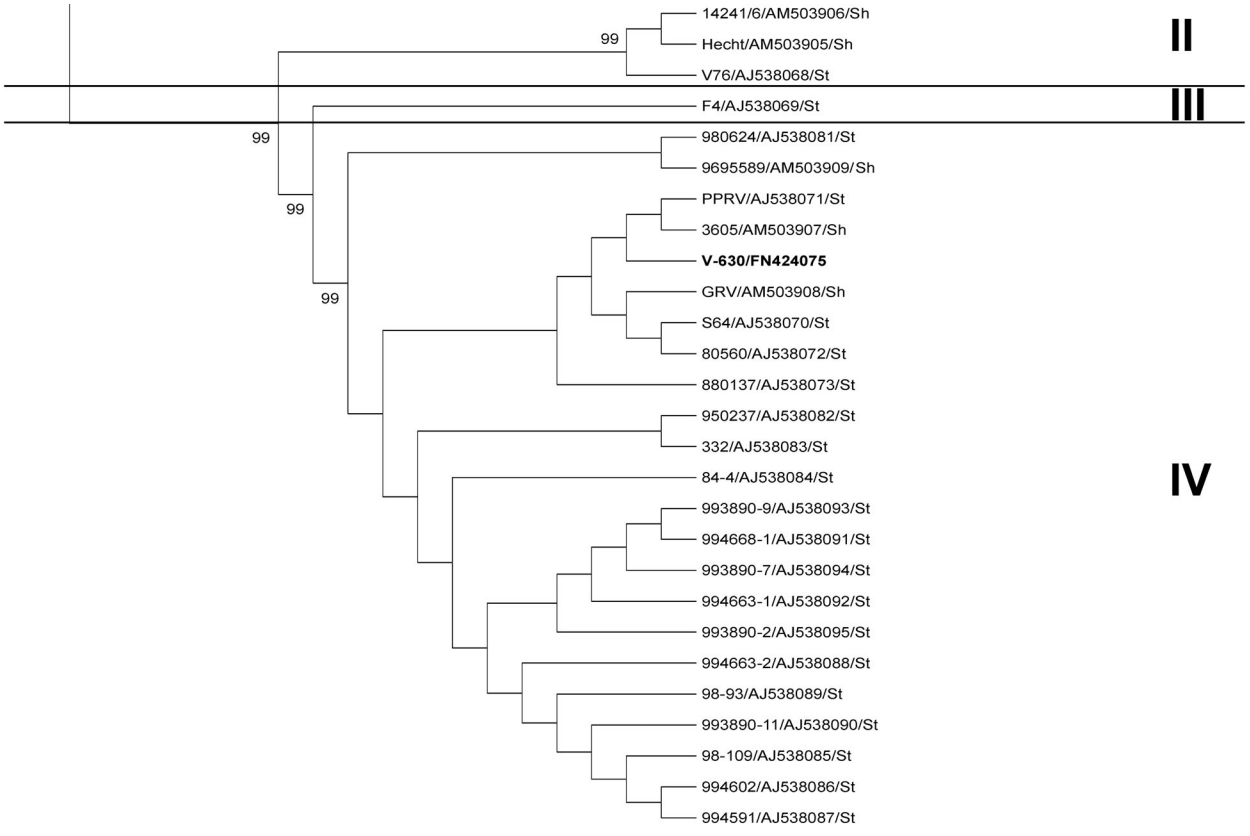

Fig. 2. Phylogenetic tree based on a 401 nt region of the G gene of the 23 Czech isolates, corresponding to the 11 unique sequences, and 72 representative piscine vesiculovirus isolates previously characterised (Bj = Björklund et al. 1996, St = Stone et al. 2003, G = Garver et al. 2007, M = Miller et al. 2007, Sh = Sheppard et al. 2007, T = Teng et al. 2007, W = Warg et al. 2007, Y = Yue et al. 2008, Ba = Basic et al. 2009) showing topology only. Bootstrap values above 70% obtained from 1000 resampled datasets are displayed only beside the main nodes on the tree. Details of the Czech isolate origins (in bold) are presented in Table 1. (•) represents isolates VM97, V-539, V-541, V-613, V-649, V-551, V-556, V-555 and V-552. Genotype I sublineages designated Ia to Id (Stone et al. 2003) and Id1 to Id3 (Basic et al. 2009) were confirmed by significant bootstrap values (>70%) in phylogenetic analyses based on 550 nt (nucleotides 3498 to 4047 of GenBank sequence U18101) and 520 nt (nucleotides 3498 to 4017 of GenBank sequence U18101) regions, respectively, of the G gene of isolates of which the relevant region was available (bootstrap data in 'Results'). The neighbour-joining distance analyses were performed using the p-distance substitution model (Mega software; Tamura et al. 2007). Gaps and missing bases were omitted using pairwise deletion

isolates. The Id isolates were mostly disseminated in 2 southern regions of the Czech Republic (Fig. 1) where mass production of common carp occurs. However, in contrast to neighbouring Austria (Basic et al. 2009), no isolates belonging to other subgroups were observed in the Czech Republic. The majority of the Id isolates originating from most of the Czech Republic regions sampled (Fig. 1) were found to be the Id1 virus type. Only 2 isolates of the Id2 type were detected in the Czech Republic, whereas Basic et al. (2009) found 7 Id2 isolates from widespread regions within Austria. These 2 identical isolates (V-636 and V-637 from koi carp and sturgeon), based on their G gene region, were sampled from the same site in South Bohemia in 2005 and 2006. There may be a link between the 2 Czech isolates and 7 Austrian isolates of the same SVCV type, which were sampled at about the same time in neighbouring regions of both countries. As these regions belong to different river catchment areas, a trade in fish might be a probable cause of this observation.

Three nucleotide substitutions were observed in the investigated G gene segment of certain Czech SVCV isolates. These substitutions resulted in replacements of AA by others of similar chemical properties, determining the biological activity of the protein. From the data, it can be inferred that no change in infectivity for host cells relating to potential changes in the viral glycoprotein G conformation occurred. Another observation on the G gene segment data was made in our study: based on the 550 nt sequences of SVCV isolates included in the phylogenetic analysis, the Val^{290}→Ile AA substitution (deduced from G→A substitution in nucleotide position 3961 of the VR-1390 G gene) was only detected in Id1 isolate sequences (data not shown)

and therefore could be considered as a marker for this SVCV type.

Sequence analyses of the same region of the G gene from the Czech Republic isolates that grouped with West-Central European SVCV in the present study, and from recent United States (US) isolates that were classified in Asian SVCV Subgroup Ia (Warg et al. 2007), reveal high nucleotide homology among the Czech Republic isolates and a high sequence homology among the US isolates. As expected, Stone et al. (2003), who compared the same gene region of SVCV isolates collected over a large time frame and from a wide geographic range found higher levels of nucleotide sequence diversity. Their data suggest that SVCV subgroups have evolved independently in distant geographical areas. Nucleotide sequence identity of the US (Warg et al. 2007) and the Czech SVCV isolates with the reference SVCV strain, representative of the West-European SVCV tree branch, was higher among the latter. At the AA level, the identities among the Czech and the US SVCV (Warg et al. 2007) showed high and almost identical levels.

To the best of our knowledge, the present study is the first confirmed report of SVCV rhabdovirus in pike (family *Esocidae*) under natural conditions. In addition, this is also the first confirmed report of the virus in sturgeon (family *Acipenseridae*), moreover with clinical signs of disease, which included internal haemorrhages, considerably enlarged, bright red spleen, and greyish-yellow liver showing tiny red spots. The sturgeon isolate was genetically identical, defined by the G gene segment, to the isolate from clinically healthy koi carp collected from the same aquaculture site. SVCV isolate PI05 had one nucleotide substitution unique among SVCV isolates (at position 3501 relative to GenBank U18101), while isolate V-590 was identical over the 550 nt of its G gene segment with 9 other SVCV isolates (see Fig. 2 for list of relevant isolates). Of the 2 newly detected naturally infected hosts, SVCV appeared to be pathogenic only for sturgeon as the infected pike did not show any signs of disease.

A single isolate from the cyprinid fish barbel was assigned to Genogroup IV (comprising pike fry-like rhabdoviruses), and this is the first confirmed report of the identification of pike fry-like rhabdovirus in the Czech Republic. In further support of this single finding, among the ELISA-positive organ samples from fish delivered to the laboratory since 1999, the barbel isolate was the only one that could not be amplified in 2-round PCR with primers specific for SVCV (Koutná et al. 2003) (data not shown). Historically, pike fry-like rhabdovirus has mainly been isolated from pike and a number of other cyprinid species (Ahne 1975, Ahne et al. 1982, Ahne & Thomsen 1986, Haenen & Davidse 1989, Rowley et al. 2001, Way et al. 2003), but not from barbel. Retrovirus-like particles and obvious retrovirus-specific pathological changes were found in the barbel but not rhabdovirus-specific symptoms. As such the susceptibility of barbel to fish rhabdovirus remains unclear.

Sequence analysis of the 401 nt region of the G gene indicated a high (≥93.7%) degree of identity between the investigated barbel isolate and the previously isolated Genogroup IV rhabdoviruses (Stone et al. 2003, Sheppard et al. 2007), although identity among all these isolates varied from 93.2 to 100%. In contrast, identity between these isolates and the PFRV isolate F4 (Genogroup III) was <83.4%, while identity to the grass carp rhabdovirus isolate V76 (Genogroup II) was even lower (<72.6%). This confirmed high sequence homology among the Genogroup IV isolates and clearly supported the categorization of the barbel isolate.

In conclusion, the Czech vesiculovirus isolates, with emphasis on isolates from sturgeon and pike, were identified by phylogenetic analysis as SVCV, with one exception; a pike fry-like rhabdovirus, the first identified in the Czech Republic. It was isolated from the cyprinid fish barbel. Based on the presence or absence of pathological signs, we suggest that pike and barbel are vectors of the investigated rhabdoviral agents and that sturgeon is a fish species potentially susceptible to SVCV. Furthermore, based on the additional sequence data provided by this study, which expands the range of the geographic origin of characterised SVCV isolates, one nucleotide (deduced AA) substitution was found to be unique in Id1 isolates and may represent a marker for this subgroup.

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LITERATURE CITED

Ahne W (1975) A rhabdovirus isolated from grass carp (*Ctenopharyngodon idella* Val.). Arch Virol 48:181–185

Ahne W (1985) Viral infection cycles in pike (*Esox lucius* L.). J Appl Ichthyol 1:90–91

Ahne W, Thomsen I (1986) Isolation of pike fry rhabdovirus from *Pseudorasbora parva* (Temminck & Schlegel). J Fish Dis 9:555–556

Ahne W, Mahnel H, Steinhagen P (1982) Isolation of pike fry rhabdovirus from tench, *Tinca tinca* L., and white bream, *Blicca bjoerkna* (L.). J Fish Dis 5:535–537

Ahne W, Kurath G, Winton JR (1998) A ribonuclease protection assay can distinguish spring viremia of carp virus from pike fry rhabdovirus. Bull Eur Assoc Fish Pathol 18: 220–224

Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR (2002) Spring viremia of carp (SVC). Dis Aquat Org 52:261–272

Basic A, Schachner O, Bilic I, Hess M (2009) Phylogenetic analysis of spring viraemia of carp virus isolates from Austria indicates the existence of at least two subgroups within genogroup Id. Dis Aquat Org 85:31–40

Bekesi L, Majoros G, Szabo E (1984) Mass appearance of a rhabdovirus in pike fry (*Esox lucius* L.) in Hungary. Magy Allatorv Lapja 39:231–234

Björklund HV, Higman KH, Kurath G (1996) The glycoprotein genes and gene junctions of the fish rhabdoviruses spring viremia of carp virus and hirame rhabdovirus: analysis of relationships with other rhabdoviruses. Virus Res 42: 65–80

Bootsma R, van Vorstenbosch CJAHV (1973) Detection of a bullet-shaped virus in kidney sections of pike fry (*Esox lucius* L.) with red-disease. Neth J Vet Sci 98:86–90

Coll JM (1995) The glycoprotein G of rhabdoviruses. Arch Virol 140:827–851

De Kinkelin P, Galimard B, Bootsma R (1973) Isolation and identification of the causative agent of 'red disease' of pike (*Esox lucius* L., 1766). Nature 241:465–467

Dixon PF, Longshaw CB (2005) Assessment of commercial test kits for identification of spring viraemia of carp virus. Dis Aquat Org 67:25–29

Fijan N, Petrinec Z, Sulimanovic D, Zwillenberg LO (1971) Isolation of the viral causative agent from the acute form of infectious dropsy of carp. Vet Arch 41:125–138

Garver KA, Dwilow AG, Richard J, Booth TF, Beniac DR, Souter BW (2007) First detection and confirmation of spring viraemia of carp virus in common carp, *Cyprinus carpio* L., from Hamilton Harbour, Lake Ontario, Canada. J Fish Dis 30:665–671

Haenen OLM, Davidse A (1989) Isolation of pike fry rhabdovirus from roach (*Rutilus rutilus*). Bull Eur Assoc Fish Pathol 9:116

Haenen OLM, Davidse A (1993) Comparative pathogenicity of two strains of pike fry rhabdovirus and spring viremia of carp virus for young roach, common carp, grass carp and rainbow trout. Dis Aquat Org 15:87–92

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Jorgensen PEV, Olesen NJ, Ahne W, Lorenzen N (1989) SVCV and PFR viruses: serological examination of 22 isolates indicates close relationship between the two fish rhabdoviruses. In: Ahne W (ed) Viruses of lower vertebrates. Springer-Verlag, Berlin, p 349–366

Koutná M, Veselý T, Pšikal I, Hůlová J (2003) Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. Dis Aquat Org 55:229–235

Miller O, Fuller FJ, Gebreyes WA, Lewbart GA and others (2007) Phylogenetic analysis of spring viremia of carp virus reveals distinct subgroups with common origins for recent isolates in North America and the UK. Dis Aquat Org 76:193–204

OIE (World Animal Health Organization) (2010) Aquatic animal health code. OIE Aquatic Animals Commission, Paris. Available at www.oie.int/international-standard-setting/aquatic-code/

Rodak L, Pospisil Z, Tomanek J, Vesely T, Obr T, Valicek L (1993) Enzyme-linked immunosorbent assay (ELISA) for the detection of spring viraemia of carp virus (SVCV) in tissue homogenates of the carp, *Cyprinus carpio.* J Fish Dis 16:101–111

Rowley H, Graham DA, Campbell S, Way K, Stone DM, Curran WL, Bryson DG (2001) Isolation and characterisation of rhabdovirus from wild common bream *Abramis brama*, roach *Rutilus rutilus*, farmed brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* in Northern Ireland. Dis Aquat Org 48:7–15

Sheppard AM, Le Deuff RM, Martin PD, Woolford G, Way K, Stone DM (2007) Genotyping spring viraemia of carp virus and other piscine vesiculo-like viruses using reverse hybridisation. Dis Aquat Org 76:163–168

Stone DM, Ahne W, Denham KL, Dixon PF and others (2003) Nucleotide sequence analysis of the glycoprotein gene of putative spring viraemia of carp virus and pike fry rhabdovirus isolates reveals four genogroups. Dis Aquat Org 53: 203–210

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Teng Y, Liu H, Lv JQ, Fan WH, Zhang QY, Qin QW (2007) Characterization of complete genome sequence of the spring viremia of carp virus isolated from common carp (*Cyprinus carpio*) in China. Arch Virol 152:1457–1465

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Warg JV, Dikkeboom AL, Goodwin AE, Snekvik K, Whitney J (2007) Comparison of multiple genes of spring viremia of carp viruses isolates in the United States. Virus Genes 35:87–95

Way K (1991) Rapid detection of SVC virus antigen in infected cell cultures and clinically diseased carp by the enzyme-linked immunosorbent assay (ELISA). J Appl Ichthyology 7:95–107

Way K, Bark SJ, Longshaw CB, Denham KL and others (2003) Isolation of a rhabdovirus during outbreaks of disease in cyprinid fish species at fishery sites in England. Dis Aquat Org 57:43–50

Wunner WH, Peters D (1991) Family *Rhabdoviridae*. Arch Virol Suppl 2:250–262

Yue Z, Teng Y, Liang C, Xie X and others (2008) Development of a sensitive and quantitative assay for spring viremia of carp virus based on real-time RT-PCR. J Virol Methods 152:43–48

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