

THE DETECTION AND DIFFERENTIATION OF PISCINE VESICULO-LIKE VIRUSES USING AN RT-PCR REVERSE HYBRIDISATION TECHNIQUE

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Introduction

Spring viraemia of carp (SVC) is a severe haemorrhagic rhabdovirus disease of cyprinids and is notifiable to the Office International des Epizooties (OIE). SVCV infected fish are subject to movement restrictions. Antigenically related viruses that are currently non-notifiable have also been isolated from a number of other cyprinid species including grass carp (*Ctenopharyngodon idella*), tench (*Tinca tinca*), roach (*Rutilus rutilus*) and common bream (*Abramis brama*), and also from pike (*Esox lucius*). Phylogenetic analysis of a 550bp region of the glycoprotein gene has led to the grouping of SVCV and related viruses into four genogroups (Figure 1). Genogroup I the *Rhabdovirus carp* group, comprising all of the isolates classified by serology as SVCV. Genogroup II the Grass carp rhabdovirus group comprising a single isolate, previously identified as PFRV. Genogroup III, the Pike fry rhabdovirus group comprising of the original PFR isolate. Genogroup IV the Tench rhabdovirus group comprising the non-SVCV-non-PFR isolates (including some previously identified as PFR by serology) (Stone et al. submitted). It is often difficult however, to distinguish between these isolates using the serological assays currently recommended by the OIE (Ahne et al. 1998, Way et al. 1998, Rowley et al. 2001), raising concerns over the accurate identification of SVCV and the reliability of some SVC diagnoses. It is important therefore to develop methods robust enough to distinguish between SVCV and other fish rhabdoviruses. A reverse hybridisation technique modelled on a commercially available line probe assay (Innogenetics) is under development. Amplicons generated in a multiplex RT-PCR reaction are captured using immobilised genogroup specific probes and detected using a BCIP/NBT alkaline phosphatase substrate kit (BIO-RAD).

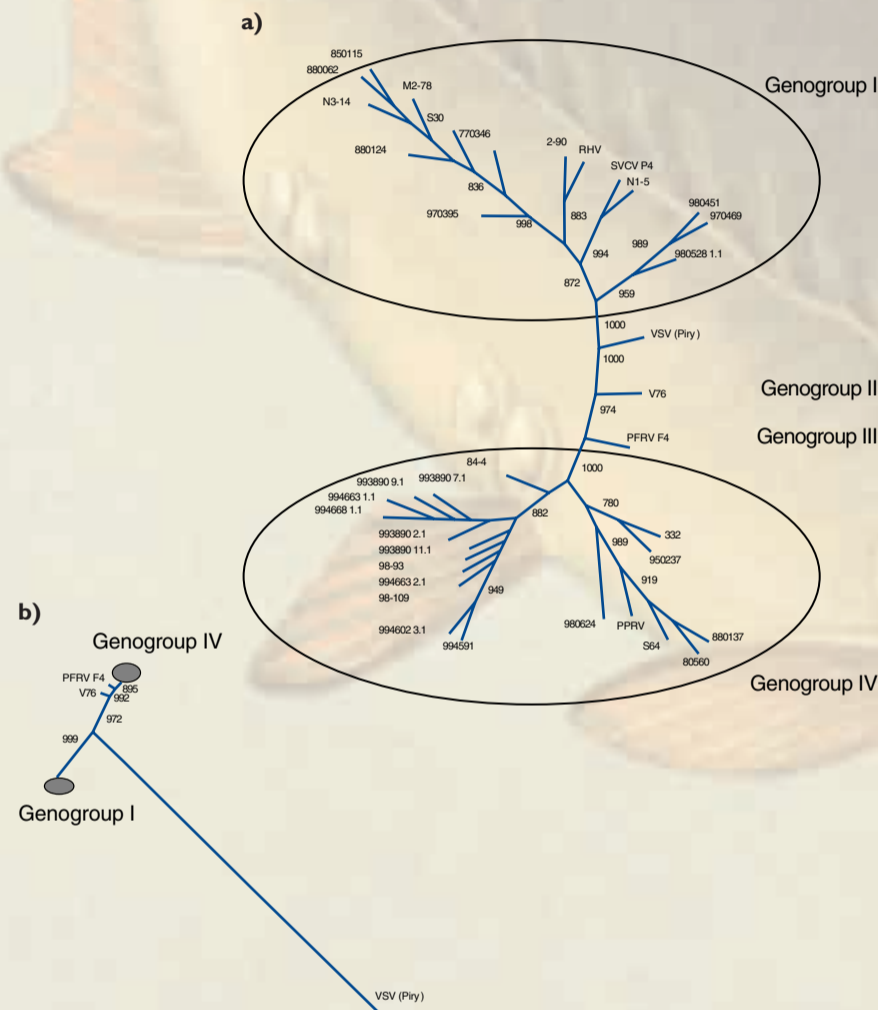


Figure 1a and 1b: Phylogenetic trees generated by maximum parsimony (A) and Neighbor-joining (B) analyses of thirty seven 550bp partial glycoprotein gene sequences from putative SVCV and PFRV isolates. The corresponding sequence of VSV Piry strain (GenBank accession no. D26175) was used as an outgroup. The circled regions in panel A represent the genogroups I and IV, and the shaded areas in panel B represent the same subgroups, that clustered in the Neighbor-joining analysis with branch lengths too short for individual labelling. Analyses were done on 1000 bootstrapped data sets and values >700 are shown on the trees. 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.

Materials and methods

Hybridisation (Figure 2)

Genotype specific PCR amplicons were used as capture probes. These were denatured before being dotted onto a nylon membrane using slot blot apparatus. The membrane was then fixed by baking at 95°C for 30 min. The membrane was cut to size and each blot was put in a small hybridisation tube containing pre-hybridisation buffer (10x Denhart's solution, 2x SSC, 1% SDS, 0.1 mg/mL SSS DNA (5'-3' Inc.)) and incubated at 55°C for 30 min. PCR products generated using test samples were labelled with biotin using BIOPRIME DNA labelling system according to manufacturers instructions (Invitrogen). 200ng of the labelled products were added to hybridisation buffer and membranes were hybridised at 55°C for 1 hour. A series of washes were performed with 0.1% SDS and 0.1x SSC. The hybridised product was then detected using Alkaline Phosphatase Conjugate Substrate Kit (BIO-RAD).

Table 1: Rhabdovirus isolates included in the studies.

Isolate identifier	Host name	Year of isolation	Country of isolation
2/90	Common Carp (<i>Cyprinus carpio</i>)	1990	Moldova
332	White Bream	1981	Germany
770346	Common Carp	1977	U.K.
80560	Roach	1983	The Netherlands
84/4	Brown Trout	1984	U.K. (N. Ireland)
860115	Common Carp	1986	U.K.
880062	Common Carp	1988	U.K.
880124	Common Carp	1988	U.K.
880137	Orfe or Ide	1988	U.K.
950237	Tench	1995	U.K.
970395	Common Carp	1997	U.K.
970469	Common Carp	1997	U.K.
980624	Orfe	1998	U.K.
98-109	Bream	1998	U.K. (N. Ireland)
980451	Common carp	1998	U.K.*
980528 1.1	Common carp	1998	U.K.*
98-93	Brown trout	1998	U.K. (N. Ireland)
983890 2.1	Bream	1999	U.K.
983890 7.1	Grass Carp	1999	U.K.
983890 9.1	Bream	1999	U.K.
983890 11.1	Roach	1999	U.K.
994591	Tench	1999	U.K.
994602 3.1	Crucian Carp	1999	U.K.
994663 1.1	Bream	1999	U.K.
994663 2.1	Roach	1999	U.K.
994668 1.1	Bream	1999	U.K.
M2-78	Silver Carp (<i>Hypophthalmichthys molitrix</i>)	1983	Moldova
N1-5	Bighead Carp (<i>Aristichthys nobilis</i>)	1986	Ukraine
N3-14	Grass Carp	1986	Ukraine
P4	Common Carp	1983	Russia
PFRV F4	Pike	1973	France
PPRV	False Harlequin	1986	Germany
RHV	Rainbow trout (<i>Oncorhynchus mykiss</i>)	1989	Ukraine
S 30	Common Carp	1971	Yugoslavia
S 64	Tench	1982	Germany
V 76	Grass Carp	1982	Germany

* Viruses were isolated during routine import checks on fish originating from China

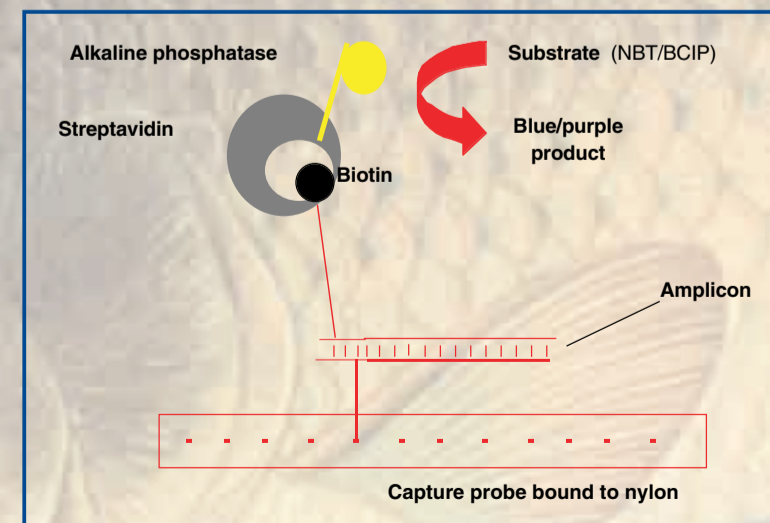


Figure 2: Schematic showing the principles of reverse hybridisation.

Extraction and RT-PCR

A simple virus extraction and amplification method has been described previously (Strømmen & Stone, 1998). Total RNA was extracted from tissue culture supernatant using the Trizol™ reagent (Gibco BRL). Reverse transcription was performed in a 20µl reaction containing 5x reaction buffer (20µM Tris-HCl (pH 7.5 at 25°C), 0.2M NaCl, 0.1mM EDTA, 1mM DTT, 0.01% Nonidet® P-40 and 50% glycerol), 200U reverse transcriptase (Promega), 40U RNasin® ribonuclease inhibitor (Promega) and 100pmol of reverse primer mix (primer locations highlighted in Figure 3) and incubated at 37°C for an hour. PCR was performed in a 50µl reaction containing 2.5µl of the reverse transcription reaction, 100pmol of primer mix (forward and reverse) and 2.5 units of Red Hot DNA polymerase (ABGene). The reaction was overlaid with mineral oil and subjected to 35 cycles of: 1 min at 95°C, 1 min at 55°C and 1 min at 72°C followed by a final extension step of 10 min at 72°C.

Results

- A wide range of putative SVCV and PFRV isolates were used in this study (Table 1). Products of the expected size were obtained for all test isolates (Figure 4).
- The preliminary results of the reverse hybridisation test are presented in Figure 5.
- Although excessive cross reactivity was observed when using the genogroup-specific probes under relatively low stringency conditions (0.1xSSC and 55°C), a stronger signal was obtained when using the appropriate capture probe/PCR product combination.

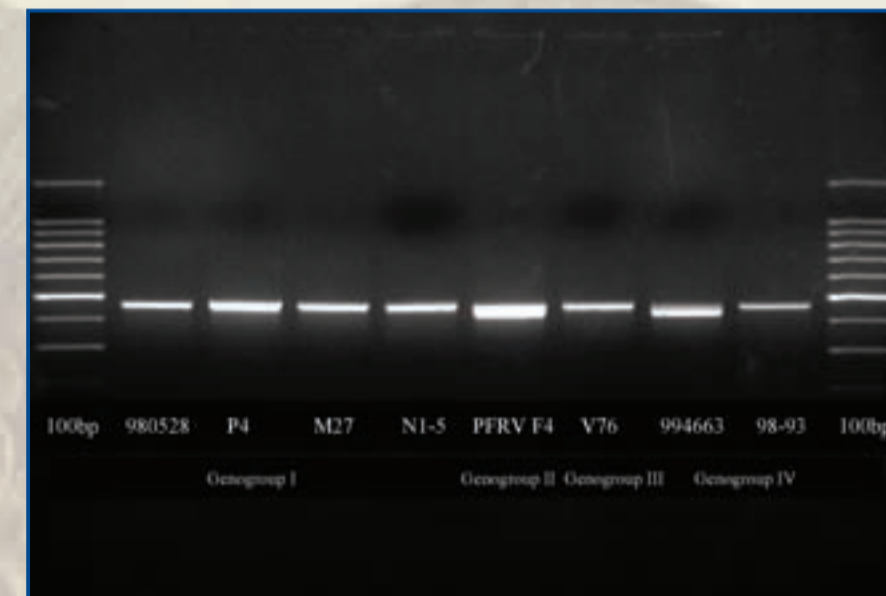


Figure 4: 2% agarose gel demonstrating the broad range of viruses in the vesiculovirus grouping that can be amplified using the designed primer sets. Including three group I isolates from different geographical locations, China, Russia and the UK. Two group IV isolates from the UK and Northern Ireland.

Isolate	Primer	Position	Sequence
V76-1	1	10	TTCACTAACC
SVCVP-1	1	10	ATCTGTGACA
PFRF4-1	1	10	CTCACTGACA
98-93	1	10	CTCACTGACA
V76-1	51	60	TTCACTGACA
SVCVP-1	51	60	TTCACTGACA
PFRF4-1	51	60	TTCACTGACA
98-93	51	60	TTCACTGACA
V76-1	101	110	GGAGAGTGCA
SVCVP-1	101	110	GGAGAGTGCA
PFRF4-1	101	110	GGAGAGTGCA
98-93	101	110	GGAGAGTGCA
V76-1	151	160	GCTCAGTATG
SVCVP-1	151	160	GCTCAGTATG
PFRF4-1	151	160	GCTCAGTATG
98-93	151	160	GCTCAGTATG
V76-1	201	210	TGACAGGGGT
SVCVP-1	201	210	TGACAGGGGT
PFRF4-1	201	210	TGACAGGGGT
98-93	201	210	TGACAGGGGT
V76-1	251	260	AATAACTTCA
SVCVP-1	251	260	AATAACTTCA
PFRF4-1	251	260	AATAACTTCA
98-93	251	260	AATAACTTCA
V76-1	301	310	CTGTGCAATG
SVCVP-1	301	310	CTGTGCAATG
PFRF4-1	301	310	CTGTGCAATG
98-93	301	310	CTGTGCAATG
V76-1	351	360	ATTGGATAGA
SVCVP-1	351	360	ATTGGATAGA
PFRF4-1	351	360	ATTGGATAGA
98-93	351	360	ATTGGATAGA
V76-1	401	410	CCAACTGTG
SVCVP-1	401	410	CCAACTGTG
PFRF4-1	401	410	CCAACTGTG
98-93	401	410	CCAACTGTG
V76-1	451	460	TTGTATGGT
SVCVP-1	451	460	TTGTATGGT
PFRF4-1	451	460	TTGTATGGT
98-93	451	460	TTGTATGGT
V76-1	501	510	GCGAGGGAA
SVCVP-1	501	510	GCGAGGGAA
PFRF4-1	501	510	GCGAGGGAA
98-93	501	510	GCGAGGGAA

Figure 3: Alignment of 550bp region of the glycoprotein gene of representatives of the four vesiculovirus genogroups highlighting the location of the species specific primers.

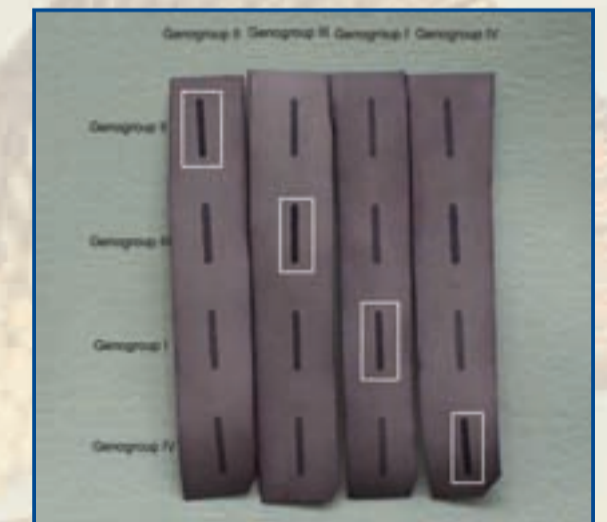


Figure 5: Early stages in the slot blot protocol development. Slots outlined in white indicate the correct capture probe/PCR product combination.

Acknowledgements

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References

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