

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

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ABSTRACT: Rhabdovirus was isolated from wild common bream *Abramis brama* during a disease outbreak with high mortality in Northern Ireland during May 1998. Rhabdovirus was also isolated at the same time from healthy farmed rainbow *Oncorhynchus mykiss* and brown trout *Salmo trutta* on the same stretch of river and 11 mo later from healthy wild bream and roach *Rutilus rutilus* in the same river system. Experimental intra-peritoneal infection of bream and mirror carp *Cyprinus carpio* var *specularis* with 2 of these isolates produced low mortality rates of $\leq 12\%$. Serological testing of these isolates by virus neutralisation indicated that they were antigenically closely related to pike fry rhabdovirus (PFRV) but not to spring viraemia of carp virus (SVCV), while testing by enzyme-linked immunosorbent assay indicated them to be antigenically different from both. Comparison of nucleotide sequence data of a 550 base pair segment of the viral glycoprotein generated by reverse transcription-polymerase chain reaction indicated a high ($\geq 96.6\%$) degree of similarity between these isolates and a previous Northern Ireland isolate made in 1984, a 1997 isolate from bream in the Republic of Ireland and an earlier Dutch isolate from roach. In contrast, similarity between these isolates and PFRV was $< 82.4\%$, indicating that these viruses belong to 2 distinct genogroups, while similarity to SVCV was even lower ($< 67.4\%$).

KEY WORDS: Rhabdovirus · Pike fry rhabdovirus · Sequence analysis · Genogroups · Fish · Cyprinids

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INTRODUCTION

Two subgroups of rhabdoviruses (family *Rhabdoviridae*) are known to infect fish (Walker et al. 2000). The first of these subgroups, provisionally assigned to the genus *Vesiculovirus*, contains spring viraemia of carp virus (SVCV) in addition to pike fry rhabdovirus (PFRV), grass carp rhabdovirus, eel virus American

(EVA) and ulcerative disease rhabdovirus (UDRV). The second subgroup, within the genus *Novirhabdovirus*, contains infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV) and hirame rhabdovirus (HIRRV). A number of other isolates, including eel virus B12 (EEV-B12), eel virus C26 (EEV-C26) and snakehead rhabdovirus (SHRV), have also been provisionally assigned to this genus. A number of these viruses are important fish pathogens, with SVCV, IHNV and VHSV being notifiable, both to

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the Office International des Épizooties (OIE) and under local legislation (Fish Health Regulations [Northern Ireland] 1998). Within the European Union VHSV and IHNV are list II pathogens and SVCV list III (Annex A, Council Directive 91/67/EEC).

It is therefore important that rhabdoviral isolates be accurately identified. This is typically performed by cross-neutralisation testing, although ELISA-based methods have also been used (Mourton et al. 1990, Way 1991). However, the need for improved methods of virus identification, particularly those based on molecular techniques such as reverse transcription-polymerase chain reaction (RT-PCR), has been recognised (Fijan 1999).

PFRV is the causative agent of red disease, an acute condition characterised by haemorrhagic lesions on the trunk, ascites and high rates of mortality, primarily in the fry of northern pike *Esox lucius* L. 1766 (de Kinkelin et al. 1973, Fijan 1999). Rhabdovirus, identified as PFRV by neutralisation testing, have also been isolated from other species including grass carp *Ctenopharyngodon idella* Val. (Ahne 1975), tench *Tinca tinca* L., white bream *Blicca bjoerkna* L. (Ahne et al. 1982), top-mouth gudgeon *Pseudorasbora parva* (Ahne & Thomsen 1986), brown trout *Salmo trutta* (Adair & McLoughlin 1986), roach *Rutilus rutilus* (Haenen & Davidsen 1989) and sheatfish *Silurus glanis* (Jorgensen et al. 1989).

In 1998, a rhabdovirus was isolated from wild common bream *Abramis brama* during a disease outbreak with high mortality in Northern Ireland (NI). Antigenically indistinguishable viruses were isolated from healthy farmed brown trout and rainbow trout *Oncorhynchus mykiss* during this outbreak and from healthy wild common bream and roach in the same river system 11 mo later. This paper describes the isolation and characterisation of these isolates using antigenic and molecular techniques, and suggests for the first time a division of PFRV-like isolates into 2 genogroups based on partial glycoprotein sequence data.

MATERIAL AND METHODS

Clinical material. Case 1: In May 1998, anglers reported high mortalities in juvenile common bream (10 to 14 cm long) in the lower section of the River Bann, which flows north from Lough Neagh in NI. The estimated eventual total mortality was in the region of 20 000. The Fisheries Conservancy Board submitted 2 samples of dead bream ($n = 14$, reference F98-94; $n = 18$, F98-109) to the Fish Disease Unit for examination over a 4 wk period. On the same day that the first bream samples were submitted, 15 brown trout and 15 rainbow trout, all clinically normal, were submitted from a fish farm drawing water from the Lower Bann (F98-93). At this time, dead bream

were present at the water inlet from the river. Data collected at a monitoring station just upstream from the fish farm showed that water temperatures during this period rose from 11 to 13.5°C.

Case 2: In April 1999, 7 wild bream, 3 roach and 1 hybrid fish caught in Lough Neagh, from which the Lower River Bann flows, were submitted for testing before export (F99-57). All fish were clinically normal.

Bacteriological and virological examination. Kidney swabs from diseased and healthy fish were inoculated onto tryptone-soya agar. From each submission, multiple pools of kidney, spleen and brain were ground with sterile sand in Eagle's minimum essential medium (MEM[E]) containing Earle's salts and sodium bicarbonate (2.2 g l^{-1}), supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 500 IU ml^{-1} penicillin, 500 $\mu\text{g ml}^{-1}$ streptomycin, 1.25 $\mu\text{g ml}^{-1}$ amphotericin B, 0.01 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid) buffer and 2% v/v foetal calf serum (Gibco-BRL, Paisley, UK), and centrifuged at $2500 \times g$ for 20 min. Duplicate 100 μl volumes of each pool, at dilutions of 1/20 and 1/50 or 1/100 in MEM(E), were adsorbed onto chinook salmon embryo (CHSE-214) and *Epithelioma papulosum carpio* (EPC) cells in a 24 well plate for 1 h. One millilitre of MEM(E) was then added to each well and plates were incubated at 15°C in 3% carbon dioxide. In addition, material from Case 1 was inoculated onto bluegill fry (BF-2) cells. In the absence of a visible cytopathic effect (CPE) after 7 d, samples were subjected to 1 freeze thaw cycle and given 1 or 2 further 7 d passages without further dilution and also diluted 1/10 in MEM(E). When a CPE was observed, selected cell cultures were further examined by negative staining using a Hitachi H7000 transmission electron microscope.

Virus neutralisation tests. Virus neutralisation tests were performed by the constant serum/varying virus method, without complement, using antisera raised against VHSV (Strain 23/75, provided by Dr P. de Kinkelin, France), SVCV (strain unknown, provided by Dr W. Ahne, Germany), PFRV (Strain F4, provided by Dr B. Hill, England) and IHNV (Strain RV, provided by Dr B. Hill, England). Equal volumes of antisera, diluted 1/50, were incubated for 1 h at room temperature with their homologous viruses and with rhabdovirus isolates from this study at dilutions of 1/100, 1/1000 and 1/10 000. All dilutions were in MEM(E).

Antigen ELISAs. Rhabdovirus isolates were tested in amplified, antigen capture ELISA systems using γ -globulin and immunoglobulin preparations from rabbit antisera specific to SVCV and PFRV, prepared as previously described (Way & Dixon 1988, Way 1998). The ELISA tests followed the procedure recommended for identification of SVCV in the OIE's *Diagnostic manual for aquatic animal diseases* (OIE 1997) and used

homologous reference strains PFRV F4 and SVCV S30 as controls. The only minor modification was that ExtrAvidin®-conjugated horseradish peroxidase (Sigma) was used rather than a streptavidin-conjugated enzyme. EPC cell grown virus isolates were tested simultaneously in both PFRV- and SVCV-specific ELISA systems, and the resulting absorbance at 450 nm used to calculate a PFRV/SVCV ELISA ratio. In this way, PFRV or SVCV isolates would be expected to yield ratios similar to those obtained with PFRV F4 and SVCV S30, respectively, with intermediate results indicating antigenic differences from both.

Sequence analysis. In the absence of published sequence data for PFRV, potential primer annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (GenBank accession no. U18101), the vesicular stomatitis virus New Jersey (GenBank accession no. V01214) and Piry strains (GenBank accession no. D26175). Primers were then designed to anneal to the regions encoding the conserved amino acids using the published sequence for SVCV (Bjorklund et al. 1996) as a skeleton, and introducing degenerate bases at the 3' termini to allow for potential differences in codon usage between SVCV and PFRV. The rationale for this approach to primer design was that if the amino acids are conserved between 3 distinct viruses, it is highly likely that they have a functional role and would be highly conserved between viruses of the same group. A 550 base pair segment corresponding to nucleotides 405 to 954 of the glycoprotein gene were amplified by RT-PCR using the primer pairs SVCVR4 (5'-CTGGGG-TTCCNCCTCAAAGYTG-3') and SVCVF1 (5'-TCT-TGGAGCCAAATAGCTCARRTC-3').

Total RNA was extracted from 100 µl of viral supernatant from infected EPC cells using the Trizol Reagent™ (BRL, Life Technologies, Paisley, UK) as described previously (Strømmen & Stone 1997). RT was performed at 37°C for 1 h in a 20 µl volume consisting of 1× M-MLV RT reaction buffer (50 mM Tris [pH 8.3], 75 mM KCL, 10 mM dithiothreitol, 3 mM MgCl₂) containing 1 mM deoxynucleotide triphosphate, 100 pmol SVCV R4 primer, 20 U M-MLV reverse transcriptase (Promega, Southampton, UK) and one-tenth of the total RNA extracted previously. PCR was performed in a 100 µl reaction volume 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0 and 0.1% Triton X-100) containing 2.5 mM MgCl₂, 200 µM deoxynucleotide triphosphates, 100 pmol each of the SVCV R4 and SVCV F1 primers, 2.5 U of RedHot Taq polymerase (AB gene, Epsom, UK) and 5 µl reverse transcription reaction mix. The reaction mix was overlaid with mineral oil and subjected to 35 temperature 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.

Aliquots (20 µl) of the amplified products were electrophoresed in a 2% (w/v) agarose/TAE (40 mM Tris-acetate, pH 7.2, 1 mM EDTA) gel containing 1.0 µg ml⁻¹ ethidium bromide and visualised by UV irradiation. PCR products were purified using GENECLAN® (Anachem, Luton, UK) and inserted into the pGEM-T vector (Promega) using the standard protocol. Both DNA strands were sequenced using the M13 universal sequencing primers and the ABI PRISM™ dye terminator cycle sequencing system (Perkin Elmer, Warrington, UK). Sequencing reactions were analysed on an ABI 310 genetic analyser and multiple alignments were analysed using Clustal V software (Higgins & Sharp 1989). Cladograms were generated using the neighbour-joining method of Saitou & Nei (1987) in the Clustal V multiple alignment package within MEGA-LIGN (DNASTAR, Inc., Madison, WI, USA).

Sequence data (unpubl.) was also included from rhabdovirus isolates 84/4 made from healthy brown trout in NI in 1984 (Adair & McLoughlin 1986), E1350 made from diseased bream in County Cavan in the Republic of Ireland (RoI) in 1997, Dutch rhabdovirus isolate 80560 from roach (Haenen & Davidse 1989), and reference strains of PFRV (F4) and SVCV (S30).

Experimental infections. Transmission studies were carried out on commercially sourced conventional juvenile (approximately 10 cm in length) bream and mirror carp *Cyprinus carpio* var. *specularis* purchased from Fish Network, Devon, England. Because of their size, these fish were not screened for previous rhabdoviral exposure. Viruses were propagated in EPC cells and harvested at maximum CPE, 4 d post inoculation (dpi). Following a single freeze/thaw cycle and clarification by centrifugation an inoculum of 0.1 ml containing approximately 10^{7.5} TCID₅₀ was administered to fish by the intra-peritoneal route. Control fish received a virus-free inoculum of MEM(E). Fish were maintained on bore-hole water at 18°C, this temperature being chosen to induce a degree of stress (McCarthy 1983). From 0 dpi to 46 dpi, when the experiment ended, dead fish were removed for post-mortem examination. In particular, spleen, kidney and brain were collected from each fish, and pools of these tissues were prepared and inoculated onto EPC cells for virus isolation as described above. Moribund fish were killed and examined in the same way. In addition, 4 to 6 clinically healthy control and inoculated fish of both species were killed and examined 0, 9, 15, 22, 37 and 46 dpi.

Pathological examination. Samples of heart, spleen, liver, kidney, pyloric caeca/pancreas, muscle and gills were taken from naturally and experimentally infected fish for histopathological examination. These samples were fixed in 10% formalin and processed by standard paraffin methods.

RESULTS

Post-mortem examination

Bream from Case 1 were found to be in poor condition on gross examination, with some haemorrhages at the base of the pectoral fins and on their sides. There was no evidence of external parasitic infection. No major gross changes were present in the internal organs, although there was virtually no body fat and ascites was present. Histopathological examinations revealed congestion of the internal organs but no specific pathological changes. However, the histopathological examination was complicated by post-mortem degenerative changes in some cases. Several fish had some renal necrosis associated with the presence of *Sphaerospora* parasites in the tubular lumina.

Bacteriology and virology

No significant bacteria were isolated from any fish. A CPE was observed in both EPC and BF-2 cells (F98-94) or in EPC cells only (F98-109) on initial passage of tissue pools from both submissions of bream from Case 1. A CPE was also observed in these cell lines on second passage of tissue pools from both brown and rainbow trout (F98-93) from this case. In Case 2, a CPE was observed on second passage in EPC cells of pools from both bream and roach. No CPE was observed in the CHSE-214 cells or in uninoculated control EPC or BF-2 cells. Electron microscopical examination of selected cell culture supernatants showing a CPE revealed the presence of virus particles 110 ± 11 nm long and 75 ± 9 nm in diameter, with typical rhabdovirus morphology and an obvious central channel in both cases (Fig. 1).

Serological typing of isolates

No neutralisation was detected between rhabdovirus isolates F98-93 and F98-94 from brown trout and bream from Case 1 and antisera to SVCV, VHSV or IHNV. However, antiserum to PFRV resulted in reductions in titre of 100-fold or greater, indicating that these isolates were most closely related to PFRV.

When EPC cell grown virus isolates were tested simultaneously in ELISA systems specific for PFRV and SVCV, ratios of 1.35 and 0.5 were observed for PFRV F4 and SVCV S30, respectively (Table 1). Three other SVCV strains tested had similar ELISA ratios to strain S30. The ratios observed for the rhabdovirus isolates F98-93 and F98-109 (bream, Case 1) and F99-57 (bream, Case 2) of 0.99, 0.94 and 0.93 were intermedi-

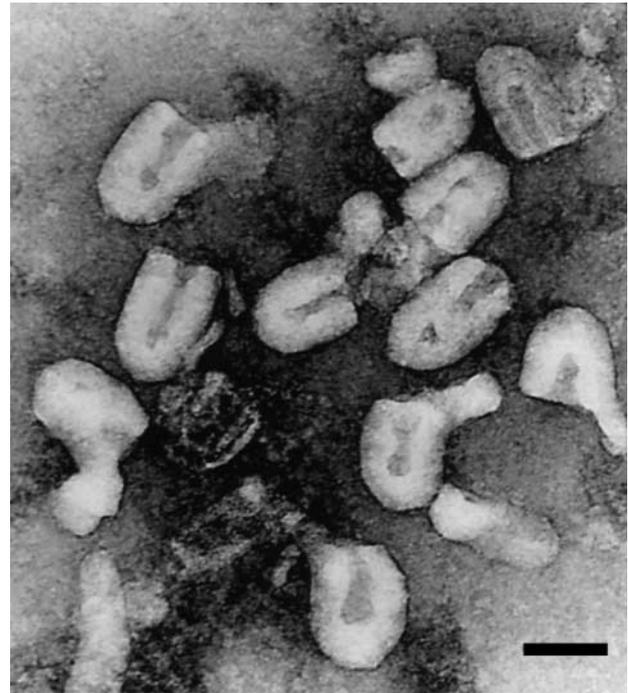


Fig. 1. Negative-stained electron micrograph of rhabdovirus F98-94, isolated from diseased bream on *Epithelioma papulosum carpio* cells ($\times 100\,000$). Scale bar = 100 nm

ate in value in comparison with PFRV F4 and SVCV S30 but similar to the ratio observed for Dutch roach isolate 80560. Rhabdovirus E1350 isolated from bream in the RoI also gave a similar intermediate value, whereas NI rhabdovirus 84-4 had a similar ratio to that of PFRV F4.

Table 1. Comparative PFRV:SVCV ELISA ratios of EPC-grown virus harvests tested simultaneously in the 2 ELISA systems. Ratios are absorbance at 450 nm (A_{450}) of virus harvest in the PFRV ELISA system divided by the A_{450} of the same harvest in the SVCV ELISA. The ratio value is the average from at least 2 separate tests

Virus isolate	Host	Origin	PFRV /SVCV ELISA ratio
SVCV M27	Carp	England	0.38
SVCV 10/3	Carp	Germany	0.46
SVCV S30 ^a	Carp	Yugoslavia	0.50
UK 88:62	Carp	England	0.42
80560	Roach	The Netherlands	0.93
F99-57	Bream	Northern Ireland	0.93
F98-93	Brown trout	Northern Ireland	0.99
F98-109	Bream	Northern Ireland	0.94
E-1350	Bream	Republic of Ireland	0.91
NI 84-4	Brown trout	Northern Ireland	1.35
PFRV F4 ^b	Pike	France	1.33

^aS30: homologous virus strain in SVCV ELISA
^bF4: homologous virus strain in PFRV ELISA

Sequence analysis

Sequence alignment revealed a high degree of homology at the nucleotide level between isolates F98-93, F98-109 and F99-57 from this study, and previous isolates F84-4 and E1350, with between 0 and 4

nucleotide differences (0 to 0.72%) found. A high degree of homology was also found between these 5 isolates and 80560 with 16 to 19 nucleotide differences (2.9 to 3.4%) distributed evenly throughout the 550 base pair region (Fig. 2). In contrast, there were 97 to 98 (>17.6%) and 178 to 180 (>32.3%) nucleotide dif-

	10	20	30	40	50	60	70
PFRV F4	CTCAGTCACA	ACAGTATCAA	ATAAGAATTA	CAAAGTTATC	GTACATCCTG	TTCATTTAGA	ACCATACCAG
84-4C..TC..G..	G..T..T..
98-93C.....C.....C..TC..G..	G..T..T..
98-109C.....C.....C..TC..G..	G..T..T..
99-57C.....C.....C..TC..G..	G..T..T..
E1350C.....C.....C..TC..G..	G..T..T..
80560C.....C.....C..TC..G..	G..T..T..
SVCV S30	...T.....	..G..G....	...CT.....	T..G...A..A	CCC...T...	G.....TGGA
	80	90	100	110	120	130	140
PFRV F4	GGTAAATGGG	TTGACCATGA	ATTTTGGGG	GGAGAATGTA	ATGTCCCGGT	TTGTGAGATG	AGAGGCAATC
84-4G.....CG..A..AG.....A.....C.....
98-93G.....CG..A..AG.....A.....C.....
98-109G.....CG..A..AG.....A.....C.....
99-57G.....CG..A..AG.....A.....C.....
E1350G.....CG..A..AG.....A.....C.....
80560G.....CG..A..AG.....A.....C.....
SVCV S30	..AC..T..A	..C..T..C..	...AAT...	..C.....C..	GA..AAAA..	G..C..A...	..A.....C..
	150	160	170	180	190	200	210
PFRV F4	ATTCAAATTTG	GCTAACAGAT	CATATCCTAA	AAGAGGAATG	CGACCAACAC	ATCGAGGAAA	CAACAGGGAC
84-4T.....T.....G..T...G.....	..T.....G..A..T
98-93C.....T.....G..T...G.....	..T.....G..A..T
98-109C.....T.....G..T...G.....	..T.....G..A..T
99-57C.....T.....G..T...G.....	..T.....G..A..T
E1350C.....T.....G..T...G.....	..T.....G..A..T
80560G.....T.....G..T...G.....	..T..A..G..A..T
SVCV S30	..C..T.....T.....	..G..T...	T.....G...	..T.....G..	TTGA...A..T
	220	230	240	250	260	270	280
PFRV F4	CATGTATGGA	AATGCTCTGA	GGGGGGATAA	TCTGTATGTA	AACAATTTTA	TCATTGACGA	TCATCATAGA
84-4T...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
98-93TCT...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
98-109TCT...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
99-57TCT...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
E1350TCT...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
80560TTT...	..A..T..C..	C.....C..G	..T.....	..A.....T..	G.....C..G
SVCV S30	T.....C...	...T..CA.	..A.....GT	AA.....CTC....	..T..A..TAG	A.....
	290	300	310	320	330	340	350
PFRV F4	GTCTACAAGT	TCCAAGATGC	CTGTGCGATG	AAATCTGTGT	GGATGGATGG	AAATCAAGTTC	CTACGTGGAG
84-4	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG.....	..T.....T..	..T..A.....
98-93	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG.....	..T.....T..	..T..A.....
98-109	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG.....	..T.....T..	..T..A.....
99-57	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG.....	..T.....T..	..T..A.....
E1350	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG.....	..T.....T..	..T..A.....
80560	..T.....A..	..A..C..GA..A.....	..G..T.....	..AG..T.....	..T.....T..	T..T..A.....
SVCV S30	..A.....GA.	..GGG..GAT	T.....AG...A.....	AT..AA.....	T..A..A.....	GCGA..A.....
	360	370	380	390	400	410	420
PFRV F4	ACTGGATCGA	GAAAA-AAGG	AGAGCTTGCA	CGAC--TTCA	TGAAAACGTA	CCGGATTGTG	CAGATGGAAAC
84-4G..T..	...G..G..	..A.....A.....CT	...G.....
98-93G..T..	...G..G..	..A.....A.....CT	...G.....
98-109G..T..	...G..G..	..A.....A.....CT	...G.....
99-57G..T..	...G..G..	..A.....A.....CT	...G.....
E1350G..T..	...G..G..	..A.....A.....CT	...G.....
80560G..T..	...GC..G..	..A.....A.....CT	...G.....
SVCV S30G..A..C..GCC	G..A..A..TA.	..A..GA...	...C..T..G	..TA..A.....	TT.....
	430	440	450	460	470	480	490
PFRV F4	CTTAATCTCA	GGGCACAAAC	CCGGTGCAGA	CTTGGTCGAC	ACAGTATTCA	ATCTAGAAAA	TCTATTGGAG
84-4	A.....C	..A.....	..T.....TG..	T.....T..TG..T..A.....A..A
98-93	A.....C	..A.....	..T.....TG..	T.....T..TG..T..A.....A..A
98-109	A.....C	..A.....	..T.....TG..	T.....T..TG..T..A.....A..A
99-57	A.....C	..A.....	..T.....TG..	T.....T..TG..T..A.....A..A
E1350	A.....C	..A.....	..T.....TG..	T.....T..TG..T..A.....A..A
80560	A.....C	..A.....	..T..C..TT.	T.....T..TG..T..	CA.....A..A
SVCV S30	G..GG.....C	..T.....CGC.	...ATT...	...A..T...C....	..T..G.....	..G..G.....A
	500	510	520	530	540	550	
PFRV F4	TTTACGCTGT	GTGAAAGTAC	GAAAGCCAAA	ATCAATCGAA	GGGAAGAGAT	AACATCCGTA	
84-4G..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
98-93G..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
98-109G..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
99-57G..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
E1350G..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
80560GG..C..	A..G..A...	G..T..C..GC	A...G..CAC	T.....A..C	
SVCV S30	..A...TT...	...G..G..	T...AGG...AA..C	AA..GA..C..	G.....A..G	

Fig. 2. Alignment of nucleotides 405 to 954 of the glycoprotein gene of isolates from Northern Ireland (NI), the Republic of Ireland (E1350) and the Netherlands, (80560) and reference strains of pike fry rhabdovirus (PFRV) (F4) and spring viraemia of carp virus (SVCV) (S30). •: Positions of sequence identity compared with the sequence for the PFRV reference strain (Stone et al. unpubl.); -: gaps introduced to improve the alignment

		10	20	30	40	50	60	70
PFRV F4	SVTTTVSNKNY	KVVVHPVHLE	FYQGKWDHE	FLGGECPAPV	CEMRGNHSIW	LTDHILKEEC	DQHIEETTGT	
84-4	·V·.....	···V·.....I	
98-93	·V·.....	···V·.....I	
98-109	·V·.....	···V·.....I	
99-57	·V·.....	···V·.....I	
E1350	·V·.....	···V·.....I	
80560	·V·.....	···V·.....	N···A···I	
SVCV S30	·····T···	R··P·S···	··G·H·I···	·N···REK·	···K·····	I·EETVQH·	AK···VE·I	
		80	90	100	110	120	130	140
PFRV F4	MYGNALRGDN	LYVNNFIIDD	HHRVYKQDA	CRMKFCGMDG	IKFLRGDWIE	KK-GELARLH	ENVPDCADGT	
84-4ENG·V··V··V··SE··	
98-93	···V····ENG·V··V··V··SE··	
98-109	···V····E	Y····NG·V··V··V··SE··	
99-57	···V····ENG·V··V··V··SE··	
E1350	···V····ENG·V··V··V··SE··	
80560	···V····ENG·	··F··V··V··	·Q······SE··	
SVCV S30	····VP··V	M·A····R	····R·GGS	·Q····NK·	···A····V·	·TA·T·TTI·	D···K·V··	
		150	160	170	180	TSV		
PFRV F4	LISGHKPGAD	LVDTVFLEN	LLEFTLCEST	KAKINRREEI				
84-4V·	I······	···V··Q·AL	···			
98-93V·	I······	···V··Q·AL	···			
98-109V·	I······	···V··Q·AL	···			
99-57V·	I······	···V··Q·AL	···			
E1350V·	I······	···V··Q·AL	···			
80560V·G·	I······	···V··Q·AL	···			
SVCV S30	·V···R·L·	·I······	VV·Y···G·	·R···KQ·KL	···			

Fig. 3. Alignment of the deduced amino acids (residues 136 to 318) of the glycoprotein gene of the NI isolates, a RoI isolate (E1350), a Dutch isolate (80560), and reference strains of PFRV (F4) and SVCV (S30). See Fig. 2 for symbols

ferences between the NI/RoI isolates and PFRV F4 and SVCV S30, respectively. This includes the deletion of 3 bases found in PFRV compared with SVCV.

A similar picture with the NI/RoI isolates was observed at the deduced amino acid level with between 16 and 18 (8.7 to 9.8%) and 56 and 57 (30.6 to 31.1%) amino acid differences relative to PFRV and SVCV, respectively. This compares with a maximum of 6 (3.3%) amino acid differences between these isolates and the roach isolate, 80560 (Fig. 3).

The cladograms based on both nucleotide (Fig. 4) and deduced amino acid (Fig. 5) sequences identified the presence of 3 distinct genogroups. SVCV and PFRV were the sole members of 2 of these genogroups, with all the remaining isolates, including 80560, clustered in the third.

Experimental transmission

No moribund or dead fish were observed among the 20 carp and 20 bream that received the control inoculum, and no virus was isolated from sequential kills of these fish. In both carp and bream inoculated with isolates F98-109 and F99-57, morbidity and mortality were low. Three of 25 bream (12%) and 4 of 40 carp (10%) inoculated with F98-109 died, with an additional moribund carp also euthanised. Deaths occurred between 4 and 11 dpi and 29 and 42 dpi, respectively. Rhabdovirus was re-isolated from dead bream but not from dead carp. Virus was re-isolated, on first passage, from bream and carp sequentially killed 9, 15 and 22 dpi, and from carp killed 37 dpi. Following inoculation with isolate F99-57, 4 of 35 bream (11%) died

between 2 and 18 dpi. Two of the 40 carp (5%) inoculated with this isolate also died, 27 and 33 dpi. In sequential kills of these fish, rhabdovirus was re-isolated, on first passage, from both carp and bream 9 and 15 dpi, and from bream killed 22 dpi.

Localised superficial haemorrhages and exophthalmos were seen in a small percentage of infected bream. No consistent pattern of pathological lesions was seen in diseased or dead fish, although histopathological findings were again complicated by post-mortem degeneration in some cases. Abnormalities observed in bream included ascites, areas of hepatocyte degeneration with hepatic necrosis, and chronic pancreatitis with fibrosis and lymphocyte infiltration. No syncytia were observed.

DISCUSSION

Rhabdovirus was isolated from both submissions of common bream *Abramis brama* from the disease outbreak with associated high mortality on the River Bann. To the best of our knowledge, these isolates, together with isolate E1350 from RoI and isolate F99-57, are the first reported in the literature from this species. In the absence of other viral, bacterial or parasitic agents known to be pathogenic in bream, this rhabdovirus appears to be the primary aetiological agent involved in the mortalities seen in Case 1. This is supported by the observation of clinical signs of external haemorrhages and ascites in this outbreak, consistent with rhabdoviral disease. In addition, the occurrence of the outbreak in the spring, when water temperatures are rising after a period of decline, is consistent

Fig. 4. A cladogram showing the genetic relationships of the NI isolates, a RoI isolate (E1350), a Dutch isolate (80560), and reference strains of PFRV (F4) and SVCV (S30) based on nucleotides 405 to 954 of the glycoprotein gene. The scale indicates percentage divergence

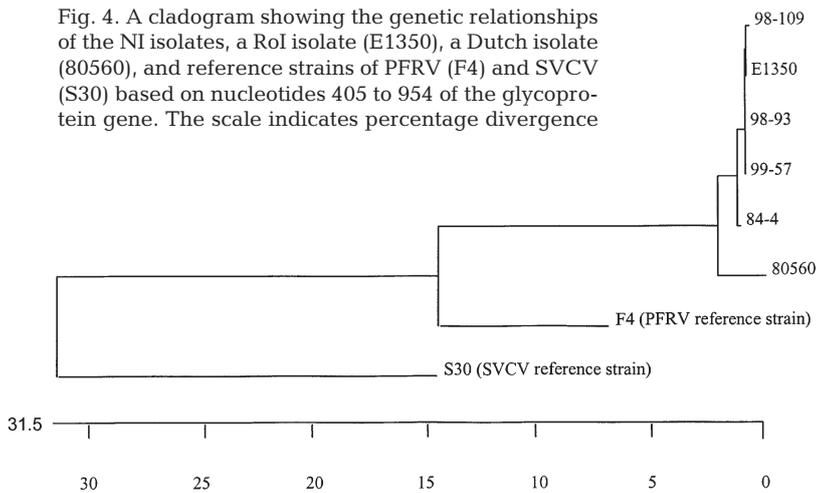
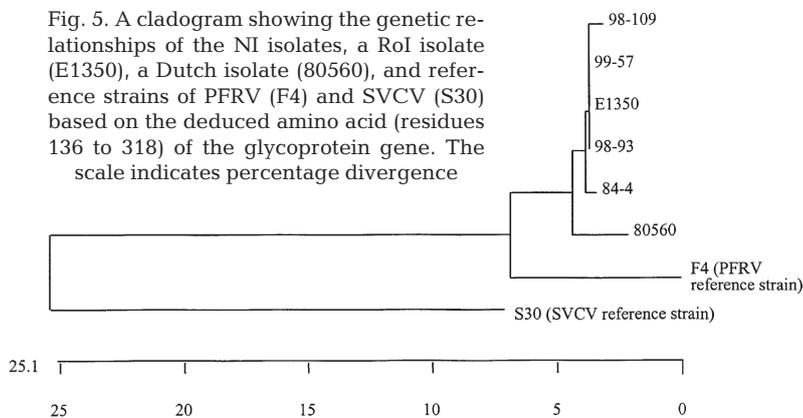


Fig. 5. A cladogram showing the genetic relationships of the NI isolates, a RoI isolate (E1350), a Dutch isolate (80560), and reference strains of PFRV (F4) and SVCV (S30) based on the deduced amino acid (residues 136 to 318) of the glycoprotein gene. The scale indicates percentage divergence



with what is known of the epidemiology of SVCV-induced disease (Fijan 1999). However, experimental intra-peritoneal infections with isolate 98-109 from this outbreak and subsequently with isolate F99-57 produced relatively low mortality rates of $\leq 12\%$ in both bream and carp. It is recognised that clinical manifestation of disease in fish may depend on interactions among the host, the pathogen and external stressors such as poor hygiene or overcrowding. In experimental infections with other rhabdoviral isolates from non-salmonids, factors including viral titre of inocula, route of inoculation, age and size of fish, and water temperature have been shown to influence mortality rates (Bootsma et al. 1975, Dorson et al. 1984, 1987, Jorgensen et al. 1993, Bovo et al. 1995, Fijan 1999). Variation of one or more of these factors from optimum levels may have been responsible for the relatively low mortalities observed in this study. Also, analysis of data from a water monitoring station on the Bann indicates a decrease in water quality coincident with the natural disease outbreak described here, and this is considered likely to have played an important role in enhanc-

ing the severity of the latter. In the absence of screening it is possible that previous rhabdoviral exposure of the fish used in the experimental infections also contributed to the low levels of recorded mortality. The isolation of a closely related virus from healthy bream and roach in Lough Neagh the following year is uncommon, and provides further evidence for the necessity of appropriate trigger factors to induce clinical disease following natural infection. The isolation in NI of a similar rhabdovirus in 1984 (Adair & McLoughlin 1986) suggests that such strains have been circulating over an extended period of time. The fact that the disease outbreak reported here is the first described in NI during this period suggests that appropriate trigger factors for disease are absent in most years.

The observation that isolate F98-93 infected, but did not produce clinical disease in, brown and rainbow trout is consistent with previous observations with isolate 84-4 (Adair & McLoughlin 1986). It is considered likely that in this instance, diseased bream were the source of infection for these species, rather than vice versa. Nevertheless, the movement of healthy, virus-infected commercial trout between sites appears to be a possible means of disseminating infection, both to other farmed fish and to wild populations. This is supported

by the subsequent isolation of an antigenically similar rhabdovirus from samples taken from commercial rainbow trout on another river system in NI during a routine health check in 1998 (authors' unpubl. data).

Isolates from this study and previous NI isolate 84-4 were shown to be serologically related to PFRV by virus neutralisation. However, these findings were only partially supported by ELISA testing. Although NI 84-4 showed a closer antigenic relationship to PFRV than to SVCV, the other NI isolates and RoI isolate E1350 showed a more intermediate relationship. However, sequence data indicate that all of the NI isolates, including NI 84-4, share a high degree of homology with each other, with RoI isolate E1350 and with geographically remote rhabdovirus isolate 80560 from roach (Haenen & Davidse 1989). This latter isolate had also been found to be serologically indistinguishable from PFRV by virus neutralisation (data not shown). In contrast to virus neutralisation results, sequencing data for the NI/RoI isolates and 80560 showed a sufficient degree of divergence to allow them to be clearly distinguished from PFRV (Fig. 4) and suggest the pres-

ence of at least 3 genogroups within the *Vesiculovirus* genus, with the possibility of more genogroups being added as sequencing is carried out on additional viruses such as EVA, UDRV and grass carp rhabdovirus. The lower degree of variation at the amino acid level may account for the high level of serological cross-reactivity observed between these 80560-like isolates and PFRV.

Despite the extended period over which the NI and RoI isolates were made, and the relatively high rate of mutation in RNA viruses, including rhabdoviruses (Benmansour et al. 1997), these viruses exhibited a high degree of sequence homology. This suggests that the sequence of the examined region of the glycoprotein gene of these viruses is subject to evolutionary constraints. The low degree of divergence ($\leq 3.4\%$) observed between these isolates and 80856 is similar to that observed with VHSV and IHNV strains of common geographical origin (Benmansour et al. 1997, Stone et al. 1997, Emmenegger et al. 2000). However, when VHSV strains of diverse geographic (European and American) origin were compared, a greater degree of divergence, approaching that observed between the 80856-like isolates in this study and PFRV, was evident. The significance of the sequence data presented here in terms of the taxonomy of these isolates is therefore unclear, but raises the possibility that they may represent 2 different viral species, typified by PFRV and 80560. More comprehensive sequence data from a wider range of isolates, as well as of other characteristics such as natural host range, cell and tissue tropisms, and physicochemical and antigenic properties, are necessary to investigate this further.

These results highlight the utility of RT-PCR in the identification of fish rhabdoviruses and its greater ability to discriminate between isolates compared with standard serological methods. Further sequencing work is required to investigate the relationship between PFRV and 80560-like isolates. The availability of these molecular tools should aid future epidemiological investigations of disease outbreaks and in identification of isolates.

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LITERATURE CITED

- Adair BM, McLoughlin M (1986) Isolation of pike fry rhabdovirus from brown trout (*Salmo trutta*) Bull Eur Assoc Fish Pathol 6:85–86
- Ahne W (1975) A rhabdovirus isolated from grass carp (*Ctenopharyngodon idella* Val.). Arch Virol 58:181–185
- Ahne W, Thomsen I (1986) Isolation of pike fry rhabdovirus from *Pseudorasbora parva* (Temminch & 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
- Benmansour A, Bernardo B, Monnier AF, Vende P, Winton JR, de Kinkelin P (1997) Sequence variation of the glycoprotein gene identified three distinct lineages within field isolates of viral haemorrhagic septicaemia, a fish rhabdovirus. J Gen Virol 78:2837–2846
- Bjorklund HV, Higman KH, Kurath G (1996) The glycoprotein genes and gene junctions of the fish rhabdoviruses, spring viremia of carp virus and hiram rhabdovirus: analysis of relationships with other rhabdoviruses. Virus Res 42:65–80
- Bootsma R, de Kinkelin P, Le Berre M (1975) Transmission experiments with pike fry (*Esox lucius* L.) rhabdovirus. J Fish Biol 7:269–276
- Bovo G, Olesen NJ, Jorgensen PEV, Ahne W, Winton JR (1995) Characterisation of a rhabdovirus isolated from carpione *Salmo trutta carpio* in Italy. Dis Aquat Org 21: 115–122
- 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
- Dorson M, Torchy C, Chilmonczyk S, de Kinkelin P, Michel C (1984) A rhabdovirus pathogenic for perch, *Perca fluviatilis* L.: isolation and preliminary study. J Fish Dis 7: 241–245
- Dorson M, de Kinkelin P, Torchy C, Monge D (1987) Sensibilité du brochet (*Esox lucius*) à différents virus de salmonidés (NPI. SHV, NHI) et au virus de la perche. Bull Fr Pêche Piscic 307:91–101
- Emmenegger EJ, Meyers TR, Burton TO, Kurath G (2000) Genetic diversity and epidemiology of infectious hematopoietic necrosis virus in Alaska. Dis Aquat Org 40: 163–176
- Fijan N (1999) Spring viraemia of carp and other viral diseases and agents of warm-water fish. In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Vol 3. Viral, bacterial and fungal infections. CABI Publishing, New York, p 177–244
- Haenen OLM, Davidse A (1989) Isolation of pike fry rhabdovirus from roach (*Rutilus rutilus*). Bull Eur Assoc Fish Pathol 9:116
- Higgins DG, Sharp PM (1989) Fast and sensitive multiple sequence alignments on a microcomputer. CABIOS 2: 151–153
- 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, Kurstak E (eds) Viruses of lower vertebrates. Springer-Verlag, Berlin, p 349–366
- Jorgensen PEV, Olesen NJ, Ahne W, Wahli T, Meier W (1993) Isolation of a previously undescribed rhabdovirus from pike *Esox lucius*. Dis Aquat Org 16:171–179
- McCarthy DH (1983) An experimental model for fish furunculosis caused by *Aeromonas salmonicida*. J Fish Dis 6: 231–237
- Mourton C, Bearzotti M, Piechaczyk M, Paolucci F, Pau B, Bastide JM, de Kinkelin P (1990) Antigen capture ELISA for viral haemorrhagic septicaemia virus serotype I. J Virol Methods 29:325–333
- OIE (Office International des Epizooties) (1997) Diagnostic manual for aquatic animal diseases, 2nd edn. OIE, Paris, p 63–73
- Saitou N, Nei N (1987) The neighbour-joining method: a new

- method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425
- Stone DM, Way K, Dixon PF (1997) Nucleotide sequence of the glycoprotein gene of viral haemorrhagic septicaemia (VHS) viruses from different geographical areas: a link between VHS in farmed fish species and viruses isolated from North Sea cod (*Gadus morhua* L.). *J Gen Virol* 78: 1319–1326
- Strømmen HK, Stone DM (1997) Detection of viral haemorrhagic septicaemia (VHS) virus in fish tissues by semi-nested polymerase chain reaction (PCR). In: Barnes A, Davidson G, Hiney M, McIntosh D (eds) *Methodology in fish diseases research*. Fisheries Research Services, Aberdeen, p 203–209
- Walker PJ, Benmansour A, Dietzgen R, Fang RX and 6 others (2000) Family Rhabdoviridae. In: Van Regenmortel MHV, Fauquet CM, Bishop DHL, Carstens EB and 7 others (eds) *Virus taxonomy*. Seventh report of the International Committee on Taxonomy of Viruses. Academic Press, London, p 563–583
- Way K (1991) Rapid detection of SVC (spring viraemia of carp) virus antigen in infected cell cultures and clinically diseased carp by the enzyme-linked immunosorbent assay (ELISA). *J Appl Ichthyol* 7:95–107
- Way K (1998) Problems arising from the use of immunoassays for the detection of viral pathogens directly in fish tissues. In: Barnes A, Davidson G, Hiney M, McIntosh D (eds) *Methodology in fish diseases research*. Fisheries Research Services, Aberdeen, p 247–256
- Way K, Dixon PF (1988) Rapid detection of VHS and IHN viruses by the enzyme-linked immunosorbent assay (ELISA). *J Appl Ichthyol* 4:182–189

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