NUCLEOTIDE SEQUENCE ANALYSIS OF THE GLYCOPROTEIN GENE OF PUTATIVE SPRING VIRAEMIA OF CARP VIRUS AND PIKE FRY RHABDOVIRUS ISOLATES REVEALS FOUR DISTINCT PISCINE VESICULOVIRUS GROUPS.

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*RT-PCR methods have been applied to the detection and sequencing of the glycoprotein gene of putative spring viraemia of carp viruses and pike fry rhabdoviruses, including the recent isolates recovered from the bream imported from Northern Ireland and their contacts. Phylogenetic analysis of a 549nt region of the glycoprotein gene identified four genogroups, I-IV. Significantly the viruses isolated recently from bream formed a distinct cluster (genogroup IV) which shared <61% nucleotide sequence similarity with the SVCV virus isolates (genogroup I). The PFR reference strain and an isolate from grass carp shared <80% nucleotide sequence similarity with representatives of genogroup IV and were considered to be sufficiently different to be assigned separate groups, genogroups II and III respectively. The viruses assigned to genogroup IV cannot be considered as SVCV (*rhabdovirus carpio*) or PFR and we propose that they are named Tench rhabdovirus or Rhabdovirus tinca after the species from which they were first isolated.*

Introduction

Pike fry rhabdovirus (PFR) was isolated from moribund pike fry (*Exos lucius*) during an outbreak of 'red disease' in a Dutch pike hatchery in 1973 [1]. There have been few additional reports of PFR isolation from pike, however, an antigenically related virus has been isolated from grass carp (*Ctenopharyngodon idella*) [2], tench (*Tinca tinca*), white bream (*Blicca bjoerkna*) [3], False Harlequin (*Pseudorasbora parva*) [4], roach (*Rutilus rutilus*) [5], sheatfish (*Siluris glanis*) [6] and Orfe (*Leuciscus idus*). Spring viraemia of carp (SVC) is a severe haemorrhagic rhabdovirus disease of cyrinids and notifiable to the Office International des Epizooties (OIE), but PFR was not made notifiable on the grounds that it is not considered to be a serious disease problem. It is sometimes difficult however, to distinguish between SVCV and PFR using the serological assays currently recommended by the OIE, raising concerns about the reliability of some SVCV and PFR diagnoses.

In 1998 a pike fry rhabdovirus was isolated following mortalities in wild bream (*Abramis brama*) stocks in the River Bann, Nothern Ireland, having been isolated from brown trout (*Salmo trutta*) in a previous year [7]. In 1999 PFR was isolated from fish of the same source and from other fish species on sites also holding bream imported from Northern Ireland. The virus reacted strongly in the ELISA using both the SVCV and PFR anti-sera but was diagnosed as PFR on the basis of a serum neutralisation assay. If the diagnosis is correct this represents the first example of PFR causing a serious haemorrhagic disease of cyprinid fish. It is important therefore, to establish the relationship between the virus recovered from bream and both SVCV and PFR because SVC is notifiable in the UK and the SVCV infected fish are subject to movement restrictions, whereas PFR infected fish are not.

We have conducted studies to determine the genetic relatedness of putative SVCV and PFR isolates from a wide range of geographical locations and from different fish species including a number of PFR isolates that cross react in the ELISA and IFAT with the SVCV anti-serum.

Materials and Methods

Oligonucleotide Primer Design

Potential SVCV primer annealing sites were identified by the alignment of the published amino acid sequences for the glycoprotein of SVCV (Genbank accession no. U18101) [8], and the vesicular stomatitis virus (VSV) New Jersey (Genbank accession no. V01214) [9], and Piry strains (Genbank accession no. D26175) [10]. Primers were then designed to anneal to the regions encoding the conserved amino acid using the published sequence [8] as a skeleton, and introducing degenerate bases and the 3´ termini to allow for potential differences in codon usage (**Figure 1.**). The rationale for this approach to primer design was that if the amino acids are conserved between three distinct viruses, it is highly likely they have a functional role and would be highly conserved between viruses of the same group.

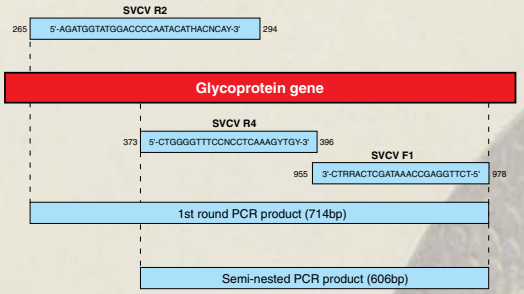

Figure 1. Schematic diagram showing the location of the SVCV-specific reverse and forward primers and the product sizes expected after the single round and semi-nested PCR reactions. A modified SVCV R4 primer (5´CCTCAAAGYTGYGGNTGGGC 3´) was designed using sequence data generated for virus isolate 880237 and was used in the semi-nested PCR in situations where SVCV R4 failed to generate a product.

Virus isolates

Table 1. Rhabdovirus isolates included in the studies

Isolate i.d.	Host name	Year of isolation	Country of origin & donor code	Comparative ELISA* (PFR EIA:SVCV EIA ratio)
2/90	Common Carp (Cyprinus carpio)		Moldova (a)	1.0
332	White Bream		Germany (b)	1.78
770346	Common Carp	1977	U.K. (d)	ND
80560	Roach	1989	The Netherlands (e)	1.17
84/4	Brown Trout	1984	U.K. (N. Ireland) (f)	2.15
98-109	Bream	1998	U.K. (N. Ireland) (f)	1.65
98-93	Brown Trout	1998	U.K. (N. Ireland) (f)	2.04
860115	Common Carp	1986	U.K. (d)	ND
880062	Common Carp	1988	U.K. (d)	ND
880124	Common Carp	1988	U.K. (d)	0.66
880137	Orfe or Ide	1988	U.K. (d)	1.14
950237	Tench	1995	U.K. (d)	1.13
970395	Common Carp	1997	U.K. (d)	ND
970469	Common Carp	1997	U.K. (d)	1.05
M2-78	Silver Carp (Hypophthalmichthys molitrix)		Moldova (a)	0.99
N3-14	Grass Carp		Ukraine (a)	1.01
N1-5	Bighead Carp (Aristichthys nobilis)		Ukraine (a)	0.92
P4	Common Carp		Russia (a)	0.99
PFRV Danil	Pike	1973	France (c)	1.97
PPRV	False Harlequin	1986	Germany (b)	2.36
RHV	Rainbow trout (Oncorhynchus mykiss)	1989	Ukraine (a)	0.99
S 30	Common Carp	1971	Yugoslavia (g)	0.83
S 64	Tench	1982	Germany (b)	2.90
V 76	Grass Carp	1982	Germany (b)	2.57
993890 2.1	Bream	1999	UK	1.44
993890 7.1	Grass Carp	1999	UK	ND
993890 9.1	Bream	1999	UK	ND
993890 11.1	Roach	1999	UK	ND
994663 1.1	Bream	1999	UK	1.71
994663 2.1	Roach	1999	UK	ND
994668 1.1	Bream	1999	UK	1.20
994591	Tench	1999	UK	ND
994602 3.1	Crucian Carp	1999	UK	1.46
980624	Orfe	1998	UK	ND

Donor codes & relevant references: (a) = I. Shchelkunov (11); (b) = W. Ahne (2, 3 & 4); (c) = P. de Kinkelin (1); (d) = CEFAS Weymouth isolate; (e) = O. Haenen (5); (f) = B. Adair (7); (g) = N. Fijan (6 & 12).
*=ratio of absorbance values from cell culture extracts tested simultaneously in a PFR and SVCV ELISA system

RNA extraction and Reverse transcription-PCR

Viral RNA was extracted from 100µl of viral supernatant from infected EPC-cells using the Trizol Reagent™ (BRL, Life Technologies) using a protocol described previously [13] and dissolved in 40µl of DEPC-treated water. Reverse transcription and the first round of PCR were performed using Ready-to-Go tubes (Pharmacia Biotech). Reverse transcription was performed at 37°C for 1hour in a 33µl volume consisting of 50mM Tris (pH 8.3), 75mM KCL, 7.5mM DTT, 10mM M_gCl_2, 0.08mg/ml RNase/DNase free BSA, 2.4mM of each dNTP, 500ng SVCV R2 primer and M-MLV reverse transcriptase (20 units). The first round of PCR was performed in a 100µl reaction volume containing the 33µl reverse transcription reaction mix, 500ng of the SVCV F1 primer and 2.5 units of *Taq* polymerase (BRL, Life technologies). The reaction mix was overlayed with mineral oil and subjected to 30 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.

Where products were not generated using a single round PCR, a second round of amplification was performed. This was performed using 5µl of the first round of amplification in a 100µl reaction volume containing 1x PCR buffer (50mM KCl, 10mM Tris-HCl pH9.0, and 0.1% Triton X-100), 2.5mM M_gCl_2, 0.25mM dNTPs, 500ng of the SVCVR4 and SVCV F1 primers and 2.5 units of *Taq* polymerase. Reaction mixes were overlayed with mineral oil and subjected to 30 amplification cycles as before.

Aliquots (20µl) of the amplified products were electrophoresed in a 2% (w/v) agarose/TAE (40mM Tris-acetate, ph7.2, 1mM EDTA) gel containing 1.0µg/ml ethidium bromide, and visualised by UV irradiation.

Sequence Analysis

PCR products were purified using the GENECLEAN® (BIO 101 inc.) and ligated into the pGEM-T vector using a standard protocol. Both DNA strands were sequenced using the universal sequencing primers (M13 -20 and M13 forward) and the ABI PRISM™ dye terminator cycle sequencing system (Perkin Elmer). Sequencing reactions were analysed on an ABI 310 genetic analyzer. A phylogenetic tree was generated using the CLUSTALV algorithm within MEGALIGN (DNASTAR inc.).

Results and Discussion

Amplification products were generated for all viruses previously confirmed as SVCV by the serological tests. However, it was necessary in most cases where the virus had been diagnosed as PFR to perform a second round of PCR, and when amplifying both the PFR reference strain, F4 and the isolate from grass carp, V76 the modified SVCV R4 primer was required. At least two independent cloning events were performed for each virus isolate to eliminate errors introduced by the *Taq* polymerase and to determine the consensus sequence within what was likely to be as complex heterologous 'quasi-species'. Phylogenetic analysis at the nucleotide level identified four main genogroups, I-IV (**Figure 2**). The SVC viruses were assigned to genogroup I. These viruses showed a higher than expected level of nucleotide sequence variation (84-99% similarity) indicating that SVCV has probably evolved independently in several different geographical areas. Indeed, isolates 980451 and 980528 which form an 'out group' of the genogroup I were recovered from carp originating in the Republic of China, whereas the other isolates used in this study originate from Europe and Russia.

Most of the other viruses studied, including many viruses previously diagnosed as PFR, were assigned to genogroup IV and showed a high degree of nucleotide sequence similarity (>93.7%). However, the PFR reference strain F4 which shared <80% similarity with representative viruses from genogroup IV was considered sufficiently different and was assigned to a separate group. (genogroup III). The grass carp isolate (V76), which was also diagnosed as PFR, shared only 70% nucleotide similarity with both F4 and representatives of genogroup IV and was assigned to genogroup II.

We propose that the viruses assigned to genogroups II and IV, are named Grass carp rhabdovirus and Rhabdovirus tinca or Tench rhabdovirus after the species from which they were first isolated [2,3]. Given that the tench rhabdovirus recovered from bream in 1998/99 was associated with large scale mortalities (H Rolli, pers. comm), and isolate 80560 was shown to be highly pathogenic for roach and common carp by bath infection [14], we believe that this virus species should be considered as a serious threat to cyprinid fish. Of particular concern is the impact the virus could have on wild fish populations. The tench rhabdovirus has already been isolated from a severely haemorrhaged chub (*Leuciscus cephalus*) caught down stream of an affected site in the UK, and crucian carp, rudd, roach and orfe are known to be a susceptible species. It should also be noted that brown trout are known carriers of the virus.

In future studies it will be interesting to establish the genetic relationship of the Perch and Lake trout rhabdovirus [15, 16] to the viruses studied here. Particularly, as both viruses have been associated with an increasing number of mortalities in several different Perch species (P de Kinkelin, pers comm and N Lorenzen, pers comm).

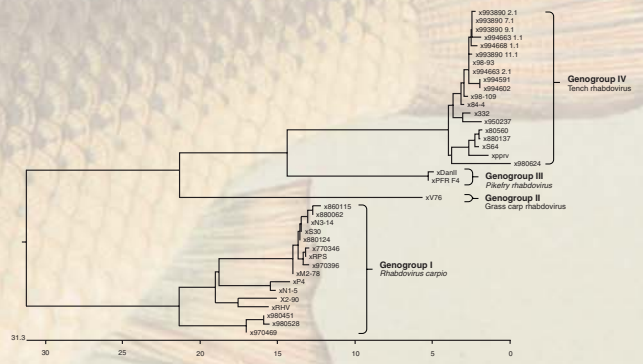

Figure 2 Dendrogram showing the genetic relationships of 38 putative SVCV and PFR isolates. The tree was based on nucleotides 405-954 of the glycoprotein gene.

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Acknowledgements

Alyson Sheppard is an industrial placement student sponsored by the Ministry of Agriculture Fisheries and Food (MAFF). Contract no. CSA3342. The work was conducted under MAFF contracts FC1105 and DP105.

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