

A RIBONUCLEASE PROTECTION ASSAY CAN DISTINGUISH SPRING VIREMIA OF CARP VIRUS FROM PIKE FRY RHABDOVIRUS

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Abstract

Thirteen rhabdovirus isolates from 10 teleost fish species as well as reference strains of spring viraemia of carp virus (SVCV) and pike fry rhabdovirus (PFRV) cross-reacted in an indirect immunofluorescence assay and were thus indistinguishable by this method. A ribonuclease protection assay (RPA) using a ³²P-labeled RNA probe made from a cloned copy of the full length SVCV glycoprotein (G) gene was able to discriminate clearly between the type strains of SVCV and PFRV and among the 13 rhabdovirus isolates. Results for the RPA were generally in agreement with standard serum neutralisation assays; however, the RPA was also able to detect genomic differences between isolates of SVCV. These results have implications for fish disease control programs for SVCV.

Introduction

Spring viraemia of carp virus (SVCV; Fijan *et al.*, 1971) and pike fry rhabdovirus (PFRV; de Kinkelin *et al.*, 1973) are listed as tentative species in the *Vesiculovirus* genus of the *Rhabdoviridae* (Wunner *et al.*, 1995). Both agents induce a generalised viraemia in a variety of freshwater teleost fishes resulting in haemorrhages in the internal organs and muscles. These viruses can cause high mortality in both juvenile and adult fish producing serious economic losses. Spring viraemia of carp is a contagious viral disease notifiable to the Office International des Epizooties (O.I.E.). The O.I.E. diagnostic manual for aquatic animal diseases recommends identification of SVCV by serum neutralisation, immunofluorescence (IF) or enzyme-linked immunosorbent assay (ELISA). However, studies have shown that SVCV and PFRV share antigenic determinants and cannot be reliably distinguished by serological approaches (Jorgensen *et al.*, 1989). In this report, we describe the use of a ribonuclease protection assay (RPA) to differentiate isolates of SVCV from PFRV.

Materials and Methods

Thirteen viral isolates from 10 teleost species obtained in different years from various locations in Europe and reference strains of SVCV and PFRV were used in this study

(Table 1). Immunofluorescence and serum neutralisation assays were performed as described by Jorgensen *et al.* (1989).

For the RPA, EPC cells (Fijan *et al.*, 1983) were grown in 6-well plates and infected with each virus at a high multiplicity of infection. After incubation at 25°C for 24 h, total RNA was isolated in a guanidinium thiocyanate solution followed by phenol/chloroform extraction and isopropanol precipitations. The RNA was quantified spectrophotometrically and analysed for quality using a denaturing (formamide/formaldehyde) gel. The RPA was carried out using a hybridisation probe comprised of ³²P-labelled, minus-sense RNA transcripts from a pBluescribe (Stratagene) clone containing a cDNA copy of the full-length glycoprotein (G) gene of SVCV (Bjorklund *et al.*, 1996). Unlabeled, plus-sense (control) RNA transcripts were also produced. The labelled probe was annealed with the target mRNA or with control transcripts. The duplexes were then digested with RNases A and T1 in conditions which allowed the enzymes to recognise and cleave mismatches. The sensitivity of the assay for the detection of complementary target RNAs was determined as described by Kurath *et al.* (1995).

Results

All 13 viral isolates were identified as rhabdoviruses by electron microscopy (Fig. 1). These European isolates and the reference

Table 1. Viruses used in this study. Reference strains of spring viraemia of carp virus and pike fry rhabdovirus as well as 13 viruses isolated from 10 different teleost fishes in Europe were tested by immunofluorescence and serum neutralisation assay using SVCV or PFRV antisera and with a ribonuclease protection assay using a probe from the G gene of SVCV.

Virus	Host	Year	Location	Immunofluorescence using antisera against		Neutralisation by antisera against		Ribonuclease protection with
				PFRV	SVCV	PFRV	SVCV	SVCV probe*
PFRV reference strain		1973	Netherlands	+	+	+	-	-
SVCV reference strain		1971	Croatia	+	+	-	+	+
1	koi carp	1993	Germany	+	+	-	+	+
2*	common carp	1982	Germany	+	+	-	+	+
3*	common carp	1974	Germany	+	+	-	+	+
4*	common carp	1974	Germany	+	+	-	+	+
5*	common carp	1977	Poland	+	+	-	+	+
6*	grass carp	1975	Germany	+	+	+	-	-
7	rasbora	1985	Croatia	+	+	-	-	-
8	rainbow trout	1972	Austria	+	+	+	-	-
9*	tench	1972	Austria	+	+	+	-	-
10	coregonus	1976	Austria	+	+	+	-	-
11	river trout	1975	Austria	+	+	+	+	-
12*	pike	1983	Croatia	+	+	+	-	-
13*	silver bream	1981	Germany	+	+	+	-	-

A (+) indicates a distinct RPA band pattern was produced (see Fig. 3); (-) indicates no RPA pattern. * relates to the 22 SVCV/PFR viruses described by Jorgensen *et al.*(1989)

strains of SVCV and PFRV cross-reacted in an indirect immunofluorescence assay (Fig. 2)

using either SVCV or PFRV antisera and could not be distinguished by this method (Table 1). However, a serum neutralisation test could clearly differentiate most of the isolates as either SVCV or PFRV (Table 1).

When the labelled SVCV G-gene probe was annealed with control transcripts that were perfectly complementary, the probe was protected from cleavage (Fig. 3, lane Tr). Target RNA from cells infected with viruses isolated from *Cyprinus carpio* e.g. koi carp (Fig. 3, lane 1) or common carp (Fig. 3, lanes 2-5) contained a number of mismatches that caused the

probe to be cleaved into several fragments. Three of the isolates from common carp of the

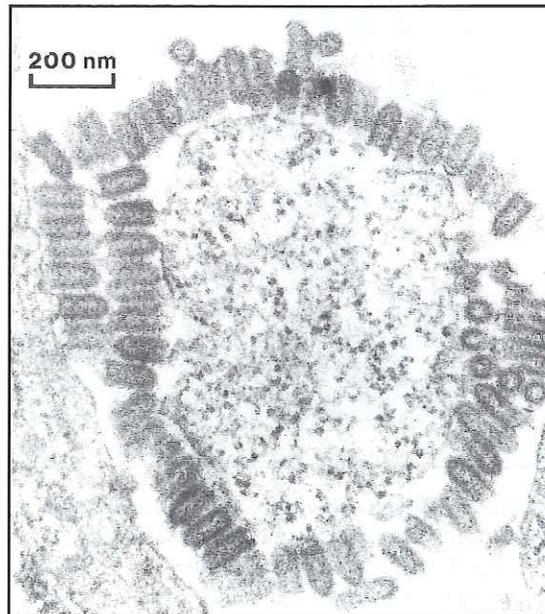


Figure 1. Electron micrograph of rhabdoviruses budding from EPC cells 24 h after infection with the coregonus isolate (58,500 x).

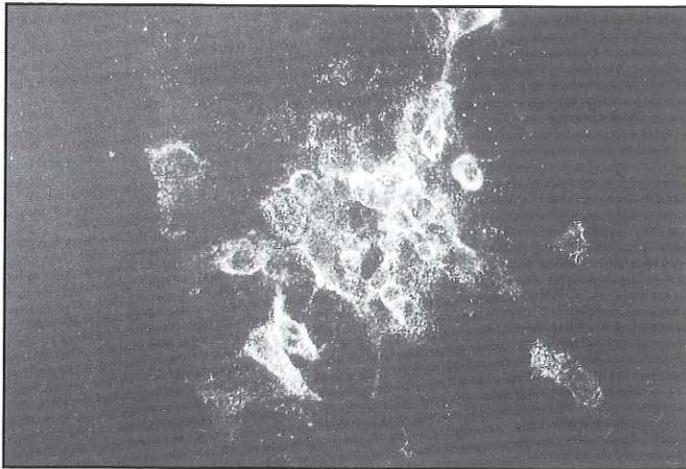


Figure 2. Indirect immunofluorescence of EPC cells 20 h after infection with the coregonus isolate. Areas of brilliant cytoplasmic fluorescence are visible in infected cells following incubation with SVCV antiserum (150 X).

same location in Bavaria (lanes 2-4) produced identical cleavage patterns, whereas one isolate from common carp (lane 5) and the isolate from koi carp (lane 1) produced unique patterns indicating genomic sequence differences from the three common carp isolates and from a reference isolate of SVCV (lane SVCV).

In contrast, the PFRV reference strain (Fig. 3, lane

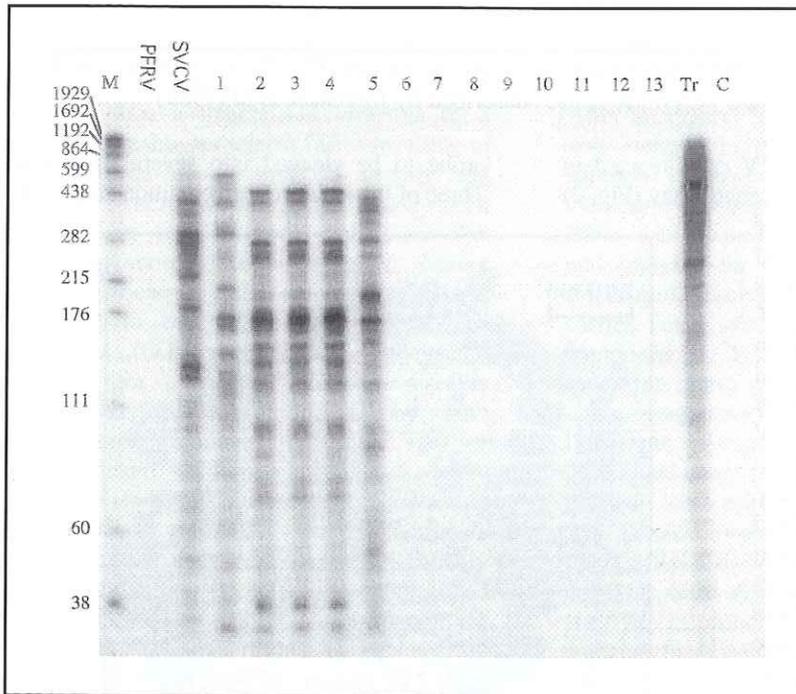


Figure 3. Autoradiograph showing results of the ribonuclease protection assay. Total RNA was extracted from cells infected with a reference strain of SVCV or PFRV or with one of the 13 rhabdoviruses isolated from teleost fishes in Europe. The RNA was hybridised to a full-length, ³²P-labeled, minus-sense RNA transcript of the SVCV glycoprotein gene and digested with RNases A and T1 to produce RPA cleavage fragment patterns. Lanes 1-13 are assays of RNAs from viruses numbered as in Table 1. M = marker, Tr = plus-sense SVCV transcript (no mismatches), C = uninfected cells.

PFRV) and the isolates from grass carp (*Ctenopharyngodon idella*) (Fig. 3, lane 6), rasbora (*Pseudorasbora parva*) (lane 7), rainbow trout (*Oncorhynchus mykiss*), (lane 8), tench (*Tinca tinca*) (lane 9), coregonus (*Coregonus alba*) (lane 10), river trout (*Salmo trutta*) (lane 11), pike (*Esox lucius*) (lane 12) and silver bream (*Blicca björkna*) (lane 13) were completely digested, resulting in blank lanes in the gel. This lack of any protected fragments longer than about 30 nucleotides (the limit of the gel) indicates the presence of a large number of sequence differences between the G gene of these isolates and the G gene of SVCV.

Discussion

The results demonstrate that the RPA is a useful tool to distinguish between relatively closely related viruses. Because SVCV and PFRV share a number of antigenic determinants, they can not be reliably distinguished by certain serological methods, especially IF or ELISA. Jorgensen *et al.* (1989) reported that only some rabbits, immunised with purified virus in Freund's complete adjuvant, were able to produce antisera that could differentiate the viruses in a serum neutralisation assay and then, only from which complement was removed. Similar results were reported by Hill *et al.* (1975) and by Clerx *et al.* (1978). Otherwise, molecular approaches including differences in the relative mobility and immunoblotting of the NS protein (Jorgensen *et al.*, 1989) or rocket immunoelectrophoresis (Clerx and Horzinek, 1978) were needed for correct identification of the viruses.

Conversely, once the RPA is established, the method can easily be used to confirm the identity of a virus and to assess genetic diversity and relatedness among different viral isolates. In addition to being able to differentiate the isolates in good agreement with the results of the neutralisation assay (Table 1), our results revealed a level of genetic diversity among isolates of SVCV from different hosts as well as isolates obtained from the major host, *Cyprinus carpio*. The patterns in Figure 3 indicate that

the three common carp isolates in lanes 2-4, which were obtained from the same location in Bavaria, may be the same, or very closely related, strains of SVCV. The fourth common carp isolate, which came from Poland (lane 5) and the koi carp isolate (lane 1) are distinct genetic types of the SVCV. Genomic variation among isolates might be due to geographic location, year of isolation or the effects of a viral population propagated under different conditions (Kurath and Palukaitis, 1990; Kurath *et al.*, 1993). These differences are important for understanding the epidemiology of viral diseases of fish.

This RPA, using a SVCV G gene probe, was capable of characterising only those virus strains sufficiently similar to the reference strain of SVCV that large fragments of the probe were protected that could be visualised on the gel. The converse assay, to generate information on the presence of strains of PFRV via formation of RPA patterns, will require a clone from the PFRV genome, which is not currently available.

Finally, fish health programs that require control of SVCV, but not PFRV, must have available a highly reliable method for distinguishing these agents. Jorgensen *et al.* (1989) have suggested that the two viruses may be considered serotypes of the same virus. Thus, agencies and organisations such as the European Union or the OIE may need to consider adding molecular methods that will assist fish health workers in confirming the identity of the viruses.

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