Detection of spring viraemia of carp virus (SVCV) with monoclonal antibodies

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ABSTRACT: Monoclonal antibodies (MAbs) against spring viraemia of carp virus (SVCV), a severe disease in cyprinid fish, were prepared. Nine MAbs were characterised using Western blotting (WB) where all reacted with glycoprotein G, except for MAb 2E1, which showed no reactivity in WB. All nine MAbs showed specificity in an immunoperoxidase test. In ELISA assays, their titres ranged between 1:32 000 and 1:128 000. A panel of SVCV isolates from different European regions were set up and examined by sandwich ELISA assay using the MAbs at a concentration of 15 µg/ml. Only MAb 4C12/3C8 showed low sensitivity in most of the isolates at an absorbance of A_{450}. Of the other MAbs, even the lowest absorbance value measured exceeded cut-off for evaluation of the whole reaction. No cross-reaction with the infectious haematopoietic necrosis virus (IHNV), viral haemorrhagic septicaemia virus (VHSV) or infectious pancreatic necrosis virus (IPNV) was demonstrated. 2E1 did not show cross-reactivity with PFRV classified in genogroup III–IV and reacted with a Czech SVCV isolate; its identity was confirmed by means of RT PCR assay. The others MAbs reacted positively with PFRV F4 reference strain, isolated from *Esox lucius L.* (genogroup III).

Keywords: monoclonal antibodies; spring viraemia of carp; SVCV; diagnostics; ELISA

Spring viraemia of carp (SVC), a rhabdoviral disease of cyprinids which manifest as an acute haemorrhagic and highly contagious viraemia, was first described in Europe in the 1930s (Wolf, 1988). The disease affects predominantly common carp (*Cyprinus carpio*) which has been for a long time considered the most important freshwater market fish in the Czech Republic (Tesarcik et al., 1977), forming up to 85% of yearly fish production. Besides common carp, SVC infections have been detected in crucian carp (*Carassius carassius)*, sheatfish (*Silurus glanis)*, silver carp (*Hypophthalmichthys molitrix)*, bighead carp (*Aristichthys nobilis)*, and tench (*Tinca tinca)* (Fijan, 1984; Jorgensen et al., 1989; Shchelkunov and Shchelkunova, 1989; Ahne et al., 1998). Accordingly, there is a high probability that the other species of Cyprinidae may also be susceptible to this infection under particular conditions.

The disease occurs during spring at water temperatures between 10°C to 17°C, affecting fish of all age categories independent of their health status, virulence of the infectious agents, the environment and fish density (Ahne, 1978; Rodak et al., 1993). Susceptibility of a fish population to the infectious agent is influenced by low humoral immunity. At higher temperatures, the infected carp develop humoral antibodies that can neutralize the spread of virus and protect against re-infection.

SVCV has been registered in the List of Contagious Diseases notifiable to the OIE (see www.oie.int), and diagnostic tests have been described in the OIE Manual of Diagnostic Tests for Aquatic Animals (OIE, 2006).

SVC is caused by an RNA virus, *Rhabdovirus carpio*, a member of the genus *Vesiculovirus,* in the family Rhabdoviridae. The bullet-shaped viral particle is 70–180 nm in size and contains 5 struc-

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tural proteins: L-polymerase, spike glycoprotein G, nucleoprotein N, phosphoprotein P, and membrane protein M (Ahne et al., 2002). In rhabdoviruses, glycoprotein G forms trimeric peplomers or spikes on the surface of the virion and is responsible for its binding to cellular receptors and induction of viral endocytosis (Gaudin et al., 1992, Nagata et al., 1992; Coll, 1995; Lorenzen at al., 1999). Surface glycoprotein is an important viral antigen and its antigenicity has been extensively studied for a number of years; as a result, in rabies virus, a related rhabdovirus, two primary antigenic sites (site II and site III), one minor site and a few isolated epitopes have been defined (Dietzschold et al., 1990; Maillard and Gaudin, 2002).

Despite the fact that SVC virus is serologically distinct from the other known piscine rhabdoviruses, it shares antigenic determinants with PFRV (pike fry rhabdovirus) that are likewise classified in the *Vesiculovirus* genus, which is however, not registered in the OIE list of contagious diseases (see www.oie.int). Serological studies have confirmed cross-reactivities between PFRV and SVCV by immunofluorescence and ELISA methods (Jorgensen et al., 1989; Dixon and Longshaw, 2005). Vesiculo-like viruses have been classified by Stone et al. (2003) into four genogroups, according to the partial G-gene sequence analysis. Due to these facts, reliable molecular methods for identification of the virus have been included in the OIE fish health control programmes that require SVCV testing.

Spring viraemia of carp disease can affect entire fish culture facilities, where high density of fish is an unfavourable factor with regard to spreading of the virus. At present, no targeted prophylactic or therapeutic measures are recommended; protection of fish cultures consists exclusively in elimination of the infected fish population. Therefore, a reliable diagnosis is necessary and plays a key role in the control of infection; rapid diagnostic procedures for identification of viral pathogens are important in order to reduce losses in relation to outbreaks, and to prevent spread of the pathogens due to trade with live fish. The methods suitable for epidemiological tracing aiming at determination of the origin of infections are also required – isolation of the virus in susceptible cell lines, immunoassays such as immunofluorescence, ELISA systems and virus neutralisation and/or gene amplification asays (Rodak et al., 1993; Einer-Jensen et al., 2002). Regarding the fact that rhabdoviral glycoproteins can in most cases induce production of neutralizing antibodies in the infected host, as confirmed by data from the literature (Volk et al., 1982; Lorenzen et al., 1990; Luo et al., 1990; Ristow, 1991), there is the possibility of preparing a successful vaccine to SVC virus, i.e. recombinant subunitary vaccine.

The purpose of our study was to prepare MAbs to viral glycoprotein, to test their basic biochemical characteristics and confirm their specificity against a panel of SVCV isolates from various European regions, with special attention given to the isolates detected in the Czech Republic. Such information can be important for the next study of the epidemiology of specific viruses as well as antigenic variation and the mechanisms by which new strains arise. The initial and most widespread use could be in the development of improved diagnostic and virus identification assays.

MATERIAL AND METHODS

SVC and PFR viruses

Twenty SVCV isolates (Koutna et al., 2003) and eight PFRV isolates used in this study are described in detail in Table 1 and 4. The isolates were propagated in cell line EPC (epithelioma papulosum cyprini) (Fijan et al., 1983) in maintenance Eagle's Minimal Essential Medium (EMEM) (Sigma Biosciences, USA) containing 2% bovine foetal serum (BOFES) (GIBCO, Scotland) and a standard concentration of antibiotics (penicilin 100 IU/ml, streptomycin 100 μg/ml). The cells were cultured at 15°C for 4–7 days until the cytopathic effect (CPE) became apparent, then cell cultures were frozen until further examination. All isolates were tested by virus titration on cell line EPC.

Virus titration on cell line EPC

The cell line EPC (at cell concentration 5×10^6/ml) was cultured in Tris-(hydroxymethyl)-aminometan MEM (Minimal Essential Medium, pH 7.6), supplemented with 10% BOFES (bovine foetal serum) on 96-well microtitre plates at growth temperature 20°C. After 24-h incubation, 20 μl of pre-diluted isolates were added into the wells in descending dilutions 10^{-1}–10^{-8}, so that in corresponding wells the dilution was higher by one order of magnitude. The plates were incubated at 15°C for 7 days and examined for CPE each day. After termination

Table 1. Spring viraemia of carp rhabdoviruses (SVCV). Isolates used in this study

Isolate No.[a]	Virus isolate	Year	Host	Country of virus isolation
1	V-612	2002	carp	Czech Republic
2	V-500	1996	carp	Czech Republic
3	V-556	1999	carp	Czech Republic
4	V-609	2001	carp	Czech Republic
5	V-539	1997	carp	Czech Republic
6	V-590	1999	pike	Czech Republic
7	V-552	1999	carp	Czech Republic
8	V-541	1998	nd	Czech Republic
9	V-613	1999	carp	Czech Republic
10	V-551	1999	carp	Czech Republic
11	Fijan ref.	1969	carp	Croatia (Fijan et al., 1971)
12	287	nd	carp	nd
13	17314/5	nd	carp	Hungary
14	12840/7	nd	carp	Hungary
15	455	nd	carp	Germany
16	17312/5	nd	carp	Hungary
17	Kp 1	1984	carp	Russia
18	Zl 4	1991	carp	Russia
19	KKK	1986	carp	Russia
20	No.3	1986	grass carp	Ukraine

[a]Czech isolates 1–10 were sampled from various localities (except isolates 5 and 10 which came from the same pond) Isolates 13–16 and 18–20 were kindly provided by Dr. N. Lorenzen of the National Veterinary Institute, Technical University of Denmark, Denmark and Dr. I. Shchelkunov of the All-Russian Research Institute of Freshwater Fisheries, Russia, respectively

Year = year of virus isolation, carp = *Cyprinus carpio*, grass carp = *Ctenopharyngodon idella*, nd = no data, pike = *Esox lucius*

of culture, the virus dilution causing infection of 50% of the inoculated cell line ($TCID_{50}$) was determined using the Reed-Muench method (Mahy and Kangro, 1996).

Preparation of SVC virus for immunization of mice

The thawed SVCV 539 used for immunisation of BALB/c mice, had cell debris removed by centrifugation at 10 000g in a rotor JS 7.5 of the centrifuge J2-21 (Beckman Instruments, Palo Alto, USA). Viral suspension was then concentrated by centrifugation in a rotor Sw 28 for 1.5 h at 120 000 g at 5°C (Beckman Instruments, L8-80M). The resulting sediment was re-suspended in 1/100 of 0.15M PBS, pH 7.2 and used for immunisation of mice.

Preparation of monoclonal antibodies

BALB/c mice, aged 6–8 weeks, were immunised *i.p.* with three doses of SVCV 539 in Freund's complete adjuvans (FCA) in 21 day intervals. The last dose was administered *i.v.* and after three days mice

were sacrificied (3 *i.p.* + 1 *i.v.*). For intraperitoneal application was used dose 10 µg/mouse, for intravenous application 50 µg/mouse. Hybridomas were prepared by the Galfre and Milstein (1981) procedure using myeloma cells Sp 2/0 Ag14 (provided by the Institute of Molecular Genetics, Prague, Czech Republic). Hybridoma colonies producing antibodies to glycoprotein G were selected by ELISA, Western blotting, and administered *i.p.* to mice which had been primed with mineral oil two weeks in advance. The obtained ascitic fluids were further tested by immunoperoxidase test and ELISA for the detection of 20 different SVCV isolates.

MAb typing

The determination of class or subclass of produced MAbs was performed by the ELISA method with anti-mouse peroxidase conjugates (Mouse-hybridoma Subtyping Kit, Boehringer Mannheim) according to manufacturies recommendation.

Preparation of polyclonal antisera

Polyclonal antisera against SVCV were obtained from rabbits by the procedure described by Reschova et al. (2001). Non-specific antibodies were removed by saturation, using an immunosorbent (CNBr Sepharose 4b, Pharmacia) with bound proteins of homogenate of non-infected EPC cells (Rodak et al., 1993).

Western blot analysis

Purified SVCV 539 was solubilised in sample buffer under reducing conditions, and after separation in 10% polyacrylamide gel (Laemmli, 1970), viral proteins were transferred onto a nitrocellulose membrane (Towbin et al., 1979). After drying, the nitrocellulose strips were incubated with ascitic fluids containing MAb diluted 1:1 000. The reaction was detected using rabbit anti-mice IgG antibodies, labelled with horseradish peroxidase (DAKO Cytomation, Denmark) and visualized with carbazole substrate solution, according to manufactures instructions.

Immunoperoxidase test

MAb specificity was tested by an immunoperoxidase test using 24-well plates with an EPC cell line monolayer, infected with SVCV by a modified method of Nicholson and Henchal method (1978): fixation of infected and non infected cells was accomplishes by cold methanol. Then the endogenous peroxidase was blocked by incubation in 3% H_2O_2 in 0.1% azide PBS, washing procedure 4–5 times. Non-infected wells, treated in the same way, were used as negative controls. MAbs were tested at a dilution of 1:1 000; the conjugate used was the same as in the Western blotting procedure. The reaction was visualised with the solution of DAB substrate (3,3'-diaminobenzidin, Sigma Chemical Co, USA) and hydrogen peroxide.

Table 2. Monoclonal antibodies (MAbs) to G SVCV

MAb	Reaction against G in immunoblotting (WB)	ELISA titr	Reaction in IP test
2C1/A10/1D12	+	1:64 000	+
2C1/A10/1H11	+	1:64 000	+
2C1/A10/2G2	+	1:64 000	+
2C1/A10/1H1	+	1:128 000	+
2C1/3C9	+	1:32 000	+
2C1/3G2	+	1:128 000	+
2C1/3H1	+	1:64 000	+
4C12/3C8	+	1:102 400	+
2E1	–	1:102 400	+

Titer is the final sample dilution with absorbance value higher than 0.1 (twofold dilution started in 1:1 000)

ELISA method for the detection of SVC virus

Sandwich ELISA for the detection of SVC virus was developed. All MAbs were diluted to the same concentration of 15 µg/ml and bound to the bottom of microtitre plate wells (GAMA, Ceske Budejovice, Czech Republic). The prepared cell supernatants of SVCV isolates were applied to each MAb at a dilution of 1:2. The next layer of the enzyme-immunological reaction consisted of rabbit polyclonal antibodies against SVCV as described previously. Commercially prepared porcine antibodies against rabbit immunoglobulins, labelled with horseradish peroxidase (DAKO, Cytomation, Denmark) were used as a conjugate. The enzymatic reaction was visualised by TMB substrate with hydrogen peroxide and stopped with 1M sulfuric acid. Positive sensitivity to SVCV strains in ELISA assays were measured at 450 nm (cut-off value: $A_{450} > 0,1$).

Selected isolates of vesiculoviruses tested by ELISA method

A method for testing the cross-reactivities of MAbs with some genotypes of PFRV (Table 4) was developed. After thawing, cell debris was removed from PFRV by centrifugation at 7 000 rpm, using centrifuge J2-21 (Beckman Instruments, Palo Alto, USA) with rotor JS 7.5. Viral suspensions were pelleted using an ultracentrifuge (Beckman Instruments, L8-80M) with rotor Sw 28, for 1.5 h, at 25 000 rpm, at 5°C. The resulting pellets of the respective isolates were resuspended in 1/100 of 0.15M PBS, pH 7.2. The PFRV isolates and SVCV 539, concentrated in the same way, were diluted 1:500 and dispensed onto the bottom of microtitration plate wells (GAMA, Ceske Budejovice, Czech Republic). All MAbs diluted 1:1000 were used for screening. Commercially available horseradish peroxidase-labelled rabbit anti-mouse antibodies (DAKO Cytomation, Denmark) were used as a conjugate. The enzymatic reaction was visualised by substrate TMB with hydrogen peroxide and stopped with 1M sulphuric acid.

RESULTS

Monoclonal antibodies to SVC virus

A total of nine samples of ascitic fluid obtained from mice by immunisation with productive clones

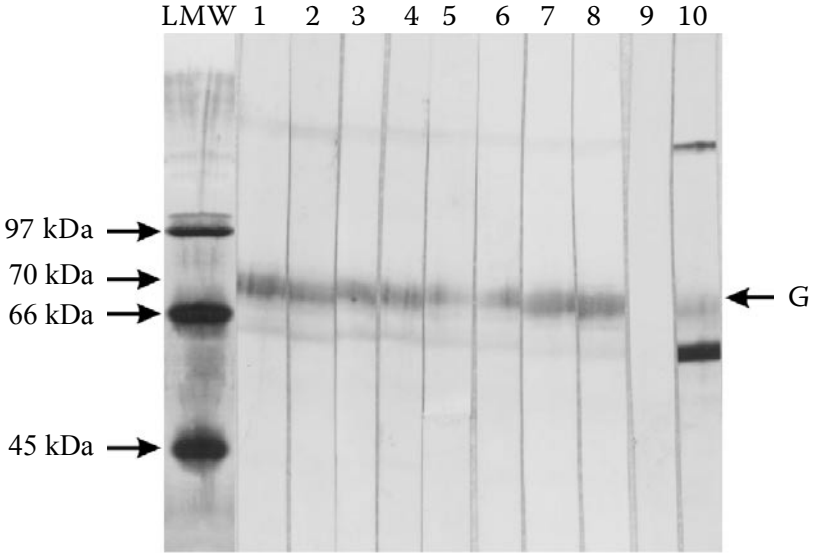

Figure 1. Western blot analysis

MAbs in dilution 1:1 000, porcine anti-mice IgG antibodies, labelled with horseradish peroxidase in dilution 1:1 000. LMW = low molecular weight standard; 1 = 2C1/A10/1D12; 2 = 2C1/A101H11; 3 = 2C1/A10/2G2; 4 = 2C1/A10/1H1; 5 = 2C1/3C9; 6 = 2C1/3G2; 7 = 2C1/3H1; 8 = 4C12/3C8; 9 = 2E1; 10 = mouse serum obtained by bleeding the immunized mice producing the hybridoms

Table 3. ELISA testing of twenty SVCV isolates with MAbs

	Distribution of viral sample absorbances (*absorbance range)			
	< 0.1	0.1–0.4	0.4–0.8	> 0.8
2C1/A10/1D12	0/20	6/20	14/20	0/20
2C1/A10/1H11	0/20	1/20	19/20	0/20
2C1/A10/2G2	0/20	9/20	11/20	0/20
2C1/A10/1H1	0/20	2/20	18/20	0/20
2C1/3C9	0/20	15/20	5/20	0/20
2C1/3G2	0/20	1/20	19/20	0/20
2C1/3H1	0/20	2/20	18/20	0/20
4C12/3C8	9/20	8/20	3/20	0/20
2E1	0/20	16/20	4/20	0/20
Polyclonal Ab	0/20	0/20	9/20	11/20

*A_{450nm} values. ELISA definition of cut-off value: A_{450nm} 0.1; for negative control (non infected EPC cell supernatant) absorbance values were in the range 0.015–0.039. MAbs concentration 15 μg/ml, dilution of viral samples 1:2

were tested. Affinity of MAb to structural glycoprotein G was demonstrated in eight of the nine fluids using Western blotting. One MAb (2E1) did not show reactivity with any viral protein (Figure 1).

All obtained MAbs were of the type IgG, subclass IgG_1. Their titres, which ranged from 1:32 000 to 1:128 000 (Table 2), were determined by ELISA. No cross reaction with IHNV, IPNV or VHSV (standard infected cell supernatants 10^{-5} $TCID_{50}$/ml) was observed in any of the resulting MAbs. Their specificity was further confirmed in immunoperoxidase test.

MAbs were used to determine their sensitivity to all of the SVCV strains (Table 3). All MAbs were analysed by means of ELISA method with the aim to detect serological reaction with other piscine

Table 4. Detection of selected fish vesiculoviruses using MAbs against spring viraemia of carp (SVCV) by ELISA method

Virus isolate	Host name	Initial identifitation	MAbs*	MAb**	Genogroup[a]
F4	*Esox lucius* L.	PFRV	+	–	III-according to Stone et al. (2003)
Hecht	*Esox lucius* L.	PFRV	–	–	nd
3605	*Ctenopharyngodon idella* V.	PFRV	–	–	nd
GRV	*Ctenopharyngodon idella* V.	PFRV	–	–	nd
14241/6	*Silurus glanis* L.	PFRV	–	–	nd
14249/5	*Silurus glanis* L.	PFRV	–	–	nd
332	*Blicca bjoerkna* L.	PFRV	–	–	IV-according to Stone et al. (2003)
V-539	*Cyprinus carpio*	SVCV	+	+	SVCV according to Koutna et al. (2003)

*MAbs: 2C1/A10/1D12, 2C1/A10/1H11, 2C1/A10/2G2, 2C1/A10/1H1, 2C1/3C9, 2C1/3G2, 2C1/3H1, 4C12/3C8 in dilution 1:1 000

**MAb: 2E1 in dilution 1:1 000

[a]isolates PFRV were kindly provided by the National Veterinary Institue, Technical University of Denmark

+ = positive reaction, – = negative reaction, nd = no data

vesiculoviruses (Table 4). Only 2E1 did not show any cross-reactivity with PFRV (classified in genogroup III–IV by Stone et al. (2003) and the others previously identified as PFRV-like by Jorgensen et al., 1989), the others MAbs reacted positively with PVRV F4 reference strain, isolated from *Esox lucius* L. All MAbs reacted with a Czech SVCV isolate; its identity was confirmed by means of RT PCR assay (Koutna et al., 2003). Specificity of primers for this method was verified by the use of 5 out of 7 PFRV strains described by Jorgensen et al. (1989).

DISCUSSION

Cultivation of field collected fish organ samples to corresponding cell lines is particularly important in virological diagnosis of fish infections. Due to the small amount of virus in tissues, passage of the sample is needed in most cases, so that the virus can be replicated to an amount detectable by other methods. The next stage is viral identification, as CPE are similar for several fish viruses. The most widely used method, based on antibody – antigen detection, is the enzyme-linked immunosorbent assay (ELISA), whose major advantage is the examination of a large number of samples in a minimum amount of time. Sensitivity and specificity of the assay are based on the quality of the reagents used. When polyclonal antibodies are used, they often exhibit high non-specificity in the background; their reactions with cell components can also lead to false positive results. Therefore, it is useful to utilise monoclonal antibodies (MAbs) against particular antigenic epitopes in ELISA and other assays. One advantage of MAbs is the continuous production of large quantities of stable antibodies, which is a prerequisite for the standardisation of the evaluation method. In our study, the characterisation of the prepared MAbs and their use in SVCV diagnosis were evaluated.

Eight MAbs out of nine showed affinity to the structural glycoprotein G (molecular weight 70 kDa) in WB; only one monoclonal antibody (2E1) did not exert any reaction in this method. The reason might be, as stated by Nagata et al. (1992), dependence of linear epitopes on the secondary structure of the virion. The majority of anti-G MAbs obtained by the immunisation of mice can recognise the structure of appropriate epitopes. However, in some cases, overlapping epitopes of various degrees, may inhibit or decrease antibody-antigen binding. Destabilisation of trimeric G occurs under changed external conditions (pH, detergents, temperature), with consequent dissociation to a monomeric form and alterations in spatial arrangements of G protein epitopes (Gaudin et al., 1992; Coll, 1995). Most probably, broad antigenic variations exist regarding virion configuration (Luo et al., 1990; Ristow, 1991), therefore neutralisation mechanisms have not been clarified yet. Interestingly, MAb 2E1 did not react in Western blotting even after separation with non-reducing conditions.

SVCV isolates were examined by ELISA using selected MAbs. Comparison of absorbance values in the examined strains revealed marked differences among the monoclonal antibodies, the most pronounced difference being in MAb 4C12/3C8, whose sensitivity was very low for most of the viral isolates. In the other MAbs, the lowest absorbance value obtained exceeded the cut-off limit for positive evaluation. Absorbance values of the examined strains, obtained by means of polyclonal antibody, were markedly higher in all viral isolates. However, different antibodies bind to different viruses and this binding depends on the number of isotypes and antibodies with various avidites as well as multiple antibodies specificities. Most theories involve either steric hindrance resulting from the antibody blocking critical sites on the surface of virion, or molecular perturbation of the viral capsid after interaction with an antibody (Volk et al., 1982).

The ELISA method can only demonstrate the presence of viral immunogenic particles, and not infectivity of the virus, therefore, no significant correlation was attributed to infectious titre of particular isolates and the ELISA test, due to differences existing in relative amounts of incomplete or decomposed viral particles (Ristow, 1991).

Cross-reactivity with PFRV isolates was tested with MAbs. SVCV and PFRV share antigenic determinants with G, N and M proteins and their differentiation by means of serologic tests using polyclonal or monoclonal antibodies is often problematic. Recent studies (Ahne et al., 2002) documented that PFRV can be distinguished by means of ribonuclease protection assay, using a G-gene probe. Stone et al. (2003) used sequence analysis of 550 nucleotide domains of G-gene for comparison of 36 SVCV and PFRV isolates from various fish species. Based on this analysis, the isolates have been classified in four genogroups. In the present study, we used PFRV isolates from groups III–IV

and PFRV-like isolates. All MAbs cross-reacted with reference strain PFRV F4, only MAb 2E1 did not show any cross-reactivity with pike fry rhabdovirus (PFRV). Indirect ELISA was selected for this test; moreover for the indirect ELISA test, sample viruses are coated directly on the ELISA plate and the investigated MAbs are used as detection antibodies. Despite the fact that a generally lower sensitivity is known for this type of method, in comparison with capture ELISA (Shieh and Chi, 2005), we used this procedure for determination of cross-reactivity with PFRV as we did not intend to use secondary polyclonal antibodies against SVC virus that may markedly increase non-specificity of the entire reaction. However, the facts mentioned above do not diminish the significance of the prepared MAbs from an aspect of detected specificity, because an exact identification of SVCV isolates was performed by validated serological methods and by means of RT-PCR. Due to the fact that another heterologous reaction with the other rhabdoviruses was not detected, we expect that MAbs prepared in our laboratory might be applied in experimental studies using the verified SVCV isolates. It is not negligible that the use of MAbs, prepared in our laboratory, within long-term experimental studies may result in a decrease of financial costs, which would otherwise have paid for commercial kits that may be quite expensive.

MAbs were prepared by *in vivo* cultivation of hybridomas in the peritoneal cavity of mice as ascitic tumours, which produced antibody concentration of the order of 20 mg/ml. The simplicity, efficiency and high concentration of the final product made ascitic fluid the preferred choice for our study requiring relatively small amounts of a large number of MAbs. However, this method of producing the MAbs is ethically very questionable and should not be used in the future.

Testing of SVCV isolates originating from different locations and countries, confirmed also the presence of a common glycoprotein G, which is responsible for virus attachment to cell receptors, and for reaction with neutralising antibodies (Coll, 1995). For diagnosis by within immunological reactions, antigen – antibody binding, combination of MAbs with a wider range of structural antigens, or combination with a polyclonal antibody might be more significant, however, in our study we focused on MAbs against glycoprotein G, which offer the possibility of developing vaccination against spring viraemia of carp.

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