

Development and characterization of monoclonal antibodies to spring viraemia of carp virus

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Abstract

Five monoclonal antibodies (mAbs) against spring viraemia of carp (SVCV0504, isolated from common carp in China) were produced from mice immunized with purified virus preparations. The virion of SVCV contains five structural proteins, representing the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA-dependent RNA polymerase (L). Western blotting analysis revealed that three mAbs (1H5, 1E10, and 1H7) recognized specifically to a single protein of 47 kDa (N), the mAb 3G4 reacted with two SVCV0504 proteins of 69 kDa (G) and 47 kDa (N), while the mAb 1A9 reacted with three SVCV0504 proteins of 69 kDa (G), 50 kDa (P), and 47 kDa (N). By indirect ELISA, two mAbs (1H5 and 1H7) showed cross-reactivity with pike fry rhabdovirus (PFRV), but no cross-reactions with the *Siniperca chuatsi* rhabdovirus (SCRV), *Scophthalmus maximus* rhabdovirus (SMRV), *Paralichthys olivaceus* rhabdovirus (PoRV) were demonstrated with the five mAbs. Indirect immunofluorescence showed intense fluorescence in the cytoplasm of the SVCV0504-infected epithelioma papulosum cyprini (EPC) cells in areas corresponding to the location of granular structures. The sucrose gradient-purified SVCV0504 particles could be detected successfully by these mAbs using immunodot blotting. mAb 1A9 could completely neutralize 100 TCID₅₀ (50% tissue culture infective dose) of SVCV0504 at a dilution of 1:8. This is the first report of development of the neutralizing mAbs against SVCV. The mAb 1A9 was analyzed further and could be used to successfully detect viral antigens in the infected-EPC cell cultures or in cryosections from experimentally infected crucian carp (*Carassius auratus*) by immunohistochemistry assay. Furthermore, a flow cytometry procedure for the detection and quantification of cytoplasmic SVCV0504 in cell cultures was developed with mAb 1A9. At 28 h after inoculation with the virus (0.01 PFU/cell), 10.12% of infected cells could be distinguished from the uninfected cells. These mAbs will be useful in diagnostic test development and pathogenesis studies for fish rhabdovirus.

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1. Introduction

Spring viraemia of carp (SVC), an important disease affecting cyprinids, mainly common carp (*Cyprinus*

carpio), originally occurred in European carp culture (Ahne et al., 2002), recently to USA (Goodwin, 2002; Dikkeboom et al., 2004; Warg et al., 2007), China (Liu et al., 2004) and Canada (Garver et al., 2007). The causative agent of SVC is a rhabdovirus, spring viraemia of carp virus (SVCV), which is presently classified as a member of the genus *Vesiculovirus* of the family *Rhabdoviridae* (Fauquet et al., 2005). The virus causes an acute hemorrhagic and contagious disease

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that has led to SVCV infections being listed as notifiable in the International Aquatic Animal Health Code of the Office International des Epizooties (OIE, 2006). The properties, epizootiology, experimental infection, in vitro multiplication, serological examination, and genomic sequence of SVCV have been well studied (Ahne et al., 2002; Stone et al., 2003; Hoffmann et al., 2002; Teng et al., 2007). As with other members of the genus, the virion of SVCV contains five structural proteins: N protein (nucleoprotein), P protein (phosphoprotein), M protein (membrane protein), G protein (glycoprotein) and L protein (RNA-dependent RNA polymerase) (Hoffmann et al., 2002; Teng et al., 2007).

Outbreak of SVCV can result in great economic losses and restrict the development of fisheries. Although some researches had been showed that DNA vaccines could elicit a protective immunity against the SVCV in experimental fish (Kanellos et al., 2006), there is no commercially available vaccines or antiviral drugs to control SVCV infection. Attempt to prevent and control SVCV relied primarily on detection, diagnosis and removal of infected fish. Many methods have been developed for detection and diagnosis of SVCV, including cell culture isolation and recognition of virions by electron microscopy, immunoassays such as immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry, and PCR techniques (Ahne et al., 1998, 2002; Oreshkova et al., 1999; Koutná et al., 2003). Monoclonal antibodies (mAbs), as useful tools, have been used widely for development of effective diagnosis of fish viral pathogens, such as infectious hematopoietic necrosis virus (IHNV; Schultz et al., 1985), infectious pancreatic necrosis virus (IPNV; Wolski et al., 1986), viral hemorrhagic septicemia virus (VHSV; Sanz et al., 1992), epizootic hematopoietic necrosis virus (EHNV; Monini and Ruggeri, 2002), and so on. Concerning SVCV, mAbs specific against the SVCV had been recently developed and applied to detect the viral antigens by sandwich ELISA (Bing et al., 2007; Reschova et al., 2007). However, problems with mAbs specificity still need to be resolved (Dixon and Longshaw, 2005; Reschova et al., 2007), and precise reports on immunoassays such as virus neutralization assay, immunodot blotting and immunohistochemistry assay with mAbs against SVCV were rare. Furthermore, neutralizing mAbs against SVCV have yet not been reported.

In this study, we describe the production and characterization of five novel mAbs directed against spring viraemia of carp (SVCV0504, isolated from common carp in China). One neutralizing mAb was

obtained and applied successfully to detect virus antigens in the cell cultures and experimental fish tissues by flow cytometry and immunocytochemistry assays.

2. Materials and methods

2.1. Virus strains

Five rhabdovirus strains, including spring viraemia of carp virus (SVCV0504, isolated from common carp in China, Chen et al., 2006), pike fry rhabdovirus (PFRV, isolated from pike, Wolf, 1988), *Siniperca chuatsi* rhabdovirus (SCRV, isolated from mandarin fish, Tao et al., 2007), *Scophthalmus maximus* rhabdovirus (SMRV, isolated from turbot, Zhang et al., 2007), and *Paralichthys olivaceus* rhabdovirus (PoRV, isolated from flounder, Gui et al., 2007), were used in this study.

2.2. Cell culture and SVCV0504 purification

Epithelioma papulosum cyprini (EPC) cells were maintained in medium 199 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum at 25 °C to propagate the SVCV0504. Production of SVCV0504 stocks and purification were performed as following steps. Infected cell cultures showing cytopathic effects (CPE) were freeze-thawed thrice. Cell debris was removed by centrifugation at 4000 × g for 20 min and the supernatant was then ultracentrifuged in a Beckman SW41 centrifuge at 32,000 rpm for 2 h. The virus pellet was resuspended in PBS (1.4 M NaCl; 0.014 M KH₂PO₄; 0.08 M Na₂HPO₄) and further purified by using discontinuous sucrose (20, 30, 40, and 50%, w/v) gradient centrifugation at 30,000 rpm for 1.5 h. The virus particle band was collected, and sucrose was removed by centrifugation at 32,000 rpm for 2 h in PBS. The purified virus was finally resuspended in PBS and stored at –20 °C until used.

2.3. Monoclonal antibody production

Eight-week-old BALB/C mice were each immunized intraperitoneally with a mixture containing 250 μl purified SVCV0504 (containing 100 μg viral protein) and an equal volume of Freund's complete adjuvant (Sigma, St. Louis, MO). On days 14, 28 and 42 after the initial injection, booster immunizations were administered with the same amount of antigen in Freund's incomplete adjuvant. Three days before cell fusion, a final booster was given via intrasplenic

injection to each mouse with 200 μl purified virus (containing 75 μg viral protein) without adjuvant. Hybridoma fusion was performed using the method described earlier (Zhou et al., 2006). Using purified SVCV0504 (1:100 diluted in PBS) as antigen, antibodies produced in hybridomas supernatants were measured by indirect ELISA. The indirect ELISA principally comprised the following steps: ELISA plates were coated overnight at 4 °C with 100 μl per well of purified SVCV0504 at 5 $\mu\text{g ml}^{-1}$ diluted in bicarbonate coating buffer (pH 9.6) and then blocked with 5% bovine serum albumin (BSA) in PBS for 30 min at 37 °C. The wells were drained and incubated with hybridomas supernatants (original, 100 μl per well) for 1 h at 37 °C. After three washes with PBS containing 0.05% Tween-20 (PBS-T), horseradish peroxidase conjugated goat anti-mouse IgG (GAM-HRP, 1:2000 diluted in PBS-T) was added and incubated for 1 h at 37 °C. After washing, substrate solution (0.1 M citrate/phosphate buffer, pH 5.0; 0.04% OPD; 0.14% H_2O_2) was applied for 15 min at room temperature. Reactions were stopped by addition of 50 μl per well 2 M H_2SO_4 and optical densities (ODs) were measured at 492 nm with a microplate reader (Bio-Rad, Hercules, CA). Absorbance values twice higher than the background level reactivity on BSA were considered to be positive.

Positive hybridomas were subsequently cloned three times by limiting dilution with one cell distributed per well, and the screening was repeated until hybridoma clones producing a strong reactivity with SVCV0504 were observed. The mAbs isotypes were determined using the mouse monoclonal antibody isotyping kit (Sigma) according to the instructions of the manufacturer.

2.4. Virus neutralization assay

The virus neutralization assay was carried out on 24-well cell culture plates. Two hundred microlitres of SVCV0504 (4×10^2 TCID₅₀) was mixed with an equal volume of serial 2-fold dilutions (from 1:2 to 1:64) of supernatants of mAbs or the mock medium (negative control). The mixtures were incubated at 25 °C for 1 h, and then added into EPC cells in 24-well plates, 100 μl per well. After 1 h adsorbing at 25 °C, growth medium with 5% fetal calf serum was added and the plates were incubated at 25 °C for 6 days. The plates were examined microscopically every day for evidence of CPE. For 1A9, the plate was fixed with formaldehyde by 6 days post-infection, and then stained with crystal violet.

2.5. Immunodot blotting and Western blotting

Polyvinylidene difluoride (PVDF) transfer membrane (Millipore, Bedford, MA) was cut into strips according to the number of antigens and samples to be tested and washed for 15 min in PBS-T with agitation. The membrane was then immersed in transfer buffer (25 mM Tris; 192 mM glycine) for 30 min and placed onto filter papers. After air drying, 1 μl sucrose-purified SVCV0504 and normal EPC cell lysates (negative control) were dotted onto the membrane and incubated at room temperature overnight. Antigen-blotted membranes were blocked with 2% BSA in PBS for 30 min at room temperature. The strips were saturated with mAbs (1:50 diluted in PBS) or mouse polyclonal antibodies (pAbs) against SVCV0504 diluted 1:100 in PBS and incubated at room temperature for 1 h. After washing, the strips were incubated with alkaline phosphatase-conjugated goat-anti-mouse IgG (GAM-AP, 1:2000 diluted in PBS) for 1 h at room temperature, and washed three times as described above. Reactions were developed with substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 5 min, and stopped by rinsing the strips with distilled water.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified virus was performed according to the methods as described before (Tao et al., 2007). The gels were stained with Coomassie blue R-250 or blotted onto PVDF transfer membrane. The membranes were blocked with 5% skim milk in TBS (0.02 M Tris-HCl pH 7.4; 154 mM NaCl) for 1 h, then washed with TBS containing 0.1% Tween-20 (TBS-T) three times, incubated for 1 h at room temperature with mAbs (1:50 diluted in PBS) or mouse pAbs (1:1000 diluted in PBS), respectively. The bound antibodies were detected by GAM-AP diluted 1:2000 in TBS-T for 1 h at room temperature, and washed three times in TBS-T, followed by substrate NBT/BCIP. The color development reaction was halted at the desired level by rinsing strips with distilled water.

2.6. Determination of mAbs specificity

The indirect ELISA described above was modified to testing the specificity of mAbs. SVCV0504 viral suspensions were coated on ELISA plates with the same does (1×10^6 TCID₅₀) as PFRV, SCR, SMRV, and PoRV viral suspensions. Normal cell suspensions serving as negative controls were done in the same way. All mAbs diluted 1:50 were used for screening. The steps of indirect ELISA referred to the procedures

described in Section 2.3. Absorbance values twice higher than the background level reactivity on normal cell suspensions were considered to be positive.

2.7. Immunoperoxidase staining and indirect immunofluorescence assay (IFA) of SVCV0504-infected cells

EPC cells grown in a 24-well plate were infected with SVCV0504 at a multiplicity of infection (MOI) of 0.01 and incubated at 25 °C. After 0, 6, 12 and 24 h post-infection, monolayers were then rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 1 h at 4 °C, followed by permeabilization with absolute ethanol for 7 min at –20 °C. After washing, undiluted mAb 1A9 and GAM-AP (1:1000 diluted in PBS) were successively applied to the monolayers and incubated at 37 °C for 1 h, respectively. After each incubation, cells were washed three times with PBS. The presence of virus replication foci was shown by reaction with fresh substrate solution NBT/BCIP.

For indirect immunofluorescent staining, paraformaldehyde-fixed cells grown on glass coverslips were first incubated with mAbs (original), subsequently stained with FITC-conjugated anti-mouse IgG antibody (Pierce Biotechnology, Rockford, IL) diluted 1:200 in PBS. After washing, the stained slides were mounted in buffered glycerin and examined under a fluorescence microscope (Leica Microsystems).

2.8. Immunohistochemistry

Each crucian carp (weight of about 5 g) of the infection group was injected intraperitoneally with 0.2 ml of SVCV0504 (1×10^7 TCID₅₀). Six kinds of tissue (gills, spleen, kidney, heart, liver, and intestine) were collected 8 days post-infection. The tissues were fixed in 4% paraformaldehyde overnight at 4 °C and subsequently saturated with 30% sucrose. Cryosections (about 6 μm thick) were prepared from these tissues on a Leica CM 1900 microtome (Leica, Germany). Sections were then transferred to microscope slides treated with poly-L-lysine. Two incubations were successively performed for 1 h each with mAb 1A9 (1:100 diluted in PBS) and GAM-HRP diluted 1:1000 in PBS. After antibody incubations, three washes in PBS were carried out. The sections were then incubated with Hoechst 33258 (a kind of fluorescent dye to detect cell nuclei) diluted 1:1000 in PBS for 15 min at room temperature. Peroxidase reaction was developed with freshly prepared sub-

strate solution (0.1 M citrate/phosphate buffer, pH 5.0; 0.04% OPD; 0.14% H₂O₂). Healthy crucian carp tissues serving as negative controls were the same as above.

2.9. Flow cytometry (FCM)

The flow cytometry procedure was performed according to the method described by Qin et al. (2005). EPC cells were infected with SVCV0504 at an m.o.i. of 0.01 and incubated at 25 °C. After 28 h post-infection, the cells were fixed in 1% paraformaldehyde with 0.2% Tween-20 and incubated at 4 °C overnight, following washed with PBS and blocked with PBS containing 1% BSA. The permeabilized cells were incubated with mAb 1A9 diluted 1:100 in PBS for 1 h at 37 °C, washed three times in PBS, then incubated with FITC-conjugated anti-mouse IgG antibody diluted 1:200 in PBS for 1 h at 37 °C. The stained cells were washed again and resuspended in 1 ml PBS containing 0.5% BSA. The uninfected EPC cells were also done as control. The infection level was analyzed by flow cytometry (Epica Altra; Beckman Coulter, Fullerton, CA).

3. Results

3.1. Generation of mAbs against SVCV0504

A number of successful fusions were achieved using spleen cells from mouse immunized with purified SVCV0504 as described under methods. Approximately 80% of fusion rate (120 hybridoma cultures) was achieved in seeded wells. There were 56 hybridomas producing antibodies against SVCV0504 detected by indirect ELISA. Five mAbs (1H5, 1E10, 1H7, 3G4, and 1A9) were eventually isolated and expanded for further characterization study. Isotype determination revealed that 1E10 and 1A9 were of subclass IgG2b, 1H7 and 3G4 were of subclass IgM, and 3G2 was of subclass IgG1 (Table 1).

3.2. Titration and virus neutralizing reactivities of mAbs

The ELISA titers of mAbs ranged from 1:256 to 1:2048 (Table 1). Only mAb 1A9 had neutralizing ability against SVCV0504 and could be diluted to 1:8 and completely neutralize infectivity of SVCV0504. When diluted to 1:16, even to 1:32, the mAb 1A9 still showed partial neutralizing activity to SVCV0504 (Fig. 1).

Table 1
Characterization of mAbs to SVCV0504 with different methods

mAb	Isotype	Different methods			
		ELISA titer	Neutralizing activity	Western blotting	Immunofluorescence
1A9	IgG2b	1:2048	+	N, P, G	+
1E10	IgG2b	1:1024	–	N	+
3G4	IgM	1:1024	–	N, G	+
1H5	IgG1	1:512	–	N	+
1H7	IgM	1:256	–	N	+

3.3. Immunodot blotting and Western blotting

For immunodot blotting assay, mouse pAbs and five mAbs showed positive reactions with SVCV0504, except that the mouse pAbs displayed faint cross-reactivity with normal EPC lysates, no positive signal was observed in normal EPC lysates with mAbs (Fig. 2).

Five viral structural proteins-L (238 kDa), G (69 kDa), P (50 kDa), N (47 kDa) and M (25 kDa) were detected from Coomassie blue staining of purified SVCV0504 (Fig. 3A). By Western blotting analysis, all five mAbs reacted with the N protein, 3G4 and 1A9 could also bind to the G protein, except that mAb 1A9 still reacted with the P protein. The mouse pAbs presented more than 10 positive binds in Western blotting including five viral proteins bands and EPC cellular proteins (Fig. 3B).

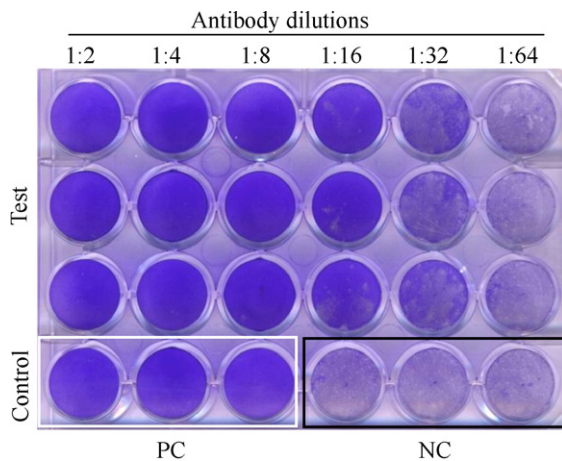


Fig. 1. Virus neutralization analysis of the mAb 1A9. Serial 2-fold dilutions (from 1:2 to 1:64) of the mAb 1A9 were reacted with equal volumes of suspension containing 1×10^2 TCID₅₀ (50% tissue culture infective dose) of SVCV0504, then added to EPC cells in 24-well plate. After 6 days post-infection, the plate was fixed with formaldehyde and then stained by crystal violet. PC: positive control (white box); NC: negative control (black box).

3.4. Cross-reactivity of mAbs

By indirect ELISA, all mAbs reacted positively with SVCV0504, and two mAbs (1H5 and 1H7) showed positive reactions with PFRV, whereas no positive reactions with SCR, SMRV, and PoRV were detected with all five mAbs (Table 2). The results indicated that mAbs 1H5 and 1H7 had cross-reactivity between SVCV0504 and PFRV.

3.5. Immunostaining of SVCV0504-infected EPC cells

Immunostaining of SVCV0504-infected EPC monolayers with mAb 1A9 at different times during infection revealed a progressive development of distinct foci of virus replication within the layer of host cells, indicating that the antibodies recognized proteins of viral origin which are present inside of cells as early as 6 h post-infection. The infected cell became rounded and

Table 2
Cross-reactivity of mAbs against SVCV0504 by indirect ELISA

Virus isolates	mAbs				
	1A9	1E10	3G4	1H5	1H7
SVCV0504	+	+	+	+	+
PFRV	–	–	–	+	+
SCR	–	–	–	–	–
SMRV	–	–	–	–	–
PoRV	–	–	–	–	–

+: positive reaction; –: negative reaction.

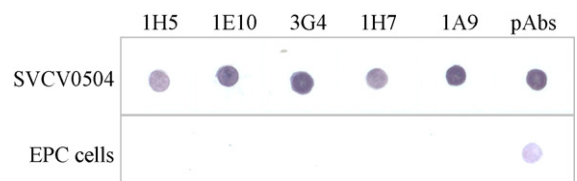


Fig. 2. Detection of SVCV0504 from sucrose gradient-purified viral particles with different mAbs and mouse polyclonal antibodies (pAbs) by immunodot blotting analysis.

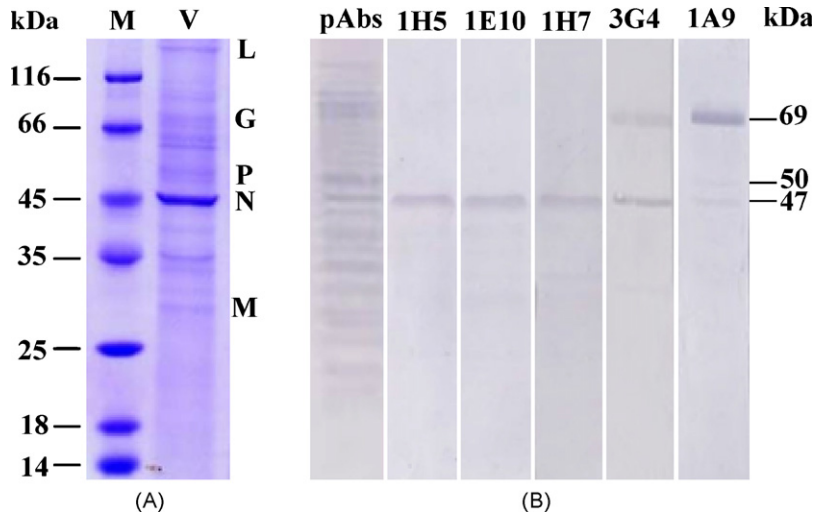


Fig. 3. SDS-PAGE and Western blotting analysis of SVCV0504 proteins. (A) SDS-PAGE gel stained with Coomassie blue. M, molecular weight marker; V, purified SVCV0504. (B) Western blotting analysis of SVCV0504 with mAbs (1H5, 1E10, 1H7, 3G4 and 1A9) and mouse polyclonal antibodies (pAbs).

desquamated from the monolayers by 12 h post-infection. Until 24 h after infection, more and more positive signals (dark color) were observed in the infected cells. Normal EPC cells displayed compact monolayer, and no positive signals were demonstrated (Fig. 4).

All of five mAbs showed positive reaction by IFA of virus infected EPC cells. The specific fluorescence signals dispersed in the cytoplasm and granular

structures around the peripheral zone of cells cytoplasm were observed. No fluorescence staining was observed in negative control cells (Fig. 5).

3.6. Immunohistochemistry

Immunohistochemistry with mAb 1A9 on frozen tissue sections from experimentally SVCV0504-infected

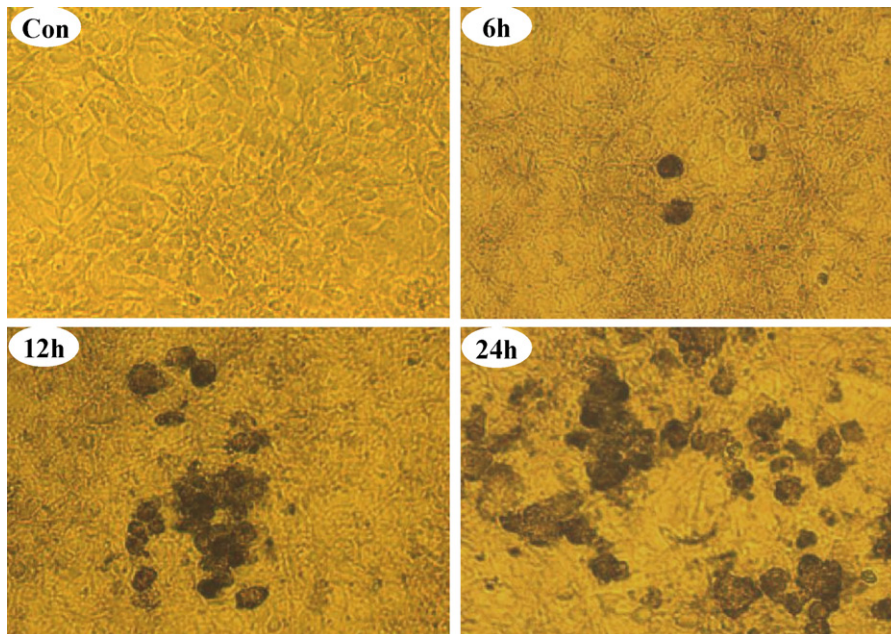


Fig. 4. Immunoperoxidases staining of SVCV0504-infected EPC cells at different times post-infection with an m.o.i. of 0.01 PFU/cell. Photographs, showing the progressive development of foci of virus replication, were taken at 6, 12 and 24 h post-infection (200×). Con: normal EPC cells.

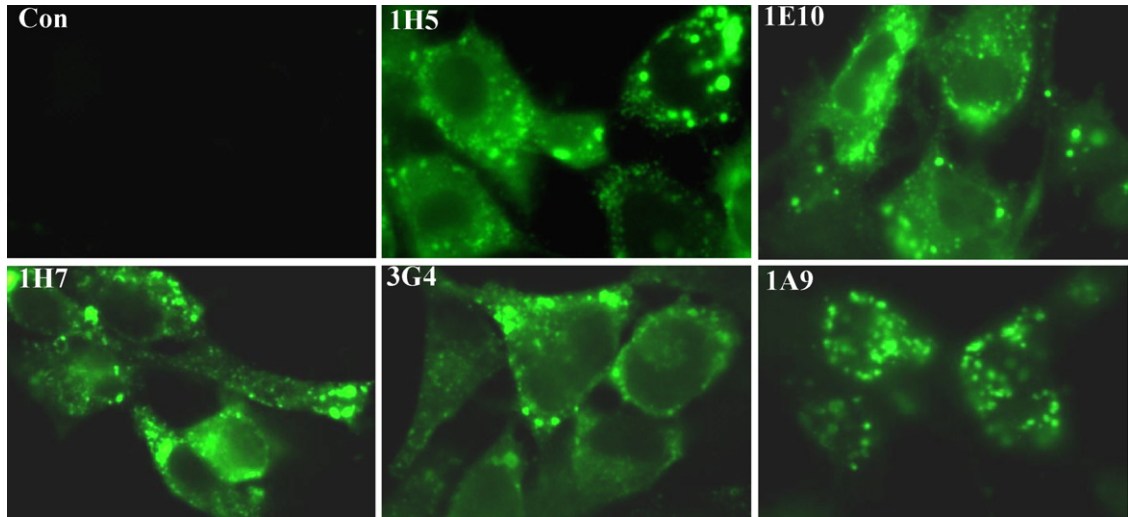


Fig. 5. Immunofluorescence staining of SVCV0504-infected EPC cells with different mAbs. Con: normal EPC cells. Photographs were taken at 24 h post-infection (1000×).

crucian carp (*Carassius auratus*) revealed positive reactions in gill, kidney, heart, liver, spleen, and intestine. The results of the infected gills and kidney were shown in Fig. 6. Normal gill cells arrangement appeared to the regular state and no positive signal was seen (Fig. 6A),

but infected gill cells were damaged and strong positive signals were observed in the area near the epidermis (Fig. 6C). Compared to the normal gill cells (Fig. 6B), the nuclei of infected gill cells disappeared (Fig. 6D). In the normal kidney, the tubules displayed a round or elliptical

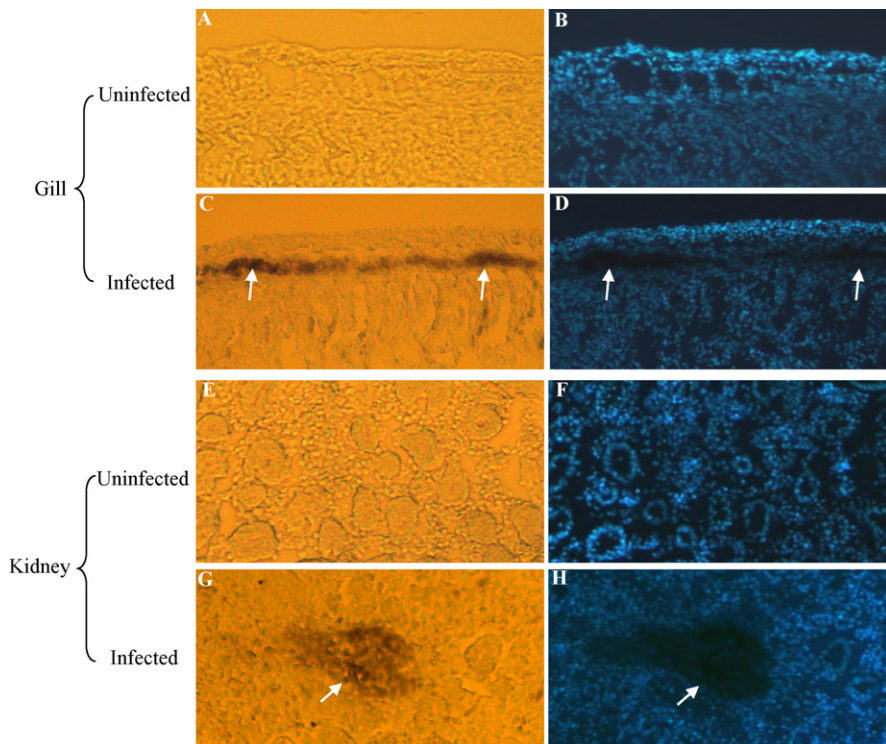


Fig. 6. Immunohistochemical analysis of tissue cryosections from fish infected with SVCV0504 by light microscopy (left) and fluorescence microscopy (right). Positive signals (arrows) were showed in the hemocytes and necrotic cells of infected gill and kidney (C and G, respectively), and the nuclei of infected cells were even undetectable (D and H, respectively). No positive reactions appeared in normal crucian carp gill (A and B) and kidney (E and F). Original magnification, 100×.

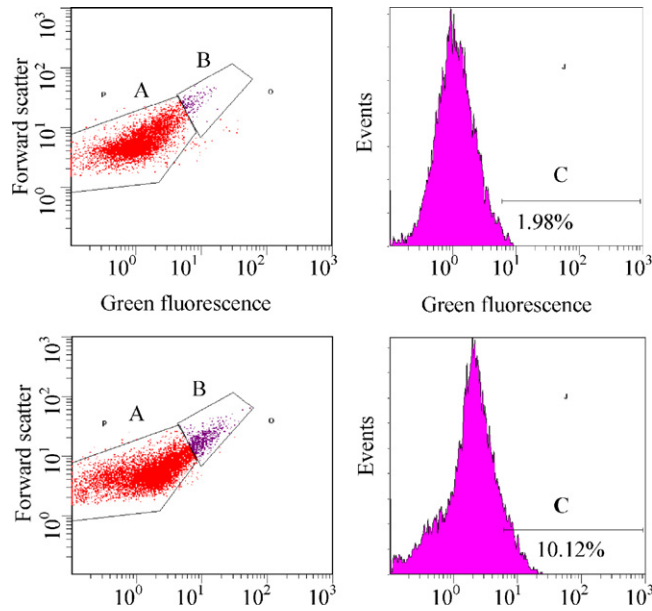


Fig. 7. Flow cytometry results of the pathogenic SVSV0504 in infected EPC cells 28 h post-infection. Top: normal cell culture, control uninfected with virus. Bottom: SVCV0504-infected cells 28 h post-infection, virus-infected cell population (gated area of B) could be distinguished from uninfected cells (gated area of A), and 10.12% of EPC cells (scale of C) were infected with SVCV0504. Each histogram contains 100,000 events.

shape and were arrayed regularly, and no positive signal was observed (Fig. 6E), whereas the tubules of infected kidney were damaged and immunostained positively (Fig. 6G). The nuclei of infected kidney cells were not visible (Fig. 6H) in comparison with the normal kidney cells (Fig. 6F).

3.7. Flow cytometry

The percentage of SVCV0504-infected cells was determined using flow cytometry with mAb 1A9. Gated areas A and B in Fig. 7 represent uninfected and infected cell populations, respectively, and the scale of histogram portions C denotes the percentage of infected cells. At 28 h post-infection, the infected cell population (gated area B) could be distinguished from uninfected cells (gated area A), and 10.12% of EPC cells (scale of C in Fig. 7, bottom) were infected with SVCV0504, whereas approximately 16,000 cells were counted. The corresponding value of control EPC cells came to only 1.98% in view of the unspecific reaction.

4. Discussion

Five monoclonal antibodies against SVCV0504 were established, and identified as belonging to three subclasses (IgG1, IgG2b and IgM), and two mAbs (1H5 and 1H7) showed cross-reactivity with pike fry rhabdovirus (PFRV). Among five mAbs, the mAb

1A9 showed neutralizing activity to SVCV0504. This is the first report of development of the neutralizing mAbs against SVCV. Neutralizing mAbs have been applied in the study of rhabdoviral proteins structure and function and in defining neutralizing epitopes on the mammalian rhabdoviruses, rabies virus (Lafon et al., 1983; Benmansour et al., 1991) and vesicular stomatitis virus (VSV; Lefrancois and Lyles, 1982; Luo et al., 1988). With regard to fish rhabdoviruses, neutralizing mAbs also have been developed against isolates of IHNV (Winton et al., 1988; Ristow and Arnzen, 1991; Huang et al., 1994), VHSV (Lorenzen et al., 1988, 1990) and SHRV (Kasornchandra et al., 1992), which all belong to the *Novirhabdovirus* genus, whereas neutralizing mAbs against viruses from the *Vesiculovirus* genus have not been reported. The rhabdoviral G protein has been confirmed that it was able to induce neutralizing antibodies (Coll, 1995). Different from the previously reported, the mAb 1A9 could recognize three structural proteins (G, N and P) by Western blotting, suggesting that the mAb 1A9 may be useful in developing specific and sensitive diagnostic assays, providing potential information about the nature of the epitopes on the SVCV and understanding virus host cell interactions.

All of five mAbs could recognize the N protein of SVCV0504 in Western blotting, suggesting that the N protein played an important role in the viral antigens which induce production of antibodies. Generally, one mAb reacts with one protein in Western blotting

analysis, but in the present work, two mAbs (3G4 and 1A9) reacted with more than one protein. The inconsistency is probably due to the complexity of the antigens, suggesting that mAbs may identify a common epitope present in more than one protein. Similar results were reported in mAbs studies against SGIV (Shi et al., 2003), LCDV (Cheng et al., 2006), and SMRV (Zhou et al., 2006). Immunofluorescence assay (IFA) can provide visualized evidence regarding epitopes localization in infected cells. By IFA, the mAbs gave distinct immunofluorescent staining patterns characterized by dispersing in the cytoplasm and intense staining of cytoplasmic granular structures in SVCV0504-infected EPC cells, suggesting that these three viral proteins were localized within the cytoplasm of infected cells. The model for rhabdoviral replication displays that virus replication occurs in some sites or microdomains of the cytoplasm that are favorable for rhabdovirus assembly and sometimes appears as characteristic cytoplasmic inclusion bodies (Ni et al., 1996; Jayakar et al., 2004). The fluorescent staining patterns observed in the present study were consistent with these conclusions.

Monoclonal antibodies have been used in protocols developed for the rapid and sensitive detection and diagnosis of SVCV, for example, antigen capture ELISA, sandwich ELISA and IFA (Dixon and Longshaw, 2005; Reschova et al., 2007). In order to further characterization of the mAb 1A9, it was applied to detect virus antigen in infected cell cultures and fish tissues by immunocytochemistry and immunohistochemical assays. Immunostaining of EPC cell monolayers at various times during infection showed a progressive development of distinct *foci* of virus replication, and even the antibodies could recognize proteins of viral origin as early as 6 h post-infection. With immunohistochemical analysis, positive reactions were observed in six tissues of infected fish. Compared with other four kinds of tissues (heart, liver, spleen, and intestine), more immunostaining signals were observed in the gills and kidney, suggesting that they were important targeted organs to SVCV0504. It has been demonstrated that apoptosis is involved in death of EPC cells caused by SVCV with typically morphological changes, like reduction of cell volume, nuclear fragmentation, and formation of apoptotic bodies (Bjorklund et al., 1997). The obvious morphological change that the nuclei of infected tissue cells could not be visible by fluorescence staining in our experiments, may be related to the cell apoptosis induced by SVCV0504.

Flow cytometry has allowed sensitive and rapid detection and quantification of viral infection by immunofluorescence staining (Mcsharry, 1994). For rhabdovirus, the use of flow cytometry assay for detecting the intracellular virus has been reported (Bordignon et al., 2002; Zhou et al., 2006). In the present study, using mAb 1A9 as capture reagents, SVCV0504 viral antigens were detected quantitatively in virus-infected EPC cells by flow cytometry. The background in samples from uninfected EPC cells was remarkably low. The result shows that the flow cytometry system using mAbs could find application for detecting and quantifying SVCV antigen in virus-infected cells or tissue homogenates.

In conclusion, a panel of monoclonal antibodies against SVCV0504 were produced and characterized in this study. We believe that these mAbs will be useful for developing specific and rapid diagnostic tests, and for elucidating the studies of pathogenesis and antigenesis of SVCV.

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