Production of monoclonal antibodies against immunoglobulin heavy chain in common carp (*Cyprinus carpio* L.)

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ABSTRACT: A method for purification of carp serum immunoglobulin (IgM), intended for the production of monoclonal antibodies, was described in the present study. Hybridomas that produce antibodies against IgM heavy chain were selected by ELISA method and Western blotting. Ascitic fluids were prepared and tested by the above mentioned methods, and their typing followed. Monoclonal antibody with the highest titre of antibodies against carp immunoglobulin was selected for conjugation with horseradish peroxidase. Specificity of conjugated monoclonal antibody was tested in a panel of various fish species sera. Cross-reactivity was not detected in rainbow trout (*Oncorhynchus mykiss*) and eleven other fish species. Besides common carp, positive results were also found in goldfish (*Carassius auratus*) and bighead carp (*Aristichthys nobilis*), that are members of Cyprinidae family. Among fish other than Cyprinidae, positive results were also detected in sheatfish (*Silurus glanis*). The sensitivity in common carp was approximately 10 ng/ml.

Keywords: *Cyprinus carpio*; IgM; monoclonal antibodies; ELISA; Western blot

Fish immunoglobulins share various characteristics with mammalian immunoglobulins. However, some differences exist; those partly arise from the process of evolution from common ancestors, and partly reflect the effect of fish adaptation to a changed life environment. Studies of the teleost fish antibody repertoire have been conducted in a number of different fish species (Solem and Stenvik, 2006).

The fish immune system contains five types of immunoglobulins: IgM (Warr, 1995), IgD (Hordvik et al., 1999; Hirono et al., 2003), IgZ (Danilova et al., 2005), IgT (Hansen et al., 2005) and IgH (Savan et al., 2005). The major immunoglobulin of fish blood serum is tetrameric IgM-like molecule (Koumansvandiepen et al., 1995) consisting of eight light and eight heavy chains. Due to the fact that teleost fish IgM share some structural and functional characteristics with mammalian IgM, the ability of various teleost fish to produce heterogenous mixtures of IgM polymers, monomer (Rombout et al., 1993) and halfmer subunits (Pucci et al., 2003) is noteworthy.

Light and heavy chains exist in two alternative forms: as a membrane-bound antigen receptor on the surface of B cells, and as a secreted soluble protein in body fluids (Cheng et al., 2006). IgM heavy chains have been isolated from goldfish (*Carassius auratus*) (Wilson et al., 1985) and catfish (*Ictalurus punctatus*) (Phillips and Ourth, 1986). They were also obtained from the following teleost fish, cloned and sequenced: Atlantic salmon (*Salmo salar L.*), (Hordvik et al., 1992, 1997), rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 1993; Hansen et al., 1994), zebrafish (*Danio rerio*) (Danilova et al., 2000) and others. The aforementioned studies revealed that the IgM heavy chain of teleost fish

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showed differences in gene numbers, sequences and genetic arrangements (Cheng et al., 2006).

Detailed knowledge of the molecular structure of serum IgM heavy chains and quantification of antibody response in cyprinids, that are an economically significant component of aquaculture in our country, will be essential for further immunological studies in the field of virology and vaccinology. The majority of carp immunoglobulins are tetrameric structures (±760 kDa), composed of 25 kDa light (L) chains and 70 kDa heavy (H) chains. This study presents methods for processing and purification of common carp (*Cyprinus carpio*) immunoglobulin (CCIg), production of monoclonal antibodies against CCIg and their subsequent application in serology.

MATERIAL AND METHODS

Fish. Blood from common carp (*Cyprinus carpio*) and other cyprinids was collected during pond fishing in autumn, when high serum immunoglobulin levels may still be expected (Rijkers et al., 1980; Ahne, 1986).

Blood collection. Blood was taken from *vena caudalis,* then allowed to clot at room temperature for 1 h, incubated at 4°C and centrifuged at 1 000 × g for 15 min. Serum containing immunoglobulin fractions was collected and stored at –20°C before processing.

Purification of carp immunoglobulin (CCIg). 2% solution (v/v) of 10% dextran sulphate 500 and 10% (v/v) of 1M $CaCl_2$ were added to 200 ml of carp serum. The mixture was stirred for 30 min at 4°C and centrifuged at 15 000 × g at 4°C for 10 min. The supernatant was dialyzed against PBS overnight. The Ig fraction was precipitated with 35% (w/v) ammonium sulphate solution and the volume and protein content were measured after dialysis (Rombout et al., 1993).

The following gel filtration was performed on a Sephacryl S-300 column (Pharmacia, Sweden) (volume 500 ml), using the following elution buffer: 0.1M Tris-HCl, pH 8, containing 0.15M NaCl and 0.01% NaN_3. Approximately 200 mg of semi-purified immunoglobulins were applied onto the column. Respective flow fractions were collected and differentiated according to the protein content and measured using a spectrophotometer; readings were performed at an optical density of 280 nm and recorded on a graphic device.

CCIg purity was tested by protein separation using SDS-PAGE (Laemmli, 1970) electrophoresis.

Production of monoclonal antibodies. BALB/c mice, aged 6-8 weeks, were immunized i.p. with three doses of CCIg in Freund's adjuvant (FA) at 21 day intervals. The last dose of soluble antigen was administered i.v. and the mice were exsanguinated after three days. Hybridomas were prepared by a conventional procedure (Galfre and Milstein, 1981) using myeloma cells Sp 2/0 Ag14 (provided by the Institute of Molecular Genetics, Prague). Hybridoma colonies producing antibodies against the CCIg heavy chain were selected by ELISA method and Western blotting, and administered i.p. to mice pre-treated with pristan. The ascitic fluids obtained were further tested by immunoblotting technique and ELISA method (Reschova et al., 2001).

Western blotting. Purified CCIg was solubilized in sample buffer under reducing conditions. After separation in 10% polyacrylamid gel (Laemmli, 1970), the proteins were transferred onto a nitrocellulose membrane (Towbin et al., 1979). After drying, the nitrocellulose strips were incubated with ascitic fluids containing monoclonal antibodies (MAb) diluted 1:1000. The reaction was detected using rabbit anti-mouse IgG antibodies labelled with horseradish peroxidase (DAKO Cytomation, Denmark), and visualized using a carbazol substrate solution.

ELISA method. The ELISA method for the selection of hybridomas and their clones that produce antibodies against heavy or light chain of CCIg has been developed. The wells of microtitre plates Polysorp (Nunc) were coated with 0.5 µg/ml of purified CCIg fraction in a binding solution (0.05M carbonate buffer, pH 9.6). After incubation at 4°C overnight, the plate was rinsed with washing solution (0.15M PBS 0.1% Tween 20). Hybridoma supernatants were tested after a dilution of 1:2 with a diluent (PBS, Tween 20, LAH) and incubated for 1 h at 37°C. After rinsing, rabbit conjugate (peroxidase) against mouse Ig (DAKO, Cytomation, Denmark) diluted 1:1000 was dispensed into all wells. Then, after 1 h incubation at 37°C and subsequent rinsing, the enzymatic reaction was visualized using substrate TMB with hydrogen peroxide and stopped with 1M sulphuric acid. Optical density was measured spectrophotometrically at 450 nm.

An identical reaction process was performed when ascitic fluids containing MAb against required CCIg epitopes were tested by titration.

MAb typing. The determination of class, subclass and the type of light chains of produced MAbs was performed by ELISA method with a purified carp Ig-coated microtitration plate, binding of the examined MAbs and their typing by means of class and subclass specific anti-mouse peroxidase conjugates (Mouse-hybridoma Subtyping Kit, Boehringer, Mannheim).

Conjugation of MAb by a periodate method. The selected purified MAb (10 mg) was labelled with horseradish peroxidase (POD) by oxidation method (Farr and Nakane, 1981). The produced conjugate was tested by a direct ELISA method.

Direct ELISA method for the detection of sensitivity and specificity of conjugated MAb. The Ig fraction of MAb 1E10/2A8 was bound to the bottom of a 96-well microtitre plate Polysorp (Nunc). After incubation at 4°C overnight, the plate was rinsed with a rinsing solution (0.15M PBS, 0.1% Tween 20). Diluted carp and trout immunoglobulins were dispensed into the plate to test specificity and sensitivity of the conjugated component. After 1 h incubation at 37°C and rinsing, POD MAb 1E10/2A8 conjugate was pipetted into the wells. After a further 1 hr incubation at 37°C and subsequent rinsing, the enzymatic reaction was visualized by addition of TMB substrate with hydrogen peroxide, and stopped with 1M sulphuric acid. Optical density was measured spectrophotometrically at 450 nm.

Identical enzymatic reaction was performed with a panel of Ig fractions obtained from various species of Cyprinidae.

RESULTS AND DISCUSSION

The major component of the immunoglobulin fraction in common carp and other bony fish species is the IgM-like molecule presented mostly in tetramers. Each unit consists of light (L) and heavy (H) chains. Besides the dominant tetrameric structure, dimeric and monomeric forms are also present (Rombout et al., 1993). H and L chains are composed of variable (V) and constant (C) domains. IgL isotype designated L1A, has been identified in carp. The constant domain (CL) sequence showed the high degree of similarity to those of rainbow trout (*Oncorhynchus mykiss*) IgL1 and catfish (*Ictalurus punctatus*) G isotypes, with 59% and 55% similarity, respectively (Tomana et al., 1999). Common carp was reported to possess three distinct heavy chain constant region (CH) sequences, which share 72–95% identity at the amino acid level (Nakao et al., 1998), and show serologic heterogeneity in IgH molecules (Egberts et al., 1983).

Due to the fact that polyclonal antibodies against fish immunoglobulins are not sufficiently specific (Siwicki et al., 1994), we prepared a panel of seven anti-CCIg MAbs with the aim to further detect the presence of circulating antibodies against carp pathogens, which may be a response to natural infection or vaccination.

The method according to Rombout et al. (1993) was selected for purification of carp immunoglobulin; the first step of this method is serum delipoidization using dextran sulphate. A similar procedure was effective for preparation of the immunoglobulin

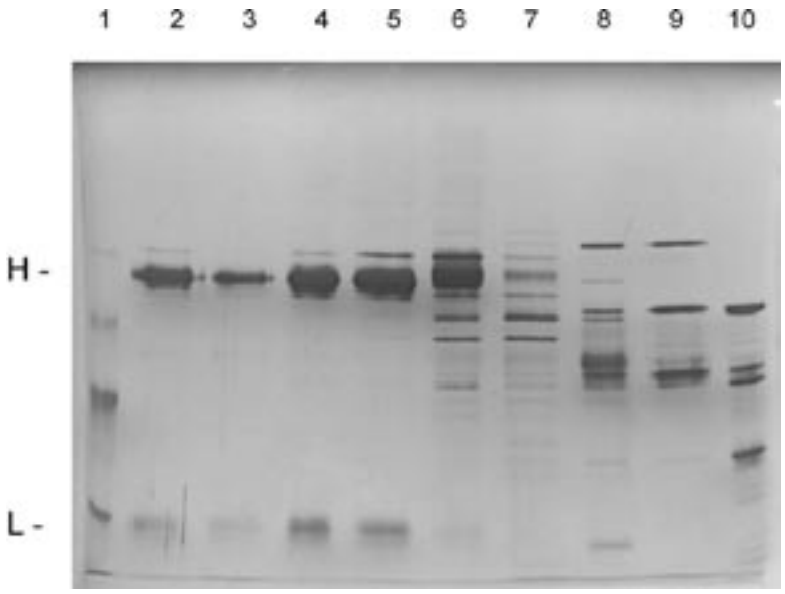

Figure 1. SDS PAGE of CCIg fractions. H = heavy chain of CCIg; L = light chain of CCIg; 1 = LMW (Pharmacia); 2–4 = fractions 1–3 after separation on Sephacryl S-300; 5 = combined fractions 1–3; 6–10 = fractions 4–8 after separation on Sephacryl S-300

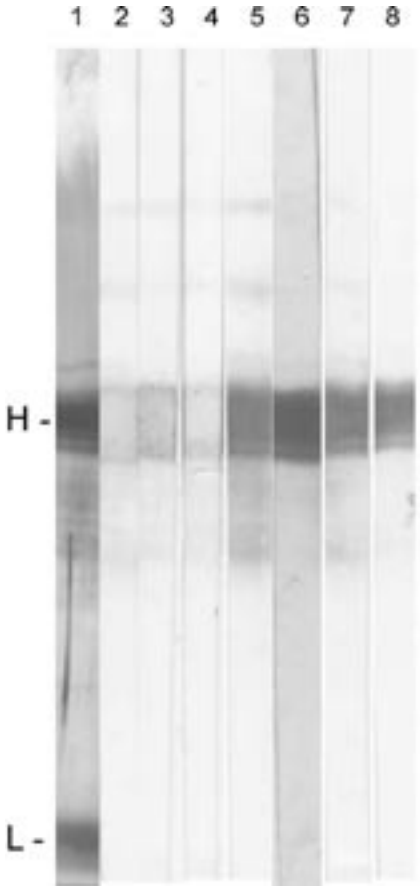

Figure 2. Western blotting. H = heavy chain of CCIg; L = light chain of CCIg; 1 = CCIg in PAGE stained with colloid gold solution; 2 = MAb 1A10/2A9; 3 = MAb 2E7/2G8; 4 = MAb 2E7/3D12; 5 = MAb 1E10/2H9; 6 = MAb 1E10/2A8; 7 = MAb 3F10/2G1; 8 = MAb 3F10/1G3

fraction IgY from egg yolk intended for diagnostic purposes (Pokorova et al., 2000). After separation on the column Sephacryl S-300, the purity of respective fractions was tested by electrophoresis in polyacrylamid gel (Figure 1). It was shown that the first fractions contained almost pure CCIg. Combined fractions 1, 2, 3 with marked bands in the region of heavy (70kDa) and light chains (25kDa) were used for the immunization of mice.

After administration of the purified globulin CCIg fraction to mice, and fusion of mouse splenocytes with myeloma cells, seven different hybridomas that produce anti-CCIg MAbs were selected. Their specificity was tested by Western blotting; all of them reacted with the heavy chain of CCIg (Figure 2).

Using a kit with isotype conjugates (Mouse-hybridoma Subtyping Kit, Boehringer, Mannheim) MAbs were classified as IgG1 class and light chain κ isotype (Figure 3).

Selected hybridomas were used for preparation of ascitic fluids with increased concentrations of MAbs against the heavy chain (70kDa). After semi-purification of ascitic fluids by precipitation using ammonium sulphate (40% saturation) and subsequent desalting, Ig fractions were analysed by ELISA method; their titres were 1: 256 000–1: 2 000 048, except for MAb 2E7/3D12 where the ELISA titre was lower (Table 1) and corresponded to the titres of native ascitic fluids.

MAb with the highest ELISA titre (1: 2 000 048) and specificity to heavy Ig chain – 1E10/2A8 detected by immunoblotting was selected for conjugation with horseradish peroxidase.

The selected MAb 1E10/2A8 was conjugated using the periodate technique, and subsequently tested for sensitivity and specificity in a direct ELISA test. Specificity was tested in a panel of sera collected from various freshwater fish species (Table 2).

Horseradish peroxidase labelled conjugate POD MAb 1E10/2A8 did not cross-react with coagulated trout serum. Its sensitivity was tested with a purified carp immunoglobulin, and showed to be

Table 1. Characterisation of MAbs

MAb designation	Protein concentration (mg/ml)	ELISA titre	Immunoblotting reaction in the region of 70 kDa
1A10/2A9	9.8	1:256 000	+
2E7/2G8	9.0	1:256 000	+
2E7/3D12	8.4	1:32 000	+
1E10/2H9	8.15	1:1 000 024	+
1E10/2A8	11.45	1:2 000 048	+
3F10/2G1	8.25	1:1 000 024	+
3F10/1G3	8.8	1:1 000 024	+

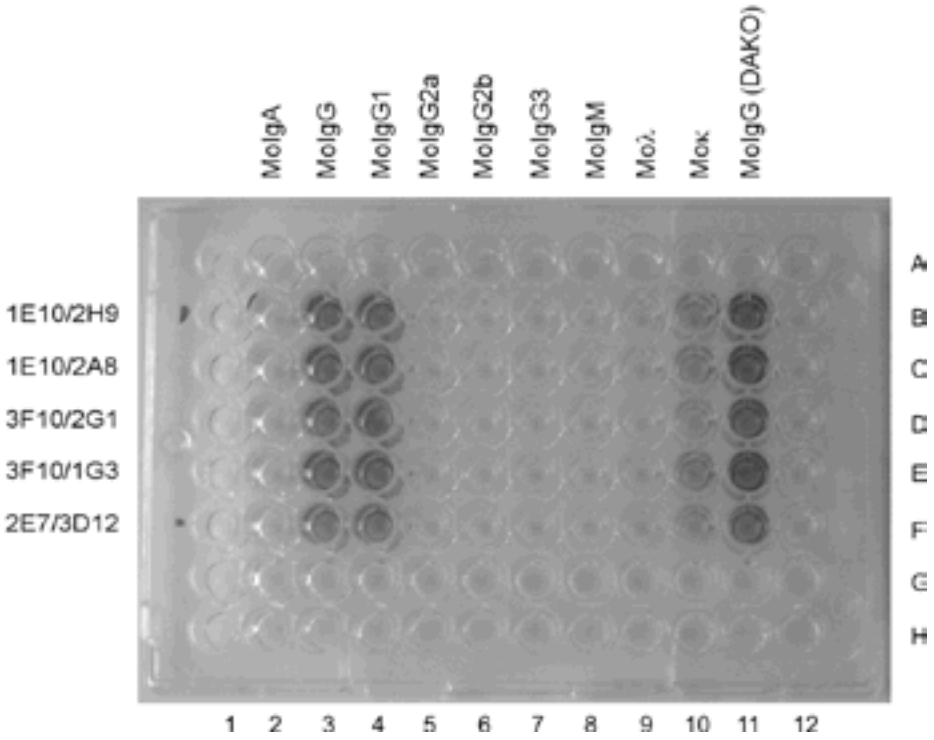

Figure 3. Isotyping of MAbs. B–F rows = individual MAbs; column 2–11 = antibodies labelled with POD to respective classes and subclasses of mouse immunoglobulins

approximately 10 ng/ml. A positive response was detected among Cyprinidae family members only in goldfish and bighead carp up to the concentration of 12.5 µg/ml, and in sheatfish, that is not a member of Cyprinidae family, up to the concentration of 6.25 µg/ml.

In a panel of immunoglobulin fractions obtained from sera of the above mentioned species of Cyprinidae family and from a few fish species of a different family, cross-reactivity with immunoglobulin of goldfish and bighead carp of Cyprinidae family; and with Ig of sheatfish that is a member

Table 2. Specificity of peroxidase labelled MAb 1E10/2A8 in a panel of fish Ig fractions

Latin designation	Fish species	Family	Positivity in the ELISA test (OD ≥ 0.1)
Cyprinus carpio	common carp	*Cyprinidae*	up to 10 ng/ml
Carassius auratus	goldfish	*Cyprinidae*	up to 12.5 µg/ml
Scardinius erythrophthalmus	rudd	*Cyprinidae*	–
Tinca tinca	tench	*Cyprinidae*	–
Hypophthalmichthys molitrix	silver carp	*Cyprinidae*	–
Aristichthys nobilis	bighead carp	*Cyprinidae*	up to 12.5 µg/ml
Ctenopharyngodon idella	grass carp	*Cyprinidae*	–
Leuciscus idus	ide	*Cyprinidae*	–
Rutilus rutilus	roach	*Cyprinidae*	–
Abramis brama	bream	*Cyprinidae*	–
Blicca bjoerkna	white bream	*Cyprinidae*	–
Perca fluviatilis	perch	*Cyprinidae*	–
	buffalo	*Cyprinidae*	–
Stizostedion lucioperca	pike perch	*Cyprinidae*	–
Silurus glanis	sheatfish	*Siluridae*	up to 6.25 µg/ml
Oncorhynchus mykiss	rainbow trout	*Salmonidae*	–

of Siluridae family, was detected by ELISA testing. Due to the fact that monoclonal antibodies are viewed as highly specific in the recognition of small deviations in epitopes, close similarity of epitope in the structural part of heavy chain is likely to exist in carp, goldfish and bighead carp of Cyprinidae family and sheatfish from another family.

Comparable results were described by (Siwicki et al., 1994). MAbs against light and heavy chain of goldfish in their study reacted in Western blotting with purified Ig both from carp and goldfish. Cross-reactivity between MAbs produced against Ig H of cod and Ig of relative species from Gadidae family was described (Israelsson et al., 1991).

The aim of the present study was to produce MAbs for diagnostic purposes, i.e. for the improved diagnosis of diseases of common carp, which is the most important market fish in the Czech Republic. Using the MAbs obtained, levels of circulating antibodies may be detected in carp, both after infection and vaccination. One of the priorities where MAbs produced against carp Ig may be used is the detection of specific antibodies against viruses of spring viraemia of carps (SVCV), and investigation of the status of infestation of carp breeds with viruses of spring viraemia of carps in the Czech Republic. Our further study will be focused on the development of a serologic test for these purposes.

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