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Monoclonal antibodies to snakehead, *Channa striata* immunoglobulins: Detection and quantification of immunoglobulin-positive cells in blood and lymphoid organs

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ABSTRACT

Snakehead *Channa striata* is an important freshwater food fish in many Southeast Asian countries. Three monoclonal antibodies (C9, C10 and D10) were developed against purified serum immunoglobulins of *Channa striata* (Cs-Ig) and characterized. C9 and D10 MAbs were specific to heavy chain, while C10 MAb detected only unreduced Cs-Ig in western blotting. In competitive ELISA, C9 and C10 MAbs were specific to *C. striata* Ig and showed no cross reactivity with serum Ig of other fish species i.e. *Channa punctatus*, *Channa marulius*, *Clarias batrachus* and *Labeo rohita*. D10 MAb showed reactivity to serum Ig of *C. striata* and *C. marulius*. In FACS analysis of gated lymphocytes, the percentage of Ig+ cells detected by C9 MAb was 18.2%, 27.7% and 10.3% in blood, spleen and kidney, respectively ($n = 3$, body weight 500–600 g). However, only a few cells (0.5%) were found to be Ig+ in thymus ($n = 5$). C9 MAb was also successfully employed to demonstrate Ig+ cells in blood smears and formalin fixed sections of spleen and kidney. These findings suggest that the spleen plays an important role in humoral immunity as compared to head kidney. Further, these MAbs can be useful immunological tool in monitoring health status of cultured *C. striata*.

1. Introduction

Snakehead *Channa striata* is an important freshwater food fish in many Southeast Asian countries including India. This species has an extensive natural distribution, extending from Iran to the Far East including India, China and Indonesia. In India and other Southeast Asian countries, snakeheads particularly, *Channa striata* represents a significant component of the freshwater fish catch and fetches relatively high prices as compared to carps. Another positive aspect associated with this fish species is that it can be cultured even in oxygen-depleted waters at high densities, due to its air breathing nature. But intensification of its culture has led to increased occurrence of diseases and health related problems e.g. epizootic ulcerative syndrome [1], which merits attention.

Studies on immune system of *C. striata* could pave way in planning strategies for effective health management measures. B-lymphocytes are important cells of immune system that secrete immunoglobulins (Ig) in blood and other body fluids and play a vital role in conferring humoral immunity. Monoclonal antibodies (MAbs) to Ig have been used as cell specific marker for differentiation of B cells from Ig- lymphoid cells. Moreover, MAbs to fish Ig have been proven to be useful diagnostic reagent for detection of pathogens in immunoassays [2].

The present study was carried as a part of overall programme of our institute on development of MAbs against serum immunoglobulins of important freshwater finfish of India. Production of MAbs to serum immunoglobulins of *Labeo rohita* was our pioneer effort of this programme to cater carp aquaculture [3]. In continuance, MAbs to Ig of *C. striata* will serve to monitor the health needs of snakeheads. We have already reported purification and characterization of serum Ig of *C. striata* [4]. Previously, purification of Ig [5] and development of MAbs to serum Ig of *C. striata* has been reported from AAHRI, Thailand [6]. These MAbs were used for assessment of relative antibody concentration in *C. striatus* [7]. Commercially

Abbreviations: BSA, bovine serum albumin; c-ELISA, competitive ELISA; Cs-Ig, *Channa striata* immunoglobulins; DAB, 3-3′ diaminobenzidine; FITC, fluorescein isothiocyanate; FSC, forward scatter; HC, heavy chain; Ig, immunoglobulins; LC, light chain; MAbs, monoclonal antibodies; MNCs, mononuclear cells; OD, optical density; OPD, ortho-phenylenediamine; PBS-T, phosphate buffer saline with 0.05% Tween-20; SDS-PAGE, sodium dodecyl sulphate- polyacrylamide gel electrophoresis; SSC, side scatter.

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also, anti-snakehead IgM MAb is available with Aquatic Diagnostics Ltd, University of Sterling, Scotland, U.K. However, to the best of our knowledge, complete information on reactivity of MAbs against Ig positive cells in different lymphoid organs of *C. striata* needs to be established, besides their other applications. In view of this, our present attempt will not only provide us a continued source of diagnostic reagent, but also generate important immunological information of this important fish species.

2. Materials and methods

2.1. Fish serum samples

Standard procedures were used for blood collection, processing and storage of fish serum in this study. Blood was collected from the caudal vein of the fish and allowed to clot overnight at 4 °C. It was then centrifuged at low speed to collect the serum. The harvested serum was stored at −20 °C prior to use. Twenty-five serum samples (5 no./species) from adult *C. striata* (200–250 g), *Channa marulius* (600–850 g), *Channa punctatus* (30–40 g), *Clarias batrachus* (100–120 g) and *L. rohita* (250–300 g) were collected and used subsequently in the competitive ELISA (c-ELISA) as field serum samples.

2.2. Production and characterization of monoclonal antibodies

Serum immunoglobulins of *C. striata* (Cs-Ig) were purified by affinity chromatography as per the method described by Sood et al. [4]. Briefly, 10 fish (180–220 g) were immunized with 1 mg of bovine serum albumin (BSA) emulsified with adjuvant by intraperitoneal route and boosted thrice at 2 weeks intervals. Blood from hyper-immunized fish was collected and anti-BSA antibodies were purified from the serum using BSA-CL agarose column. Purity of the immunoglobulins was checked by non-reducing and reducing sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE).

Monoclonal antibodies were produced as per the standard procedures using purified Cs-Ig as an immunogen. Well-to-well screening of hybridoma clones; employing an indirect ELISA (described below) was performed at appropriate stage of growth (25% of the well surface covered by cells). Positive clones were amplified, followed by single-cell cloning and sub-cloning using the limiting dilution method. Single-cell cloned hybrids were again screened by indirect ELISA at the appropriate stage of growth. Single cell cloning was repeated at least twice so as to prove the monoclonality of the hybridoma culture supernatant. Isotyping of MAbs was carried out on selected clone supernatants using a self-indicating dipstick kit (Sigma).

2.3. Indirect ELISA

An indirect ELISA was performed for titration of mice serum for anti-Cs-Ig antibodies and screening of the wells containing Cs-Ig positive hybridoma. The ELISA plates (Nunc, Medisorp) were coated with 1 μg/ml of purified Cs-Ig (50 μl/well). The plates were incubated at 37 °C for one hour (h) at stationary phase. Unbound antigen was washed thrice with PBS-T (phosphate buffer saline with 0.05% Tween-20) in each well. Further, either serial two-fold dilutions of mice serum (50 μl) were added to each well or 50 μl of hybridoma culture supernatants were transferred from cell culture plate to ELISA plate wells in the same format. The plates were incubated for 1 h at 37 °C and washed. Anti-mouse IgG peroxidase conjugate (Sigma) diluted 1:10,000 in dilution buffer (PBS-T with 1% BSA) was added at the rate of 50 μl to each well. Plates were further incubated for 1 h at 37 °C under constant shaking. Plates were washed again and 1 mg/ml of ortho-phenylenediamine (OPD) containing H_2O_2 (50 μl/well) was added. Colour reaction was allowed to develop for 5 minutes (min) followed by stopping of the reaction with equal volume of 2 N H_2SO_4. The absorbance of the wells was read at 492 nm (A_{492}) in an ELISA reader (Tecan). The hybridoma culture supernatants that gave at least five times the A_{492} values of 0-day mouse serum were considered positive for anti-Cs-Ig antibodies or hybridomas measuring an absorbance (A_{492}) of 0.5 or more were selected. This absorbance corresponded to approximately five times the absorbance of the background (0-day mice serum).

2.4. Western blotting

Standard procedures of western blotting were used to determine the reactivity of the selected MAbs (C9, C10 and D10) in hybridoma culture supernatants to reduced Cs-Ig and *C. striata* serum on 12% acrylamide gel. A non-reducing gradient SDS-PAGE (3–12% gel) of Cs-Ig and serum followed by western blotting was carried out for C10 MAb that did not show reactivity to reduced Cs-Ig.

2.5. Competitive ELISA: Demonstration of specificity of MAbs to Cs-Ig

The optimal concentration of antigen and dilution of MAb to be used in c-ELISA was determined through standard checkerboard titration using doubling dilutions to obtain an expected optical density (OD) range of 1.0 ± 0.2. ELISA plates were coated with 50 μl/well of optimal concentration of the antigen (Cs-Ig). The plates were incubated at 37 °C for 1 h at stationary phase. Serial dilutions (1/200 to 1/51,200) of field *C. striata* serum samples as test species or field sera of other test fish species (*C. marulius*, *C. punctatus*, *Clarias batrachus* and *L. rohita*), in dilution buffer were used as competitor in solution. Competition was carried out by incubating the washed plates with 50 μl/well of serially diluted competitor along with 50 μl of optimal dilution of test MAbs (1:100) in dilution buffer, at 4 °C for overnight. Subsequently the plates were washed and 50 μl of goat anti-mouse IgG peroxidase conjugate (1:10,000) was added to the wells. After incubation for 1 h at 37 °C, plates were again washed and 50 μl substrate (OPD) was added. Wells were observed for the development of colour and reaction was stopped using 2 N H_2SO_4 to obtain an expected OD range of 1.0 ± 0.2 in MAb control wells. Percentage competition was calculated using following formula:

$$\%\,\text{Competition} = 100 - [\{\text{OD of field serum sample} \div \text{OD of MAb control}\}]100.$$

2.6. Flow cytometry: quantification of Ig-positive cells in blood, spleen, kidney and thymus

Heparinized blood was collected from caudal vein, diluted 1:1 with PBS and layered on Histopaque-1077 for separation of mononuclear cells (MNCs). Individual cell suspension of adult *C. striata* (500–600 g) spleen, kidney and thymus were prepared in DMEM, by squeezing the individual tissues sequentially through a coarse mesh and then fine (40 μm) nylon gauge cell strainer (BD falcon). Cells were washed twice in PBS and resuspended in DMEM (10^7 cells/ml), and layered 1:1 on Histopaque-1077 for separation of MNCs. Isolated MNCs of blood and individual tissues were kept on ice for flow cytometry.

2.6.1. Analysis by flow cytometry

For flow cytometry, blood, spleen, kidney and thymus MNCs (10^6 cells) were individually washed in DMEM and resuspended in 250 μl of C9 MAb (1:20 dilution in DMEM) and incubated for 30 min on ice. In control cells, C9 MAb was replaced with myeloma culture supernatant. Again, the cells were washed twice and incubated for 30 min on ice with rabbit anti-mouse fluorescein isothiocyanate (FITC) conjugate (1:100 in DMEM). Thereafter, cells were washed twice,

resuspended in 500 μl DMEM and analyzed with flow cytometer. In all, blood, spleen and kidney samples from three fish were pooled and analyzed by flow cytometry. In case of thymus, tissue samples from five fish were pooled. Analysis was carried out on a FACS Caliber (Becton Dickinson) equipped with an argon-ion laser tuned to 480 nm. Ten thousand events were acquired from each sample and data were analyzed using software. Putative lymphocytes were characterized and gated by their Forward Scatter (FSC) and Side Scatter (SSC) properties. Surface Ig+ cells of FSC vs. FL1 dot plot were enumerated as percent of total events.

2.7. Indirect immunoperoxidase test (IIPT)

Ig+ cells were detected in spleen, kidney, thymus and blood smears using indirect immunoperoxidase test [8]. The sections of spleen, kidney and thymus were dewaxed and rehydrated in descending grades of alcohol. The sections were rinsed in PBS and endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 20 min. Heat-mediated antigen retrieval was carried out in a microwave oven at 800 W for 10 min using antigen unmasking solution (Vector). After conditioning the sections in PBS-T, the slides were laid flat and blocked with normal horse serum (Vector). Thereafter, the sections were incubated overnight with C9 MAb culture supernatant at 4 °C and washed thrice with PBS-T. In control slides, C9 MAb was replaced with myeloma culture supernatant. The sections were again incubated with ImmPRESS anti-mouse Ig Reagent (Vector) for 30 min at 37 °C and washed with PBS-T. Colour was developed by adding 3-3′ diaminobenzidine (DAB) chromogen solution (BioGenex) at 37 °C for 10 min. After washing the slides, the sections were counterstained in Gill's haematoxylin, dehydrated, cleared and mounted in DPX.

Similar procedure was followed for demonstrating Ig+ cells in blood smears, except the heat-mediated antigen retrieval step.

3. Results

3.1. Production and characterization of monoclonal antibodies

Cs-Ig proved to be a good immunogen as both the immunized mice showed considerable increase in antibody titre, as compared to 0-day mice serum. Out of 672 wells of microtitre plate seeded from a single fusion, a total of 153 wells showed hybridoma clones. Hybridoma culture supernatants, of these wells at appropriate stage of screening, showed 28 positive wells with significantly high absorbance (A_{492}) values to Cs-Ig in contrast to 0-day mice serum dilution (1/100) in indirect ELISA. Out of these, three strongly positive primary hybridoma clones (C9, C10, D10) were subjected to single cell cloning and sub-cloning by limiting dilution. Monoclonality of a clone was accepted only when all the wells of a microtitre plate with growing cells gave positive reaction in indirect ELISA after repeated sub-cloning. The polypeptide specificity of these clones to reduced Cs-Ig was determined by western blotting. Two clones (C9, D10) were specific to heavy chain (HC) of Cs-Ig and corresponding band in serum (Fig. 1), while the other clone (C10) failed to react with either the HC or light chain (LC). However, clone C10 recognized the unreduced Cs-Ig and corresponding band in serum in immunoblot analysis (Fig. 2). The MAbs secreted by these clones were designated as C9, D10 and C10 and all belonged to subclass IgG1.

3.2. Competitive ELISA

Competitive ELISA was carried out to demonstrate the specificity of MAbs to Cs-Ig or indirectly as the cross-reactivity pattern of MAbs with whole serum Ig of different species of fish i.e. *C. marulius*, *C. punctatus*, *Clarias batrachus* and *L. rohita*. Percent competition of purified Cs-Ig with sera of *C. striata*, *C. marulius*, *C. punctatus*, *Channa batrachus* and *L. rohita* as detected by MAbs C9, C10 and D10 is shown in Fig. 3. The results indicate that C9 and C10 MAbs were highly specific to *C. striata* and showed no competition with any of the other tested fish species at any serum dilution [Fig. 3 (A), (B)], whereas, D10 MAb showed competition to serum Ig of *C. marulius* [Fig. 3(C)].

3.3. Flow cytometry: quantification of Ig-positive cells in spleen, kidney and thymus

MNCs from blood, spleen, kidney and thymus of *C. striata* were individually analyzed for FSC and SSC patterns representing size and granularity of cells, respectively. Lymphocytes were presumed

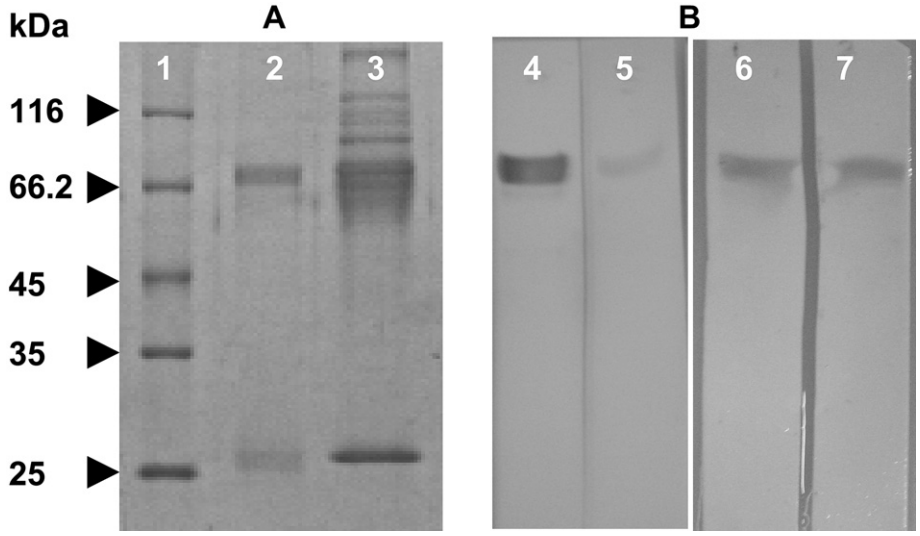

Fig. 1. Demonstration of reactivity of monoclonal antibodies (MAbs) to *Channa striata* purified Ig (Cs-Ig) and serum by Western blotting. A. SDS-PAGE of Cs-Ig and serum on 12% gel under reducing conditions; Lane 1: Molecular weight marker (Fermentas). Lane 2: Cs-Ig. Lane 3: *C. striata* serum. B. Western blotting of Cs-Ig and serum; Lane 4 and 5: Reactivity of C9 MAb and D10 MAb (1: 20 dilution) with reduced Cs-Ig, respectively. Lane 6 and 7: Reactivity of C9 MAb and D10 MAb (1: 20 dilution) with reduced serum, respectively.

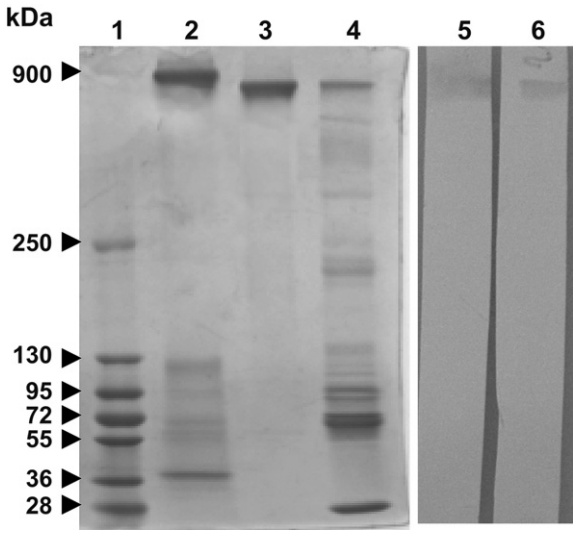

Fig. 2. SDS-PAGE and immunoblotting analysis of purified *Channa striata* purified Ig (Cs-Ig) on 3–12% gradient gel under non-reducing conditions. Lane 1: Prestained protein molecular weight marker (Fermentas). Lane 2: Bovine IgM. Lane 3 and 4: Cs-Ig and *C. striata* serum (Non-reduced). Lane 5 and 6: Reactivity of C-10 MAb with Cs-Ig and *C. striata* serum, respectively.

to be represented by medium sized cells with less granularity, in all analyzed lymphoid tissues. The mononuclear cell population in all analyzed tissues had relatively homogenous FSC [200–400] properties [Fig. 4(A), (C), (E) and (G)]. The percentage of putative B-lymphocytes in blood and lymphoid tissues of adult *C. striata* was determined by incubating the cells with C9 MAb and subsequently staining with anti-mouse IgG FITC conjugate. Representative flow cytometry histograms of negative control and C9 MAb stained lymphocytes in blood, spleen, kidney and thymus are shown in Fig. 4(B), (D), (F) and (H), respectively. The percentage of Ig positive cells in blood, spleen, kidney and thymus was found to be 18.2%, 27.7%, 10.3% and 0.5% of gated lymphocytes, respectively.

3.4. Indirect immunoperoxidase test

3.4.1. Spleen

Spleen is an elongated lymphoid organ in *C. striata*, located near greater curvature of stomach. It has a thin connective tissue capsule and trabeculae are absent. The major constituents of spleen are red and white pulp and ellipsoids along with random distribution of melanomacropahge centres (MMCs). In the tissue sections, Ig+ cells were observed in white and red pulp of spleen. These cells were scattered predominantly as single cells and sometimes as cluster of a few cells. The concentration of Ig+ cells was more in mantle layer of white pulp [Fig. 5(A),(B)].

3.4.2. Kidney

Kidney is a paired organ in *C. striata* performing haematopoietic, excretory and endocrine functions. The anterior kidney has lymphoid and haemopoietic tissue with a number of MMCs. In head kidney, the Ig+ cells were scattered mainly in lymphoid tissue and occasionally in the hematopoietic tissue of head kidney. In trunk kidney, the Ig+ cells were detected mainly in peritubular area near the basement membrane. The Ig+ cells occurred singly or in small clusters of 2–3 cells [Fig. 5(C)]. The reaction was occasionally observed in glomeruli [Fig. 5(D)] and blood vessels. Overall, less number of Ig+ cells were observed in kidney sections than in spleen sections.

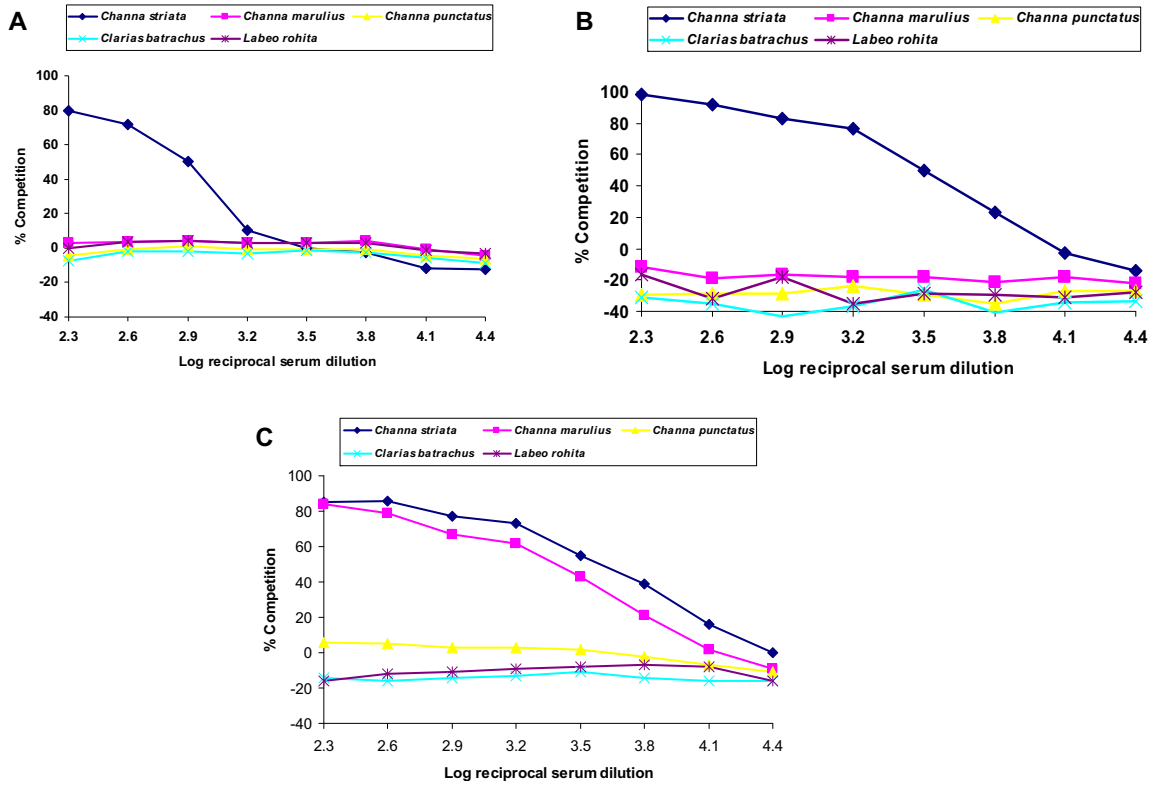

Fig. 3. Demonstration of antigenic relatedness of *Channa striata* Ig (Cs-Ig) with serum Ig of heterologous fish species by indirect competitive ELISA. Competition was carried out on Cs-Ig coated plates with serial dilutions of test fish serum along with fixed quantity of C9 (A), C10 (B) and D10 MAb (C), respectively.

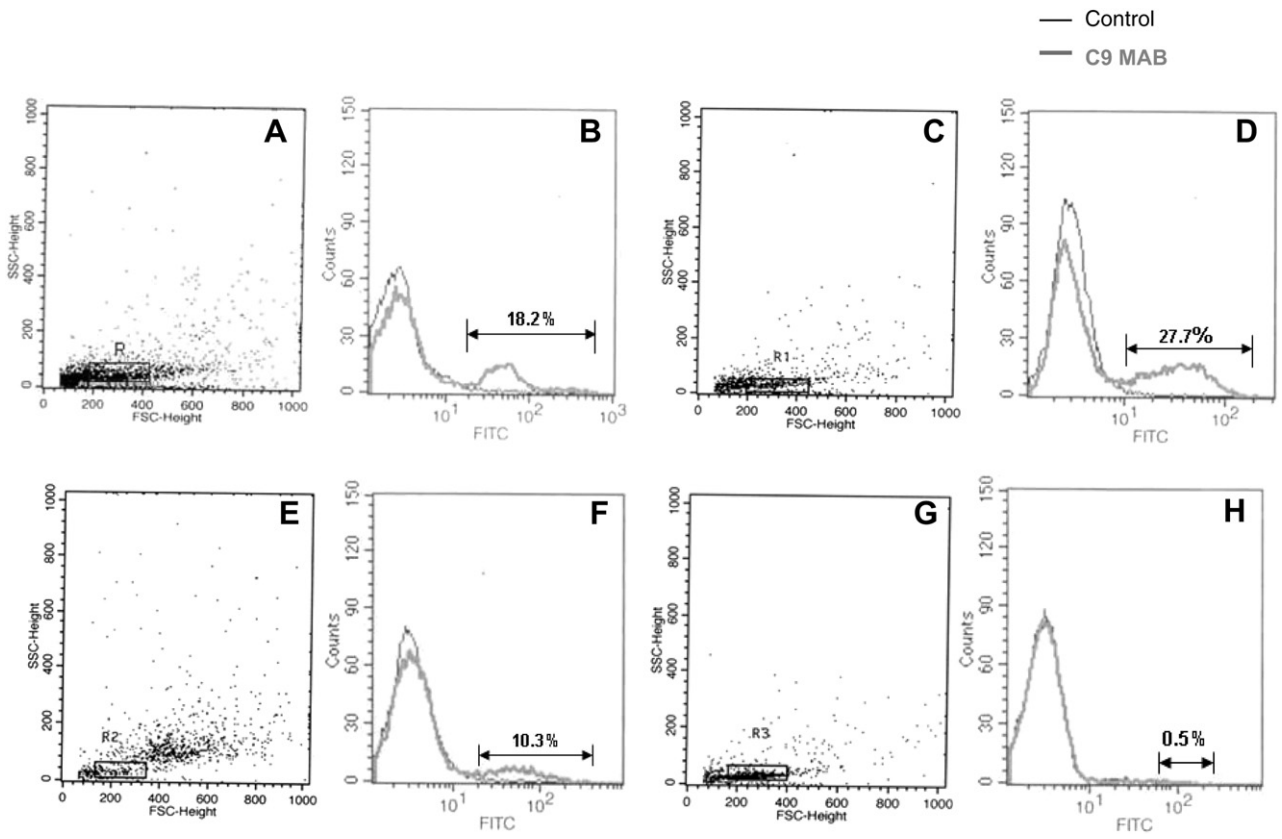

Fig. 4. Quantification of Ig+ mononuclear cells in blood and lymphoid organs of *Channa striata* by flow cytometry. FSC/SSC dot plot of mononuclear cells of blood, spleen, kidney and thymus respectively, showing gated lymphocytes (A, C, E, G). Fluorescence histogram of gated mononuclear cells in blood (B), spleen (D), kidney (F) and thymus (H) without MAb (black line) and with C9 MAb (grey line).

3.4.3. Thymus

In *C. striata*, thymus is a paired organ located in dorsolateral position in opercular cavity. It has a well developed capsule and aggregation of small lymphocytes with macrophages, epithelial nests and MMCs. No reactivity of C9 MAb was demonstrable in thymus sections [Fig. 5(E)].

3.4.4. Blood cell smears

In these smears, MAb reacted with round cells that had large nucleus [Fig. 5(F)]. No reactivity of MAb was observed with erythrocytes and granulocytes.

In control slides, Ig+ cells were not observed in spleen, kidney and thymus sections and blood smears.

4. Discussion

In this study, we report the development of monoclonal antibodies specific to Ig of *C. striata* and the hybridomas were obtained by immunizing Balb/c mice with purified Cs-Ig and positive clones were identified by indirect ELISA, where Cs-Ig was coated as antigen. Three stable clones (C9, C10 and D10) were selected for further characterization. It was later observed that though all three clones were positive in indirect ELISA, however C10 clone failed to react to reduced Cs-Ig and serum in western blotting. This was probably because it recognized a particular conformation of the epitope, which involves S-S bonding on the Ig molecule. This particular conformation of the molecule was altered in the presence of reducing agent [9]. The remaining two clones C9 and D10 recognized the heavy chain (HC) of Cs-Ig in western blotting. No reactivity was seen with the light chain (LC) even at higher concentration of MAbs. Previous studies have also shown that HC is more antigenic in nature as compared to LC, as most of the MAbs against fish Ig, are specific for the Ig HC [2]. The specificity of these MAbs was clearly demonstrated by western blot of purified Ig and also with whole serum of *C. striata*. These MAbs can be considered suitable for the detection of pathogen-specific antibodies by means of an indirect ELISA and can be used in assessing antigen-specific response to infection or for demonstrating the efficacy of vaccination on antibody production.

In competitive ELISA, no cross reactivity of C9 and C10 MAbs was observed with serum Ig of related fish species namely *C. marulius* and *C. punctatus,* even at lower serum dilution. However, D10 MAb showed almost equivalent reactivity to *C. marulius* as well. All the three MAbs were not reactive to serum of other distantly related fish species belonging to *L. rohita* and *C. batrachus*. Our results suggest that the epitope of Cs-Ig recognized by D10 MAb is partially conserved among related fish species of same Channidae family i.e *C. marulius*, which leads to varying degree of cross reactivity. MAbs directed against Ig have shown cross-reaction to Ig of other phylogenetically related species of same family or order [10–12]. So it is not unexpected to get a similarity between the Ig molecules of *C. marulius* in our results, as they belong to one common family.

The percentage of Ig+ MNCs in blood, spleen, kidney and thymus was investigated by flow cytometry using C9 MAb. These reacting cells would be B-lymphocytes, as also judged from their low scores of FSC and SSC in the dot plot FACS analysis [11,13]. The non-reacting cells could be T lymphocytes or thrombocytes, as fish thrombocytes are nucleated and appear mixed with lymphocytes. In the present study, 18.2%, 27.7% and 10.3% of gated lymphocytes in blood, spleen and kidney were reactive with C9 MAb, respectively. Wide variations in percentage of Ig+ cells in blood and lymphoid organs have been observed in fishes (Table 1). This may result from

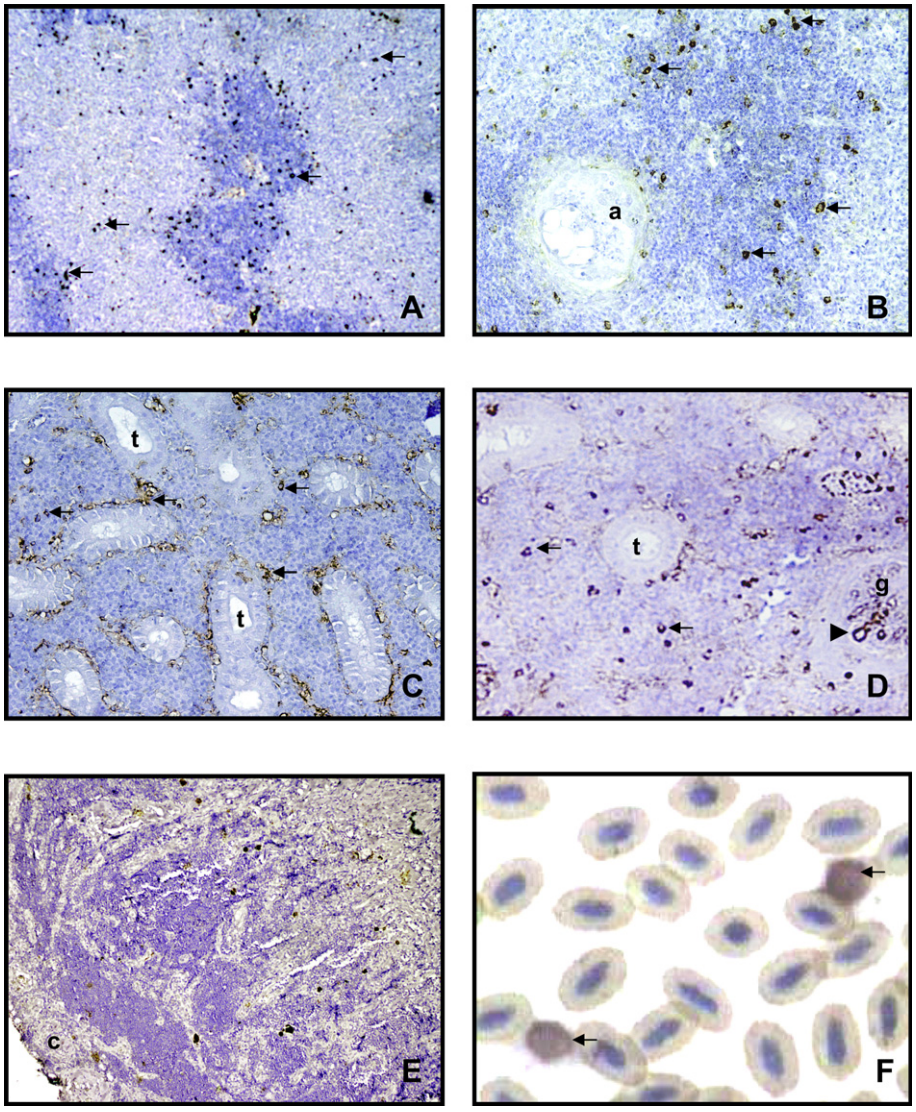

Fig. 5. Immunohistochemical detection of Ig+ cells in formalin fixed paraffin embedded tissue sections. (A) Spleen (200×), Ig+ cells seen as scattered cells in white and red pulp (arrows). (B) Spleen (400×), Ig+ cells were observed predominantly as single cells in mantle layer of splenic corpuscles (arrows); a- arteriole. (C) Kidney (200×), C9 MAb reactivity was observed in peritubular areas (arrows), t- renal tubule. (D) Kidney (400×), Ig+ cells seen in lymphoid areas as single cells (arrows). C9 MAb reactivity was also observed in glomerular basement membrane (g) (arrowhead). (E). Thymus (100×), the reactivity of C9 MAb was not appreciable in thymus. Only a few melanin containing cells were seen in thymus, c- thick connective tissue capsule. (F). Blood smear (1000×), Ig+ cells were seen as small round cells, resembling lymphocytes (arrows).

binding of MAb to a subset of B-lymphocytes or its whole population [14]. In addition, environmental factors and physiological conditions such as age, sex, weight, reproductive and health status of the fish can also affect serum Ig levels [15,16]. The low percentage of Ig+ cells in the present study could be attributed probably to C9 MAb recognizing a subpopulation of B-lymphocytes.

Lowest reactivity (0.5%) of C9 MAb was seen in gated mononuclear cells of thymus, as also observed in earlier studies [9,11,25]. This indicates that most of the thymocytes are Ig- cells and could be presumed to be T-cells.

In immunoperoxidase studies also, C9 MAb reacted strongly with lymphoid cell population in formalin fixed sections of spleen

Table 1
Percentage of Ig+ cells in blood and lymphoid organs of fishes.

Fish	MAbs	PBL	HKL	Spleen	Thymus	Reference
Paralichthys olivaceus	JFW20, JFW21	5.2–26.1	1.9–8.3	N.D.	N.D.	Matsuyama et al., [17]
Paralichthys olivaceus	2D8	40.48	9.67	17.32	N.D.	Li et al. [18]
Paralichthys olivaceus	FIM 511	N.D.	49	24	N.D.	Jang et al. [19]
Paralichthys olivaceus	FB17	N.D.	10.3	14.1	N.D.	Tokuda et al. [20]
Oncorhynchus mykiss	Four MAbs	35–51	N.D.	28–35	1–2	Thuvander et al. [21]
Oncorhynchus mykiss	Twelve MAbs	3.2–18.1	6.1–27.4	0.6–28.1	5.7–46.5	Sanchez et al. [22]
Salmo salar	MAb Type II	33	N.D.	N.D.	N.D.	Magnadottir et al. [23]
Dicentrarchus labrax, L.	DLIg3	19.7	12.2	22.2	N.D.	Scapigliati et al. [9]
Dicentrarchus labrax, L.	DLIg3	21	33	30	2	Romano et al. [24]

PBL, Peripheral Blood lymphocytes; HKL, Head Kidney Lymphocytes; N.D., Not done.

and kidney and in fixed smears of blood cells, as also reported in other fishes [26]. The DAB reactivity was observed as uniform dark brown colour and appreciable mainly as unipolar reaction on cytoplasmic membranes of lymphoid cells. In contrast, the melanin-possessing cells had black to brown granular cytoplasm. In spleen, Ig+ cells were particularly more abundant in mantle layer of splenic corpuscles. It is presumed that most of these reacting cells would probably be B-cells or plasma cells, but macrophages, neutrophils and non-specific cytotoxic cells have been also reported to be Ig+, probably through binding of Ig to Fc receptors [26]. Ig+ cells were observed mainly as individual cells and rarely as clusters of a few cells in above tissues, in accordance with earlier reports [14,26,27]. The number of Ig+ cells was more in spleen than in kidney. This finding correlates well with the results obtained in flow cytometry of spleen and kidney MNCs in our experiment and in previous studies involving flounder [18], puffer fish [11] and rohu [3]. In contrast, Fournier-Betz et al. [27] and Petteresen et al. [14] observed more Ig+ cells in kidney than in spleen by immunoperoxidase test. In kidney, glomerular basement membrane also showed reactivity with C9 MAb and this might be due to deposition of immune complexes in the basement membrane. An immune-complex glomerulonephritis of Chinook salmon, *Oncorhynchus tshawytscha* suggestive of type III hypersensitivity [28] and immunoglobulin-positive deposits in glomerular basement membrane of bacterial kidney disease infected rainbow trout [29] also support the present observation. A few cells in blood vessels of kidney showing reactivity to C9 MAb are presumed to be circulating B-lymphocytes.

C9 MAb did not show any reactivity in thymus sections. On the contrary, Fournier-Betz et al. [27] observed a few Ig+ cells near Hassall's corpuscles in turbot thymus. In blood smears, C9 MAb showed binding to a population of MNCs in IIPT and these cells were presumed to be B-lymphocytes. The reactivity of MAb was not observed with erythrocytes, granulocytes and a few MNCs. The presence of Ig+ cells in preparations of fish peripheral blood leukocytes has been demonstrated earlier for carp [30], sea bass ([31] and salmon [14].

Our findings show that the developed MAbs can be successfully employed for detection and quantification of Cs-Ig in immunological studies.

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