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Short Communication

Monoclonal antibody to serum immunoglobulins of *Clarias batrachus* and its application in immunoassays

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ABSTRACT

Serum immunoglobulins of *Clarias batrachus* (Cb-Ig) were purified by affinity chromatography using bovine serum albumin as capture ligand. Under reducing conditions in SDS-PAGE, Cb-Ig was composed of a heavy (H) chain (68.7 kDa) and two light (L) chains (27.4 and 26.3 kDa). Purified Cb-Ig was used to produce a monoclonal antibody (MAb) designated E4 MAb that belonged to IgG1 subclass. In Western blotting, this MAb showed binding to H chain of purified Cb-Ig and putative H chains in reduced sera of *C. batrachus*, *Clarias gariepinus* and *Heteropneustes fossilis*. However, no binding was observed with serum protein of *Labeo rohita* and *Channa striata*. Cross-reactivity of anti-Cb-Ig MAb was observed with serum of *C. batrachus*, *C. gariepinus* and *H. fossilis* in competitive ELISA. In immunoblotting of non-reduced Cb-Ig with E4 MAb, four bands assumed to be tetrameric, trimeric, dimeric and monomeric form were observed. In flow cytometric analysis of the gated lymphocytes, the number of surface Ig-positive (Ig+) cells in blood, spleen, kidney and thymus of *C. batrachus* was determined to be 50.1 ± 3.1, 55.1 ± 3.36, 42.4 ± 4.81 and 5.1 ± 0.89%, respectively, using E4 MAb. Ig+ cells were also demonstrated in formalin-fixed paraffin embedded tissue sections of spleen, kidney, thymus and smears of blood mononuclear cells in indirect immunoperoxidase test. The developed MAb was employed to detect pathogen-specific immunoglobulins in the sera of *C. batrachus* immunized with killed *Edwardsiella tarda*, by an indirect ELISA. This monoclonal antibody can be useful tool in immunological research and assays.

1. Introduction

Teleosts are the earliest evolutionary class to possess adaptive immunity which is composed of humoral and cell-mediated immunity. The adaptive immune responses are mediated by actions of 2 major groups of lymphocytes classified as B and T cells. Immunoglobulins (Ig), secreted by B cells, are the major effector molecules in humoral immunity and are directed to neutralize the pathogen or tag them for removal by the immune system. Teleost B cells produce 3 different immunoglobulin isotypes; namely, IgM, IgD and IgT. The IgM is the principal player in systemic immunity and IgT appears to be the teleost Ig class specialized in mucosal immune responses (Salinas et al., 2011). Monoclonal antibodies (MAbs) specifically reacting with Ig have proven to be a powerful tool for determining the level of total and specific Ig (Tang et al., 2010; Zhan et al., 2009) and number of Ig-positive (Ig+) cells in different tissues in healthy (Li et al., 2007) infected and vaccinated fish (Tang et al., 2010; Xu et al., 2011). Such monoclonal antibodies have also been used for immunolocalization of Ig+ cells in lymphoid organs (Sood et al., 2011; Tokuda et al., 2000) and have contributed greatly to improved understanding of the architecture and functioning of the fish immune system.

The fish *Clarias batrachus*, locally known as magur, is native to Southeast Asia. The natural distribution range of the species includes India, Bangladesh, Sri Lanka, Pakistan, Myanmar, Malaysia, Singapore, Philippines, Borneo, Java and Thailand (Talwar and Jhingran, 1992). The fish can occur in all types of freshwater but are more abundant in derelict and swampy waters with high turbidity. *C. batrachus* is a popular and valuable food fish owing to its good taste and low fat content, and fetches a high market price. Due to aerial respiration, the fish can be traded and sold live, thereby, ensuring a fresh food product. This catfish is at increased risk of developing infections by virtue of its preferential habitat in bottom zones of swampy waters, where the bacterial population may be 10–20 times higher than in water column (Lewis and Bender, 1961). There are reports of occurrence of diseases and health related problems in this species (Anonymous, 1981; Kanchanakhan, 2009).

MAbs have been raised to Ig of a number of teleost fish (Li et al., 2007) and most of the MAbs recognize the heavy chain of Ig (Scapigliati et al.,

Abbreviations: APES, 3-aminopropyltriethoxysilane; BSA, bovine serum albumin; c-ELISA, competitive ELISA; Cb-Ig, *Clarias batrachus* immunoglobulins; DAB, 3-3′ diaminobenzidine; DMEM, Dulbecco's Modified Eagle Medium; FITC, fluorescein isothiocyanate; FSC, forward scatter; HAT, hypoxanthine–aminopterin–thymidine medium; HC, heavy chain; Ig, immunoglobulins; IIPT, indirect immunoperoxidase test; LC, light chain; MAbs, monoclonal antibodies; MNC, mononuclear cells; MW, molecular weight; OD, optical density; OPD, ortho-phenylenediamine; PBS-T, phosphate buffer saline with 0.05% Tween-20; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, side scatter; TMB, 3,3′,5,5′-tetramethyl benzidine.

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1999). There is a report on purification and characterization of serum immunoglobulins of *C. batrachus* (Swain et al., 2004). However, there is no published information regarding reactivity of monoclonal antibodies in lymphoid organs of *C. batrachus*. In this paper, we describe the purification of serum immunoglobulins of *C. batrachus* (Cb-Ig), production of monoclonal antibodies to the purified immunoglobulins and their characterization. It is envisaged that monoclonal antibodies against serum Ig of *C. batrachus* will help in better understanding of immune system of this species.

2. Materials and methods

2.1. Fish immune sera preparation

Fifteen apparently healthy *Clarias batrachus*, weighing 100–150 g were acclimatized in fiber-reinforced plastic (FRP) tanks and divided in two groups (test and control). The test group comprised of 10 fish, whereas, five fish served as control. The fish in test group were immunized intra-peritoneally with 100 μg of bovine serum albumin (BSA) emulsified with Freund's complete adjuvant (Sigma-Aldrich, St. Louis, USA) and subsequently boosted thrice with similar emulsion in Freund's incomplete adjuvant (Sigma-Aldrich) at 2 week intervals. The fish in control group were injected with phosphate buffer saline (PBS) emulsified in Freund's complete and incomplete adjuvant, similarly. Pre- and post-immunization blood samples (7 days after last injection) were collected from the fish and allowed to clot overnight at 4 °C. It was centrifuged at 2000 rpm to collect the serum. The harvested serum was stored at −20 °C.

Indirect hemagglutination (IHA) test was used to assess the humoral immune response to BSA in immunized fishes (Cho et al., 1976). Briefly, sheep erythrocytes (S-RBCs) were fixed in glutaraldehyde and sensitized with BSA. For the test, two-fold dilution of individual fish serum (1:2 to 1:512) was prepared in PBS in a 96 well microtiter plate, except RBC control wells. Equal volume of sensitized S-RBCs (50 μl) was added to each well, incubated for half an hour (h) and the highest dilution of serum showing agglutination was considered as IHA titer.

2.2. Purification of Ig by affinity chromatography

BSA-CL agarose column (Genei, India) was used to purify anti-BSA Ig from immunized fish following Rathore et al. (2008). SDS-PAGE was carried out under reducing and non-reducing conditions to check the purity and molecular weight (MW) of the purified Ig. Reduced samples were analyzed on 12% gel (Laemmli, 1970) while non reduced samples were analyzed on 3–12% polyacrylamide gel (Walker, 1996). The gels were subsequently stained with Coomassie Brilliant Blue to detect the polypeptides. The molecular weight of the bands in the gel was determined by LabWorks Image Acquisition and Analysis software (UVP BioImaging Systems) using appropriate MW markers.

2.3. Fish serum collection

Blood was collected from caudal vein of *C. batrachus* (100–150 g), *Clarias gariepinus* (400–500 g), *Heteropneustes fossilis* (80–100 g), *Channa striata* (200–250 g) and *Labeo rohita* (250–300 g) and allowed to clot. The serum was separated and stored at −20 °C. Serum from three individuals of each species was pooled for use in Western blotting and competitive ELISA.

2.4. Production of monoclonal antibodies

Monoclonal antibodies to Cb-Ig were raised following the standard procedure (Hamilton and Davis, 1995). Female BALB/c mice were procured from the Central Drug Research Institute, Lucknow and 2 mice were immunized with 50 μg of Cb-Ig emulsified in Freund's complete adjuvant (Sigma-Aldrich) by subcutaneous route. These mice were boosted twice with similar emulsion in Freund's incomplete adjuvant at 2 week intervals. Test bleeding of the mice was done on day 36 for testing the antibody titer. A final injection of Cb-Ig in PBS (25 μg) was given by intraperitoneal route to the mouse with higher antibody titer. Four days after last injection, the spleen cells from the mouse were collected and fused with myeloma cells (SP2/0) at a ratio of 10:1, using PEG–DMSO (Sigma-Aldrich). The fused cells were seeded in 96 well plates and grown in Dulbecco's Modified Eagle Medium (DMEM) containing hypoxanthine–aminopterin–thymidine (HAT, Sigma). The plates were checked for growth of hybridomas and positive clones were screened using indirect ELISA. The positive clones were subjected to single cell cloning and subcloning using limiting dilution method. The single clones were again checked by indirect ELISA and positive clones were further expanded. The class of monoclonal antibodies was determined by a mouse monoclonal antibody isotyping kit (Sigma-Aldrich).

An indirect ELISA was carried out for titration of mice sera and screening of wells containing hybridomas, for anti-Cb-Ig antibodies (Sood et al., 2011). The serum dilution giving 5 times OD to that of 0-day serum was considered as the titer. Similarly, the culture supernatants that gave 5 times OD to that of 0-day mouse serum on two occasions were selected for limiting dilution.

One of the strongly reacting MAbs (E4 MAb) was characterized by a number of immunological assays; (1) Western blotting to know the reactivity of anti-Cb-Ig MAb, (2) competitive ELISA (c-ELISA) to know the antigenic relatedness of Cb-Ig with whole serum of heterologous fish species, (3) flow cytometry to quantify Ig-positive (Ig+) cells in blood and lymphoid organs, (4) indirect immunoperoxidase test (IIPT) to demonstrate the reactivity of selected MAb in tissue sections of lymphoid organs and smears of blood mononuclear cells (MNCs) and (5) indirect ELISA to detect *Edwardsiella tarda*-specific antibodies in serum of immunized magur.

2.5. Western blot analysis

The reactivity of selected monoclonal antibody against reduced Cb-Ig and serum was checked using standard technique for Western blotting (Towbin et al., 1979). Briefly, after SDS-PAGE of reduced Cb-Ig and pooled sera samples of *C. batrachus*, *C. gariepinus*, *H. fossilis*, *C. striata* and *L. rohita*, proteins were electrophoretically transferred from unstained 12% gel to nitrocellulose membrane (Sigma-Aldrich) at 25 V for 2 h. After blocking in PBS with 5% skimmed milk powder, the nitrocellulose strips were incubated with 1:20 dilution of culture supernatant of E4 MAb. Following three washings, the strips were incubated with 1:4000 dilution of rabbit anti-mouse IgG peroxidase conjugate (Sigma-Aldrich) and the reaction was visualized using 3,3′,5,5′-tetramethyl benzidine (TMB) substrate solution (Sigma-Aldrich).

Immunoblotting analysis was also carried out following SDS-PAGE of non-reduced Cb-Ig on a gradient (3–12%) polyacrylamide gel.

2.6. Competitive ELISA

The optimal dilution of E4 MAb giving an OD in the range of 1.0 ± 0.2 was determined by an indirect ELISA, using doubling dilutions of MAb on Cb-Ig coated plates. ELISA plates were coated with 50 μl well^{-1} of Cb-Ig (1 μg ml^{-1}) at 37 °C for 1 h. Serial dilutions (1:200 to 1:25,600) of sera samples of *C. batrachus*, *C. gariepinus*, *H. fossilis*, *L. rohita* and *C. striata* were used as competitor. The competition was carried out by adding 50μl well^{-1} of serially diluted competitor along with 50 μl of optimally diluted E4 MAb, in duplicate. In control wells, MAb was added without any competitor. The plate was incubated overnight at 4 °C and subsequently washed. The plate was again incubated with 50 μl of goat anti-mouse IgG peroxidase conjugate (1:4000) at 37 °C for 1 h. After washing, color reaction was developed by adding OPD. The

reaction was stopped by adding 2 N sulfuric acid to obtain expected OD of 1.0 ± 0.2 in MAb control wells. Percent competition was calculated using the following formula:

$$\text{Percent competition} = 100 - [(\text{OD of well with competitor} / \text{OD of MAb culture supernatant}) \times 100)].$$

2.7. Flow cytometry

The studies were performed on 15 apparently healthy *C. batarchus* (100–150 g). Flow cytometric analysis was carried out separately for blood, spleen, kidney and thymus in triplicates and each replicate consisted of pooled tissues of 5 fish. Blood was collected from caudal vein of *C. batrachus* using ethylenediaminetetraacetic acid as anticoagulant, diluted 1:1 with PBS and layered on Histopaque-1077 (Sigma-Aldrich) for separation of MNCs. Single cell suspension of *C. batrachus* spleen, kidney and thymus was 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. The cells were washed twice with DMEM and 10^6 cells from each organ were incubated with 250 μl of E4 MAb (culture supernatant diluted 1:20 in DMEM) on ice for 30 min. In control cells, MAb was replaced with myeloma culture supernatant. After three washings with DMEM, the cells were incubated with 250 μl of 1:400 dilution of rabbit anti-mouse IgG FITC conjugate (Sigma-Aldrich) for 30 min on ice. The cells were washed again and analyzed by flow cytometer FACS CALIBER (Becton Dickinson, New Jersey, U.S.) equipped with an argon-ion laser tuned to 480 nm. Finally, ten thousand events were acquired from each sample and data were analyzed using software. Ig-positive (Ig+) cells were enumerated as percent of total events.

2.8. Indirect immunoperoxidase test

Ig+ cells were demonstrated in spleen, kidney and thymus sections and smears of blood MNCs using indirect immunoperoxidase test (Sood et al., 2011). Briefly, after rehydration, the endogenous peroxidase activity of tissue sections and blood smears was quenched with 3% hydrogen peroxide in methanol for 15 min. Thereafter, the heat-mediated antigen retrieval of tissue sections was carried out in antigen unmasking solution (Vector Laboratories, Burlingame, CA 94010) at 800 W for 20 min. After conditioning the sections in PBS-T, the slides were laid flat and blocked with normal horse serum (Vector Laboratories) followed by overnight incubation with E4 MAb culture supernatant at 4 °C. In control slides, E4 MAb was replaced with myeloma culture supernatant. After washing, the sections/smears were incubated with ImmPRESS anti-mouse Ig Reagent (Vector Laboratories) for 30 min at 37 °C and washed with PBS-T. Color was developed by adding 3–30 diaminobenzidine (DAB) chromogen solution. After washing the slides, the sections were counterstained in Gill's hematoxylin, dehydrated, cleared and mounted in DPX.

2.9. Detection of anti-E. tarda antibodies

Ten *C. batrachus* (70–80 g) were immunized intraperitoneally with 10^8 cells of ethanol killed *E. tarda* in 100 μl PBS. After 14 days, the fish were boosted with a similar dose of antigen. One week later, all the fish were bled, serum collected and stored at −20 °C for further use. The detection of specific anti-*E. tarda* Ig in the individual serum was carried out by an indirect ELISA, using anti-Cb-Ig MAb as detector antibodies. For this, wells of ELISA plate were coated with 50 μl of sonicated supernatant (protein concentration 1 μg ml^{-1}) of *E. tarda*, diluted in carbonate–bicarbonate buffer. After washing, 50 μl of pooled pre- and post-immunization sera were serially diluted (1:100–1:51,200) in the coated plate. The plates were incubated overnight at 4 °C. Following washing, optimal dilution of E4 MAb was added and incubated at 37 °C for 1 h. The binding of MAb to Cb-Ig was detected with goat anti-mouse IgG peroxidase conjugate (1:4000). Color reaction was developed by the addition of OPD substrate as mentioned earlier and OD was recorded.

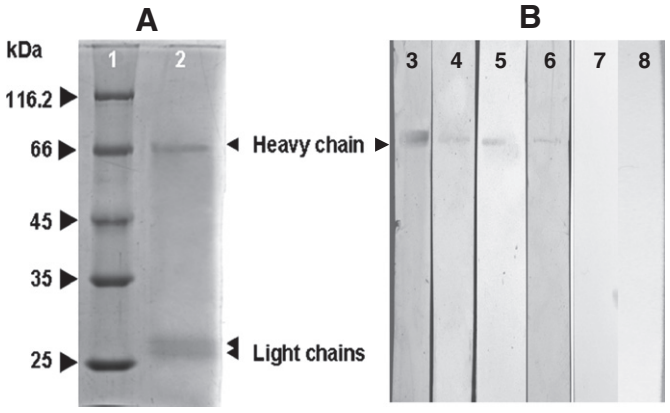

Fig. 1. Demonstration of reactivity of monoclonal antibodies (MAbs) to *Clarias batrachus* purified Ig (Cb-Ig) and serum by Western blotting. A. SDS-PAGE of Cb-Ig was carried out in 12% gel under reducing conditions and the gel was stained with Coomassie Brilliant Blue. Lane 1: molecular weight marker (Fermentas) and Lane 2: Cb-Ig. B. Western blotting of Cb-Ig and serum of *C. batrachus* and heterologous fish species (n = 3) with E4 MAb; Lane 3: Cb-Ig, Lane 4: *C. batrachus* serum, Lane 5: *Clarias gariepinus* serum, Lane 6: *Heteropneustes fossilis* serum, Lane 7: *Labeo rohita* serum and Lane 8: *Channa striata* serum.

3. Results

3.1. Purification and characterization of Cb-Ig

The anti-BSA antibody titer in immunized fish was checked by IHA test and was found to range from 1:8 to 1:128, whereas the antibody titer

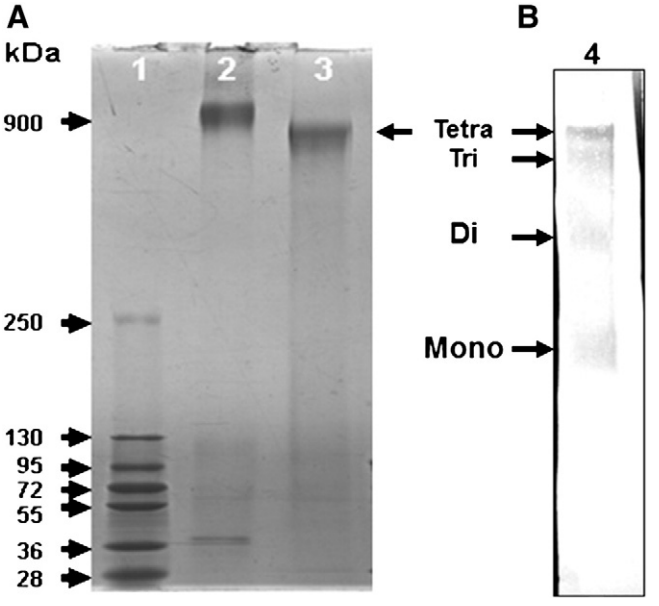

Fig. 2. SDS-PAGE and immunoblotting analysis of purified *Clarias batrachus* purified Ig (Cb-Ig) under non-reducing conditions. A. SDS-PAGE was carried out on a 3–12% gradient gel which was subsequently stained with Coomassie Brilliant Blue. Lane 1: prestained protein molecular weight marker (Fermentas). Lane 2: bovine IgM (Sigma-Aldrich) and Lane 3: purified Cb-Ig. B. Reactivity of E4 MAb with Cb-Ig (Lane 4).

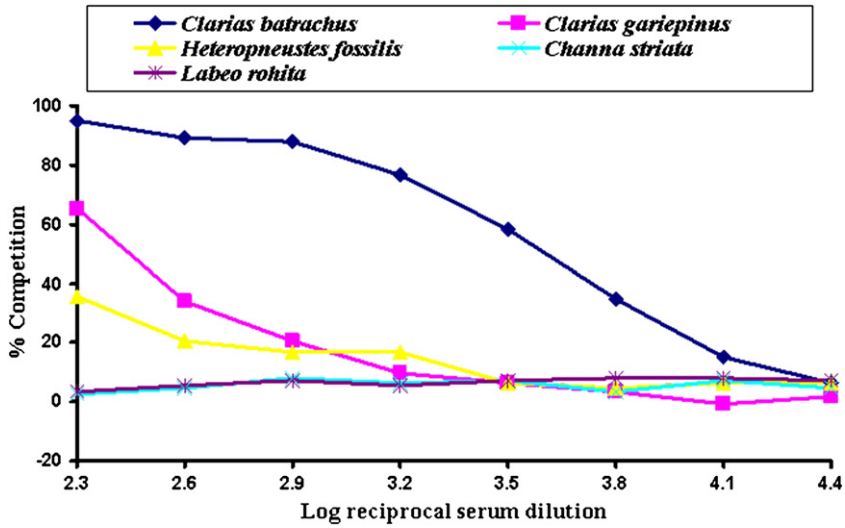

Fig. 3. Demonstration of antigenic relatedness of serum Ig of *Clarias batrachus* with serum Ig of heterologous fish species as detected by E4 MAb in competitive ELISA. Competition was carried out on Cb-Ig coated plates with 2-fold dilution of serum from heterologous fish species against fixed dilution of E4 MAb.

was <1:2 in control group and pre-immunization serum. Sera samples with antibody titer of 1:32 and more were pooled and passed through BSA-CL agarose column. A single peak was observed on elution with glycine NaOH buffer and the pH of eluted fractions was neutralized by adding 2 M Tris–HCl. Subsequently, the eluted fractions were concentrated with Centriplus-YM filter (Millipore, Billerica, Massachusetts, USA). The protein concentration of this fraction (1.4 ml) from 8 ml of hyper-immunized serum was determined to be 1.56 mg/ml. The purity of

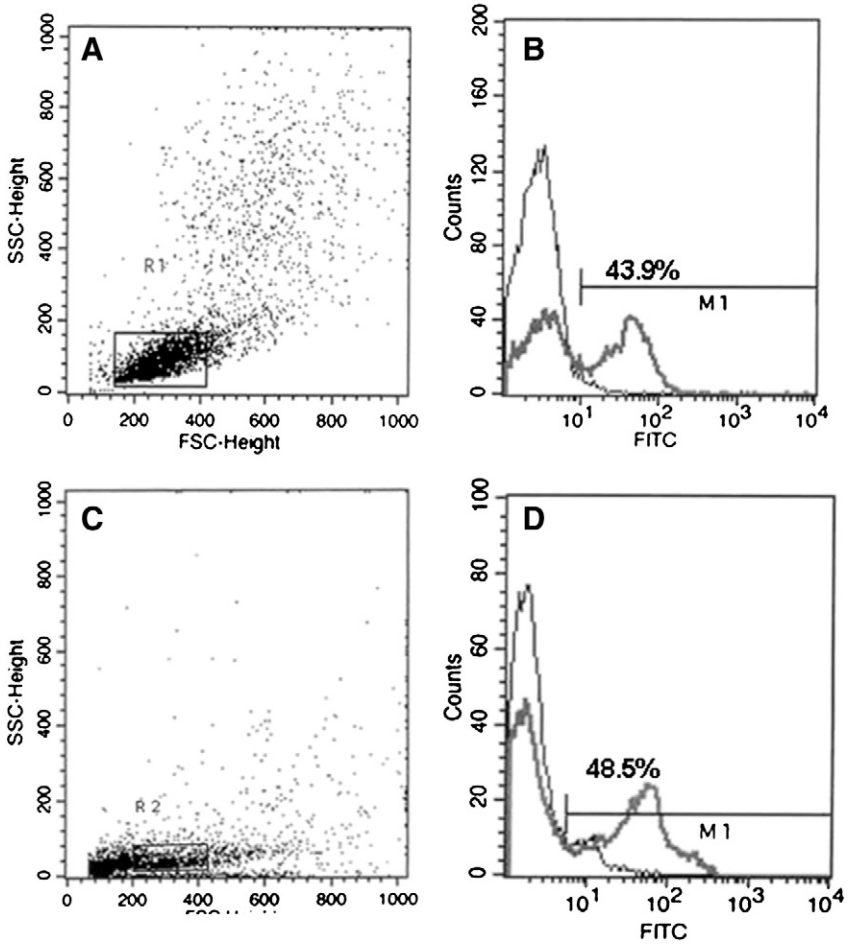

Fig. 4. Quantification of Ig-positive mononuclear cells in blood and lymphoid organs of *Clarias batrachus* by flow cytometry. FSC/SSC dot plot of mononuclear cells of blood, spleen, kidney and thymus respectively, showing gated lymphocytes (A, C, E, G). Fluorescence histogram of gated mononuclear cells in blood (B), spleen (D), kidney (F) and thymus (H) without MAb (black line) and with E4 MAb (gray line).

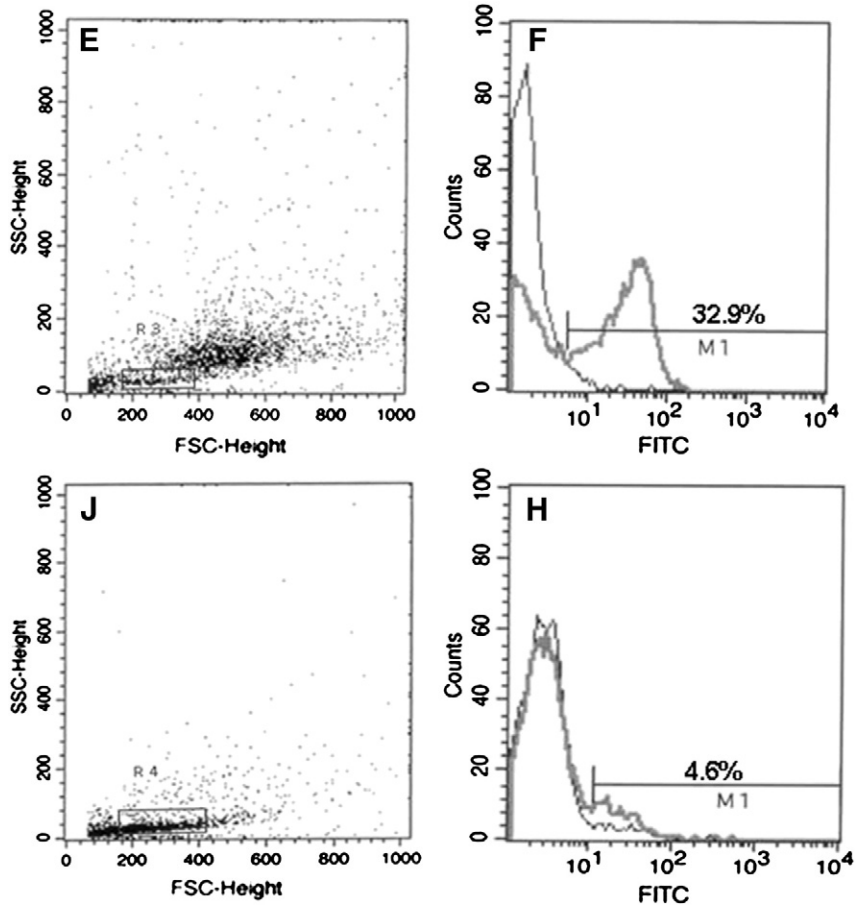

Fig. 4 (*continued*).

concentrated protein was analyzed by reducing and non-reducing PAGE. On SDS-PAGE under reducing conditions, the Cb-Ig dissociated into a heavy (H) chain and two light (L) chain subunits [Fig. 1(A)]. The molecular weight of H chain was estimated to be 68.7 kDa and that of L chain subunits was 27.4 and 26.3 kDa. In non-reducing gradient SDS-PAGE, only a single prominent band of 806.6 kDa was observed [Fig. 2(A)].

3.2. Production of monoclonal antibodies to Cb-Ig

There was a significant increase in anti-Cb-Ig antibody titer in mice sera on 36th day of immunization program with respect to 0-day mice serum, as detected by indirect ELISA. Following fusion of splenocytes from immunized mouse with myeloma cells, 322 hybridoma clones were obtained, out of which, 49 clones showed reactivity to Cb-Ig in ELISA. Two clones, selected on basis of their strong reactivity were subjected to single cell cloning by limiting dilution. The limiting dilution was repeated three times. Finally, one clone (E4) was selected and characterized. The antibodies secreted by this clone were designated E4 MAb and this MAb belonged to IgG1 subclass.

3.3. Western blotting

Fig. 1(B) shows the results of immunoblot analysis of E4 MAb with reduced Cb-Ig and serum. The MAb reacted with heavy chain of Cb-Ig and putative H chains in reduced sera samples of *C. batrachus*, *C. gariepinus* and *H. fossilis* but no reactivity was observed with reduced sera of *C. striata* and *L. rohita*.

In Western blots of non-reduced Cb-Ig, E4 MAb showed reactivity with four protein bands [Fig. 2(B)]. The molecular weight of these bands was estimated to be 806, 637, 418 and 211 kDa.

3.4. Competitive ELISA

The optimal dilution of E4 MAb giving an OD of 1 ± 0.2 was determined to be 1:64 by indirect ELISA. Antigenic relatedness of Cb-Ig was demonstrated as the cross reactivity pattern of E4 MAb with serum proteins of *C. batrachus*, *C. gariepinus*, *H. fossilis*, *C. striata* and *L. rohita* through competitive ELISA. E4 MAb showed competition with *C. batrachus*, *C. gariepinus* and *H. fossilis* serum [Fig. 3].

3.5. Flow cytometry

MNCs from blood, spleen, kidney and thymus were stained by immunofluorescence and analyzed for forward scatter (FSC) and side scatter (SSC) pattern, representing size and granularity of the cells, respectively. Cells with smaller size and less granularity were presumed to be lymphocytes [Figs. 4(A, C, E, G)]. These cells had relatively homogeneous FSC (200–400) properties. Cell debris and dead cells were observed in all samples at the extreme left with FSC value closer to 50 and were excluded from gate. Percentage of Ig+ cells was calculated by means of flow cytometric analysis. Analysis of gated cells presumed to be lymphocytes, revealed that the percentage of Ig+ cells (mean ± SE) was 50.1 ± 3.1, 55.1 ± 3.36, 42.4 ± 4.81 and 5.1 ± 0.89% cells in blood, spleen, kidney and thymus, respectively [Figs. 4(B, D, F, H)].

3.6. Indirect immunoperoxidase test

E4 MAb was successfully employed as primary antibody to demonstrate Ig+ cells in formalin-fixed paraffin-embedded sections of spleen, kidney and thymus. In the spleen, Ig+ cells were distributed uniformly in the lymphoid follicles [Figs. 5(A,B)]. The Ig+ cells were

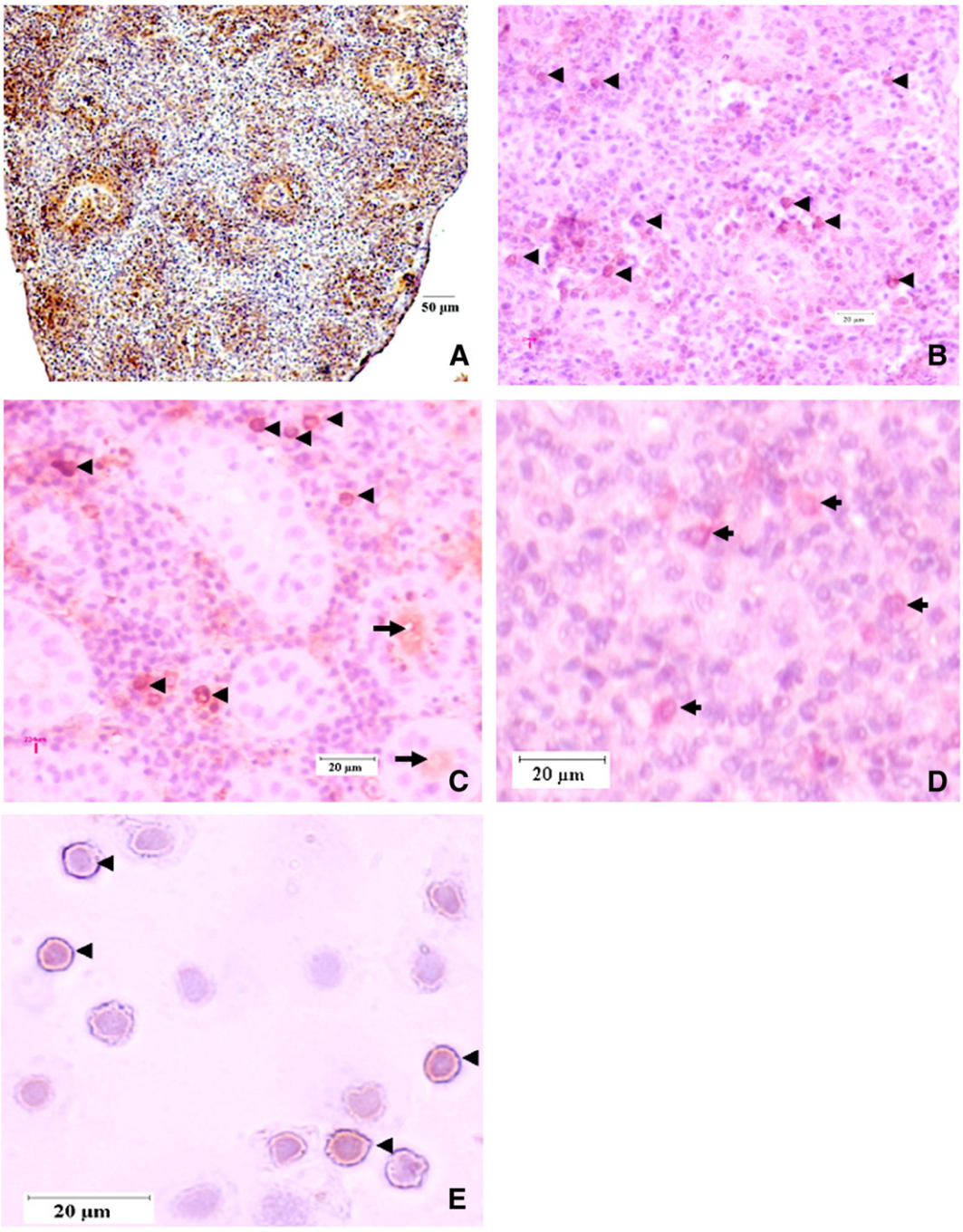

Fig. 5. Immunohistochemical staining of Ig+ cells in formalin-fixed paraffin-embedded tissue sections of *Clarias batrachus* with E4 MAb. (A) Spleen, Ig+ cells were evident in lymphoid follicles of spleen (100×); (B) spleen, higher magnification of lymphoid follicles showing Ig+ cells which were distributed predominantly as single cells (arrowheads); and (C) kidney, Ig+ cells were seen in the lymphopoietic areas (arrowheads). Mild reactivity of E4 MAb was also observed along tubular lumen at some places (arrows); (D) thymus, Ig+ cells were seen in the medullary region (arrowheads); and (E) blood MNCs smear, small round cells with large nucleus took marginal staining along the margins (arrows). Note some lymphocyte-like cells did not show marginal reactivity.

scattered in the follicles mainly as single cells and sometimes as small clusters of 2–3 cells. No specific association of Ig+ cells was observed with melanomacrophage centers. In the head and trunk kidney, E4 MAb reactivity was observed as marginal staining of individual cells in lymphopoietic areas. Mild reactivity was also observed along the luminal surface of renal tubules at some places [Fig. 5(C)]. Overall, less reactivity was observed in kidney sections as compared to spleen sections. In the thymus, very few cells in the medullary region showed reactivity with the E4 MAb [Fig. 5(D)]. In smears of blood MNCs, the lymphoid cells showed DAB staining along their margins but some negative cells were also observed [Fig. 5(E)]. The reactivity was not observed with rare erythrocytes present in the smears. No DAB staining was observed in control slides.

3.7. Detection of anti-E. tarda antibodies

E4 MAb was used as detector antibody in indirect ELISA for measuring the humoral antibody response in *C. batrachus*. An elevated and measurable immune response to *E. tarda* was observed in the sera of immunized fish [Fig. 6(A)]. There was almost a linear decline in specific antibody level with an increase in serum dilution. The non-immunized control fish did not show any increase in antibody titer [Fig. 6(B)].

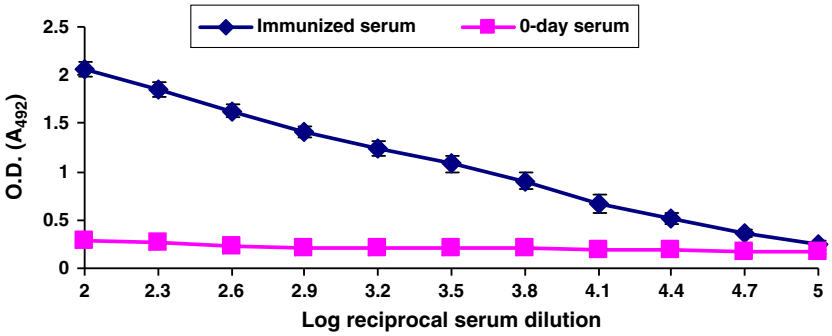

Fig. 6. Monitoring of humoral immune response in sera of *Clarias batrachus* (n = 10) immunized with killed *Edwardsiella tarda*. Plates were coated with sonicated supernatant (protein concentration 1 μg ml^{-1}) of *E. tarda* and immune response was monitored using E4 MAb as detector antibodies through indirect ELISA. An increase in pathogen-specific antibodies was clearly evident.

4. Discussion

In the present study, BSA was used as immunogen to induce anti-BSA antibodies in fish. The response to BSA was quite variable with antibody titer ranging from 1:8 to 128, as measured by IHA test and a total of 2.2 mg of *C. batarchus* Ig was purified from 8 ml (i.e. 0.275 mg/ml) of hyperimmune serum. BSA is known to produce a poor and inconsistent immune response in fish (Bag et al., 2008; Rathore et al., 2008). This necessitates administering a number of booster injections to produce adequate immune response. However, BSA is still preferred as BSA-CL Agarose columns are commercially available. The anti-BSA antibodies were successfully eluted from hyperimmunized *C. batrachus* serum by BSA-CL agarose affinity column, in accordance with earlier studies (Palenzuela et al., 1996; Pettersen et al., 2000). The yield of anti-BSA antibodies eluted from hyperimmune fish serum has been reported to be quite variable; 0.75–1.5 mg/ml in rohu (Rathore et al., 2008), 1 mg/ml serum in barramundi (Bryant et al., 1999), 0.255–0.33 mg/ml in Indian major carps (Bag et al., 2008) and 0.15 mg/ml in *C. batrachus* (Swain et al., 2004).

The SDS-PAGE of reduced Cb-Ig displayed one band at 68.7 kDa corresponding to heavy chain and two bands of 27.4 and 26.3 kDa corresponding to light chains, respectively. Previously, Swain et al. (2004) have reported *C. batrachus* Ig molecule to be composed of two heavy chain subunits with MW of 66.2 and 59.3 kDa and two light chains of 27.6 and 26.4 kDa. In the present study, only one heavy chain was observed repeatedly, though varying concentration of Cb-Ig was loaded in reducing SDS-PAGE. The gradient SDS-PAGE of purified protein under non-reducing conditions revealed a single band of 806.6 kDa. Previously, the MW of *C. batrachus* Ig has been previously reported to be 862.8 kDa in native gradient PAGE (Swain et al., 2004) and that of *C. gariepinus* Ig to be 840 kDa by gel filtration chromatography (Rathore et al., 2006). The variation in size and number of H and L chains has been reported for sea bass, *Dicentrarchus labrax* Ig (dos Santos et al., 1997; Palenzuela et al., 1996; Romestand et al., 1995; Scapigliati et al., 1996), flounder, *Paralichthys olivaceus* Ig (Bang et al., 1996; Jang et al., 2004; Li et al., 2007) and Atlantic salmon, *Salmo salar* Ig (Haverstein et al., 1988; Magnadóttir, 1990). The reasons for these variations are not obvious but may be due to different gel concentration and molecular weight markers, relative purity of the Ig and degradation of purified proteins. Since each Ig molecule is composed of 2H and 2L chains, therefore, the MW of monomeric Cb-Ig is presumed to range from 190 to 192.2 kDa. It is also reasonable to assume that a protein of 800 kDa would have a tetrameric structure and this is in accordance with observations for other teleost species Ig, where a tetrameric structure of Ig has been suggested.

In the present study, about 15% of the hybridomas produced antibodies that were reactive to Cb-Ig. Following limiting dilution, selected E4 MAb was found to be directed against epitope on heavy chain of Cb-Ig, as has been the case with most of the MAbs against fish Ig (Rathore et al., 2008; Scapigliati et al., 1999) and belonged to IgG1 class. In Western blotting, E4 MAb also showed reactivity to putative H chains in reduced sera samples of *C. batrachus*, *C. gariepinus* and *H. fossilis*. It appears that E4 MAb recognizes an epitope on heavy chain that is partially conserved in these fish species. No reactivity of MAb was observed with serum Ig of distantly related fish species *C. striata* and *L. rohita*. In c-ELISA, E4 MAb cross reacted with serum Ig of *C. batrachus*, *C. gariepinus* and *H. fossilis*. The results of c-ELISA with E4 MAb correlated well with the findings in Western blotting where reactivity was found with putative H chains in reduced sera of *C. gariepinus* and *H. fossilis*. Though the monoclonal antibodies are highly specific and can differentiate small variations in epitopes, yet close structural similarity in heavy or light chains of immunoglobulins can result in cross reactivity. Serum from heterologous fish species has been used to study the cross reactivity pattern of monoclonal antibodies raised against fish immunoglobulins (MacDougal et al., 1995; Morrison et al., 2002; Rathore et al., 2008; Thuvander et al., 1990) and it has been reported that only the immunoglobulin in the sera of other species participates in cross reaction with the antibodies (Israelsson et al., 1991). The antigenic relatedness of immunoglobulin molecules of fish species within same genus (Miyadai et al., 2004; Sood et al., 2011), across different genera (Morrison et al., 2002; Rathore et al., 2008; Thuvander et al., 1990; Vesely et al., 2006) and families (MacDougal et al., 1995; Vesely et al., 2006) has been reported earlier on basis of MAb binding.

Immunoblotting of non-reduced Cb-Ig with E4 MAb revealed the presence of four bands. Based on estimated molecular weight and reactivity with anti Cb-Ig MAb, it is reasonable to assume that these bands represent tetrameric, trimeric, dimeric and monomeric forms of Ig. However, only one band corresponding to tetrameric form was observed in SDS-PAGE of non-reduced Cb-Ig, as reported earlier (Swain et al., 2004). This implied that the concentration of trimeric, dimeric and monomeric forms was very low. The concentration of the dimeric form appeared to be the least of the four redox forms of Ig. The presence of several redox forms of IgM, as observed in the present study, seems to be a relatively common to teleost fish (Bromage et al., 2004; Grove et al., 2006; Whittington, 1993).

FACS analysis showed that MNCs from blood, spleen, kidney and thymus were reactive to E4 MAb. Most Ig+ cells were presumed to be lymphocytes, judging from their low scores of FSC and SSC in the dot plot FACS analysis. The number of Ig+ cells was higher in spleen than in the kidney with E4 MAb and this finding is in agreement with that of murrel (Sood et al., 2011), rohu (Rathore et al., 2008), Japanese flounder (Li et al., 2007; Tokuda et al., 2000) and torafugu (Miyadai et al., 2004). On the contrary, Jang et al. (2004) and Pettersen et al. (2000) reported higher number of Ig+ cells in head kidney than in spleen of Japanese flounder and Atlantic salmon, respectively. In *C. batrachus* blood, a high proportion of gated cells were reactive with E4 MAb, as has been reported earlier in rainbow trout (Thuvander et al., 1990), torafugu (Miyadai et al., 2004), Japanese flounder (Li et al., 2007) and rohu (Rathore et al., 2008). However, fewer cells (~5 to 25%)

have been reported to be reactive with MAbs against serum Ig (MacDougal et al., 1995; Matsuyama et al., 2009; Romano et al., 1997). In the thymus, there were a few Ig+ cells in accordance with earlier reports (Miyadai et al., 2004; Morrison et al., 2002; Romano et al., 1997). The quantification of Ig+ cells can be a useful tool to assess the immunocompetence of *C. batrachus*.

In IIPT, E4 MAb reacted with a lymphoid cell population in sections of spleen, kidney and thymus and smears of blood MNCs, as reported earlier (Fournier-Betz et al., 2000; Grove et al., 2006; Pettersen et al., 2000). MAb reactivity was observed in form of dark brown color along the margin of lymphoid cells. The reactivity was found to be more in spleen than in the kidney and correlated well with the results obtained in flow cytometry. These results are also in conformity with earlier studies (Rathore et al., 2008; Sood et al., 2011). On the contrary, Pettersen et al. (2000) and Fournier-Betz et al. (2000) reported more Ig+ cells in anterior kidney than in spleen. The reactivity along lumen of some renal tubules could be due to presence of secretory antibodies. In the blood MNCs, some of the cells having large nucleus with peripheral ring of cytoplasm showed DAB staining. 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 (Grove et al., 2006). The negative MNCs present in the smear were presumed to be T-lymphocytes, thrombocytes and monocytes. Since the E4 MAb detected a significant proportion of MNCs in the blood in flow cytometry and immunohistochemistry, therefore, one of the potential uses of this MAb can be to study changes in number of Ig+ cells following immunostimulation, vaccination and disease development.

MAbs to serum immunoglobulins have been successfully applied for detection of antibodies against pathogens by ELISA (dos Santos et al., 1997; Rathore et al., 2008; Thuvander et al., 1990). Similarly, E4 MAb also appeared to be suitable for the detection of pathogen-specific antibodies by means of an indirect ELISA. A clear increase in the antibody titer of immunized fish to *E. tarda* could be observed in comparison to pre-immunization sera, using E4 MAb as secondary antibody. This application of anti-Cb-Ig MAb can be utilized in evaluating antibody production in response to infection or for studying the possible effects of vaccination on antibody production.

In brief, the MAb produced in this study has proven to be useful in detecting Cb-Ig in ELISA, immunoblotting procedures and monitoring humoral immune response in *C. batrachus*. This MAb can also be used to quantify Ig+ cells in lymphoid organs, to study the ontogeny of lymphoid organs and also can have applications in studying the effect of abiotic factors on humoral immunity.

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