



Production and characterisation of monoclonal antibodies against immunoglobulins of *Cirrhinus mrigala* (Hamilton 1822)

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ABSTRACT

Serum immunoglobulins (Ig) of mrigal *Cirrhinus mrigala* (Hamilton 1822) immunised with bovine serum albumin (BSA), were purified by affinity chromatography using BSA-CL agarose column. The purified mrigal Ig (m-Ig) was characterised under reducing condition by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) which revealed two bands of 85 and 26 kDa corresponding to heavy and light chain, respectively. Following fusion of splenocytes from Balb/c mice immunised with purified m-Ig with myeloma cells, three hybridomas showing reactivity with m-Ig were cloned by limiting dilution. The monoclonal antibodies (MAbs) generated by these clones were designated as 3B2-E12, 3B2-F9 and 4C3-B2 and characterised by western blotting and isotyping. Western blot analysis of the supernatant from the three clones with purified m-Ig indicated that, all the three MAbs were specific to heavy chain. Isotyping revealed that 3B2-E12 MAb was of IgG1 isotype whereas the other two MAbs were of IgG2a isotype. Cross reactivity of anti-mrigal Ig MAb (3B2-E12) was observed with serum Ig of *Catla catla* and *Labeo rohita* indicating semi-conserved nature of Ig in Indian major carps.

Keywords: *Cirrhinus mrigala*, ELISA, Immunoglobulin, Monoclonal Antibodies, Western blotting

Introduction

Teleosts are the earliest vertebrates to possess both innate and adaptive immunity. The innate and non-specific immune parameters are the frontline fighters, first to respond to pathogens whereas the adaptive immune system armed with specificity and memory is acquired with time and provides extended immunity. Adaptive immunity in teleosts comprises both humoral and cell mediated immunity (Sood *et al.*, 2012). The immunoglobulins secreted by B cells play an important role in humoral immunity. Out of the three different Ig isotypes *i.e.* IgM, IgD and IgT, IgM is considered as the primary humoral component in most of the teleost fish (Bag *et al.*, 2009). The mrigal *Cirrhinus mrigala* (Hamilton 1822), is an Indian major carp (IMC) species endemic to Indo-Gangetic riverine systems. Owing to its consumer preference, the species forms an integral part of carp polyculture system. However, due to its bottom dwelling habit, the species faces increased risk of susceptibility to bacterial pathogens which is reported to be

10-20 times higher as compared to those species dwelling in the water column (Lewis and Bender, 1961). In addition, with regard to one of the serious oomycete fish pathogen *Aphanomyces invadans*, causing epizootic ulcerative syndrome in both freshwater and estuarine fishes, *C. mrigala* is reported to be severely affected in comparison to other species of IMCs (Parvati *et al.*, 2011; Pradhan *et al.*, 2014).

Monoclonal antibodies (MAbs) against Ig provide a powerful tool for monitoring the level of total and specific antibody production (Tang *et al.*, 2010) following an infection or vaccination (Al-Harbi, *et al.*, 2000). To date, MAbs have been established for a variety of freshwater and marine fish species. These include *Ictalurus punctatus* (Edholm *et al.*, 2010), *Paralichthys olivaceus* (Li *et al.*, 2007), *Takifugu rubripes* (Miyadai *et al.*, 2004), *Labeo rohita* (Rathore *et al.*, 2008), *Channa striata* (Sood *et al.*, 2011), *Clupea pallasii* (Purcell *et al.*, 2012), *Clarias batrachus* (Sood *et al.*, 2012), *Labeo rohita* (Rebello *et al.*, 2016), *Lateolabrax japonicus* (Yang *et al.*,

2018), *Oncorhynchus mykiss* (Hayashi *et al.*, 2019) and *Carassius auratus gibelio* (Wu *et al.*, 2020). All of these MAbs developed have numerous diagnostic applications in fish health management. With regard to mrigal, there has been report of isolation and characterisation of serum IgM (Bag *et al.*, 2009) but, MAbs against the Ig of mrigal have not yet been developed. Therefore, the present study was planned to purify serum Ig of *C. mrigala*, to produce MAbs against the purified Ig and to characterise the MAbs. It is envisaged that, MAbs against serum Ig of *C. mrigala* will help in better understanding of the immune system of the species and will act as a source of diagnostic reagent in MAb based immunoassays.

Material and methods

Immunisation of C. mrigala

Apparently healthy specimens of *C. mrigala* (N=7, 255±30 g) were collected from the freshwater fish farm of the Research Centre of ICAR-Central Institute of Fisheries Education (ICAR-CIFE) at Balbhadrapuram, Andhra Pradesh, India. After acclimatisation for two weeks, fish were immunised intraperitoneally with 200 µg bovine serum albumin (BSA) emulsified with Freund's Complete Adjuvant (FCA) (GeNei, India) @ 0.2 ml 100 g⁻¹ body weight and subsequently boosted thrice with similar emulsion in Freund's Incomplete Adjuvant (FIA) (GeNei, India) at two weeks interval. Post-immunisation (7 days after final dose), blood samples were collected and allowed to clot overnight at 4°C. The clotted blood was centrifuged @ 5000 g to separate serum and the serum collected was stored at -20°C for further studies.

Purification of mrigal Ig by affinity chromatography

Mrigal Ig was purified using an immuno-affinity BSA-CL Agarose column (GeNei, India) following Swain *et al.* (2004). The serum was diluted @ 1:3 with Tris buffered saline (TBS: 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4), filtered through 0.45 µm syringe filter and passed through affinity column. Before loading the samples, the column was washed with five-bed volumes (25 ml) of washing buffer (TBS) and then stripped with four-bed volumes (20 ml) of elution buffer (0.1 M glycine NaOH, pH 11.0) followed by washing with ten-bed volumes (50 ml) of TBS. The serum was loaded to the column slowly and the flow-through was repeatedly added to the column for efficient binding of the Igs to the column. The column was washed with TBS till the absorbance of the flow-through at 280 nm wavelength, became same as that of TBS. After washing of column, bound Ig in the column was eluted with 10 ml of elution buffer (0.1 M glycine, pH 11.0) and the filtrate was collected in 1.5 ml Eppendorf tubes. The absorbance of the fractions was measured at 280 nm using a spectrophotometer (Thermo Spectronic, USA). The

fractions showing >0.1 OD were pooled together followed by concentration in a cellulose membrane dialysis tubing (Sigma, USA). Subsequently, SDS-PAGE was carried out to check the purity and molecular weight (MW) of the purified Ig.

Characterisation of mrigal Ig (m-Ig): Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The affinity purified Ig was characterised using SDS-PAGE, following the standard protocol of Botwell and Sambrook (2002). SDS-PAGE was carried out on a 7.5 cm x 8.5 cm polyacrylamide gel of 1 mm thickness in a vertical gel electrophoresis apparatus (Technosource, India). The concentration of separating gel and stacking gel were 12 and 5%, respectively. Following electrophoresis, the gel was stained with 0.25% coomassie brilliant blue R-250 and analysed in gel documentation system (DNr Bioimaging System, Israel).

Production of anti-mrigal Ig antibodies in mice

Female Balb/c mice, eight weeks old, were procured from the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi Mumbai with the authentic permission and approval of Institutional Animal Ethics Committee, ICAR-CIFE, Mumbai. Two numbers of Balb/C mice were immunised intraperitoneally with 50 µg of m-Ig emulsified in FCA. The mice were boosted similarly with m-Ig emulsified with FIA on 14th and 28th day of first immunisation. On 36th day, mice were injected with 50 µg of purified m-Ig intravenously. After three days, the mice were anaesthetised using diethyl ether and bled for testing immune response. Serum was separated by centrifugation of clotted blood at 5000 g for 10 min and stored at -20°C for further use.

Immune response in mrigal and mice

Analysis of anti-BSA antibody in the affinity purified serum by Western blotting

Five microliters of BSA (1 mg ml⁻¹) was subjected to SDS-PAGE in three lanes along with a protein molecular weight marker on a 10% polyacrylamide gel. The gel was run initially at 70 V till the tracking dye reached the separating gel and the voltage was subsequently increased to 110 V till the dye reached bottom of the gel. The lane in the gel having molecular weight marker was transferred to 0.25% coomassie brilliant blue R-250 staining solution. The image of stained gel was analysed in Gel documentation system (DNr, Bio-imaging system, Israel). The remaining gel was used for western blotting. The unstained gel was transferred to Poly Vinylidene Difluoride (PVDF) membrane at constant current of 350 mA for 1.5 h, followed by blocking in PBS with 5% skimmed milk powder for 1 h at room temperature

(RT) and subsequently incubated with serum collected from immunised mrigal diluted at 1:1000 in PBST (PBS with 0.05% Tween-20) for 1 h at RT. The membrane was then incubated with mouse anti-mrigal Ig serum (diluted at 1:2000 with PBST) for 1 h at RT, followed by incubation with goat anti-mouse HRP conjugate (1:1000) (GeNei, India) for 1 h at RT. The membrane was incubated with substrate solution containing 10 mg of diaminobenzidine (DAB) in 30 ml of PBS having 10 µl of hydrogen peroxide, for 10 min in dark. The membrane was then washed in tap water, air-dried and analysed.

Production of monoclonal antibodies (MAbs) against mrigal Ig

MAbs against m-Ig were raised as per Hamilton and Davis (1995). Briefly, female BALB/c mice immunised with affinity purified m-Ig, were sacrificed five days after third booster injection and the spleen was collected under aseptic conditions. Spleen cells were fused with myeloma (SP2/0) cells in logarithmic phase, at a ratio of 10:1 using sterile polyethylene glycol (PEG) (Hybrimax, Sigma, USA), as fusion agent. The fused cells were grown in DMEM with 20% fetal bovine serum (FBS) and HAT (Hypoxanthine-Aminopterin-Thymidine) supplement. The cells were seeded in 96 well tissue culture plates containing feeder cells for nourishment @100 µl per well and incubated in CO₂ incubator. Feeder cells were prepared two days prior to fusion by collecting macrophages and spleen cells from mice and suitably diluted in DMEM along with 20% FBS so as to get a concentration of 10⁵ cells per well and seeded in 96 well tissue culture plates @100 µl per well followed by incubation for two days at 37°C with 5% CO₂ in incubator. The plates were observed regularly for growth of hybridomas and positive clones were screened by indirect ELISA. Positive hybridomas were expanded, followed by sub-cloning by limiting dilution method. Single clones were again screened for antibodies to mrigal Ig by indirect ELISA. Subsequently, the selected clones were characterised by western blotting and isotyping.

Screening of hybridomas by indirect ELISA

ELISA plate was coated with 100 µl of purified m-Ig (5 ng µl⁻¹) in coating buffer. The plate was incubated overnight at 4°C, washed thrice with PBST and blocked with 3% BSA in PBS at RT for 1 h. Thereafter, 100 µl of the hybridoma culture supernatant was added and incubated for 90 min at RT. Supernatant from SP2/0 myeloma cells served as negative control. Mouse serum from immunised mice at a dilution of 1:1000 was used as positive control. Mouse serum from immunised mice at a dilution of 1:1000 was used as positive control. The wells were washed with PBST followed by incubation with anti-mouse IgG HRP

conjugate (Sigma, 1:2000 dilution in PBST) for 1 h at RT. Subsequently, 100 µl of the substrate solution containing ABTS (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) in sodium citrate buffer having 0.1% H₂O₂ was added to the wells. After incubation for 10 min in dark, plate was read at 405 nm in ELISA plate reader (BioTek Power Wave 340, USA).

MAbs characterisation by Western blotting

Selected MAbs were characterised to determine the reactivity of the anti-mrigal Ig MAbs to reduced mrigal Ig following standard procedure as mentioned earlier.

Isotyping

Class and subclass of mouse Ig produced by hybridomas were determined by capture ELISA, using mouse monoclonal antibody isotyping kit (Sigma, USA) as per instructions of the manufacturer.

Cross reactivity of MAbs

The cross reactivity of anti-mrigal Ig MAbs with serum of *L. rohita* and *Catla catla* was checked employing western blotting. The serum of *C. catla* and *L. rohita* was run in 3 wells each in a SDS-PAGE gel and transferred to a PVDF membrane. The PVDF membrane was incubated with culture supernatant from each of the three clones for 1.5 h at RT. After washing and subsequent incubation with anti-mouse IgG-HRP conjugate and substrate, the membrane was washed with tap water and air dried (as mentioned earlier) and analysed.

Results and discussion

In the present study, an affinity column of 4% agarose beads coupled with BSA was used to purify Ig from mrigal hyper-immune serum. The anti-BSA antibodies were successfully eluted using BSA-CL agarose affinity column in accordance with previous reports in Atlantic salmon (Pettersen *et al.*, 2000); Asian catfish (Swain *et al.*, 2004); IMCs (Bag *et al.*, 2009) and *C. batrachus* (Sood *et al.*, 2012). Further, in this study, about 7.24 mg of Ig was recovered from total 10 ml of immunised mrigal serum @0.72 mg ml⁻¹ of mrigal serum. The yield of m-Ig in this study was higher than that reported in Asian catfish, 0.15 mg ml⁻¹ (Swain *et al.*, 2004); Southern blue fin tuna, 0.16-0.3 mg ml⁻¹ (Watts *et al.*, 2001); IMCs, 0.315, 0.33 and 0.255 mg ml⁻¹ for catla, rohu and mrigal, respectively (Bag *et al.*, 2009) and magur, 0.275 mg ml⁻¹ (Sood *et al.*, 2012). However, it was lower as compared to that reported in snapper, 0.85-1.47 mg ml⁻¹ (Morrison and Nowak, 2001) and seabass, 1 mg ml⁻¹ (Palenzuela *et al.*, 1996). The yield of immunoglobulins depends on size and age of fish as well as environment (Zilberg and Klesius, 1997).

The affinity purified m-Ig in SDS-PAGE revealed two bands of 85 kDa and 26 kDa which corresponded to heavy chain (HC) and light chain (LC), respectively (Fig. 1). Previously, Suresh *et al.* (2008) reported HC and LC of affinity purified rohu Ig as 85 kDa and 23 kDa bands, respectively. In agreement with our results, Bag *et al.* (2009) reported HC and LC for catla, rohu and mrigal Ig to be 88 and 26 kDa respectively. The molecular weight of H chain in tilapia has also been reported to be 90 kDa (Rajavarthini *et al.*, 2000) and 86 kDa in barramundi (Crosbie and Nowak, 2002). Single heavy and light chains, as observed in the present study, were also reported in Japanese eel (Uchida *et al.*, 2000), common carp (Vesely *et al.*, 2006), seabass (Scapigliati *et al.*, 1996), gilthead seabream (Palenzuela *et al.*, 1996), *L. rohita* (Rathore *et al.*, 2008) and *Clupea pallasii* (Purcell *et al.*, 2012). Western blotting of BSA with immunised fish serum and mouse anti-mrigal Ig showed a specific band at 66 kDa. This clearly indicated specific immune response in fish against BSA and in mice against m-Ig (Fig. 2).

Hybridomas producing MAbs against m-Ig were cloned on the basis of results of indirect ELISA. The wells with hybridoma culture supernatant having significantly higher OD (>0.500) compared to negative control were considered positive and chosen for further expansion. Subsequently, three stable hybridomas were cloned by limiting dilution and designated as 3B2-E12, 3B2-F9 and 4C3-B2 which were further characterised. The heavy chain isotypes of these three MAbs were determined to be IgG1, IgG2a and IgG2a, respectively. To authors' knowledge, this is the first report of development of MAbs specific to mrigal Ig. All of the MAbs, reacted strongly with the HC of mrigal Ig and no reactivity was observed with the LC. Previous studies indicated that HC is more antigenic in nature as compared to LC, as most of the MAbs against fish Ig are specific for the HC of Ig (Scapigliati *et al.*,

1999). The present findings of specific reaction of MAbs to HC corroborates with earlier studies in *Oreochromis niloticus* (Al-Harbi *et al.*, 2000); *Paralichthys olivaceous* (Jang *et al.*, 2004; Li *et al.*, 2007); *Sebastes schlegeli* (Shin *et al.*, 2006) *Pagrus auratus* (Morrison *et al.*, 2002); *Takifugu rubripes* (Miyadai *et al.*, 2004); common carp (Vesely *et al.*, 2006); *L. rohita* (Rathore *et al.*, 2008; Suresh *et al.*, 2014); *C. pallasii* (Purcell *et al.*, 2012) and *C. batrachus* (Sood *et al.*, 2012). Class and subclass of MAbs developed in this study were determined using a capture ELISA-based mouse Ig isotyping kit, which revealed that 3B2-E12 MAb is of isotype IgG1 whereas, 3B2-F9 and 4C3-B2 MAbs are of isotype IgG2a. Out of the seven anti-*O. niloticus* MAbs developed by Al-Harbi *et al.* (2000), four were of IgG2b, two were IgG2a and one was IgG3. Two anti-rohu Ig MAbs developed by Rathore *et al.* (2008) belonged to subclass IgG2b and IgG1.

The MAbs designated as 3B2-F9, 3B2-E12 and 4C3-B2 showed specific reaction with the heavy chain of purified m-Ig at molecular weight of 85 kDa (Fig. 3). Antigenic relatedness of m-Ig with serum Ig of other IMCs, namely catla and rohu, was demonstrated by the cross reactivity pattern of MAbs with serum of *C. catla* and *L. rohita*. After immunoblotting, it was found that MAbs (3B2-E12) showed specific reaction with serum of mrigal, rohu and catla at the position of HC, suggesting cross-reactivity of developed MAbs to serum of phylogenetically related species in the same family (Fig. 4). Our results reveal that the epitope of mrigal Ig recognised by MAbs is shared with catla and rohu Ig. The antigenic relatedness of fish Ig within species of the same family, as shown by MAbs binding in the present study, has previously been reported for puffer fish (Miyadai *et al.*, 2004), rohu (Rathore *et al.*, 2008) and magur (Sood *et al.*, 2012).

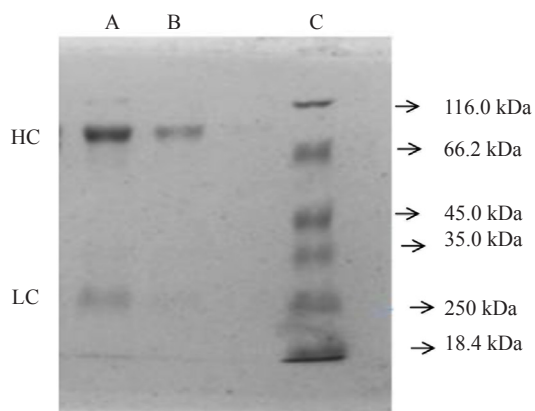


Fig. 1. SDS-PAGE gel image of purified anti-BSA Ig from *C. mrigala*. Lane A and B: Mrigal Ig; Lane C: Molecular weight marker; HC: Ig Heavy chain; LC: Ig Light chain

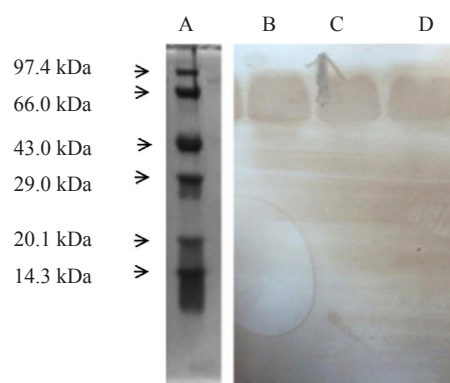


Fig. 2. Western blot image of antiserum from *C. mrigala* directed against BSA. Lane A: Stained molecular weight marker; Lane B, C and D: BSA western blotted with anti-mrigal Ig serum

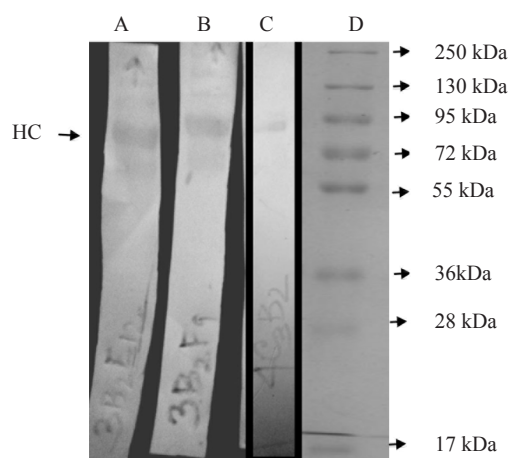


Fig. 3. Western blot image of anti-mrigal Ig MABs
Lane A, B and C: Immunoblot of purified mrigal Ig with anti-mrigal Ig MABs; Lane D: Molecular weight marker

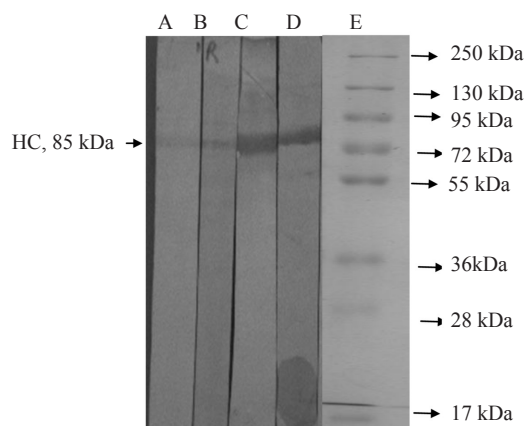


Fig. 4. Antigenic relatedness of mrigal Ig MAb to catla and rohu serum Ig
Lane A: Immunoblot of catla serum with anti-mrigal Ig MAb (3B2-E12); Lane B: Immunoblot of rohu serum with anti-mrigal Ig MAb; Lane C: Immunoblot of mrigal serum with anti-mrigal Ig MAb; Lane D: Immunoblot of mrigal Ig with anti-mrigal Ig MAb; Lane E: Molecular weight marker

It is concluded that the affinity purified mrigal Ig comprised single HC and LC and the produced MABs were specific for m-Ig HC. These can be effectively utilised for sero-monitoring of pathogens in mrigal. Since one of the MAB cross reacted with catla and rohu Ig, it can be used to monitor the immune response in these species following infection and vaccination. The anti-mrigal Ig MABs will have applications in developing antibody-based assays that can be successfully employed in disease diagnosis for effective fish health management.

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