روشی ساده برای تولید آنتی بادی های منوکلونال بر علیه IgM در ماهیان

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A Simple Method for the Production of anti-Fish IgM Monoclonal Antibodies

Abstract

Immunoglobulin M (IgM) is the predominant antibody isotype in the serum of teleost fish and plays a central role in systemic immune responses. Monoclonal antibodies (mAbs) targeting fish IgM are essential tools for immunological research and disease management in aquaculture. However, conventional mAb production protocols require purified IgM, which is difficult to obtain due to the complexity of purification procedures in teleost species. This study introduces a simplified method for generating anti-IgM mAbs by immunizing mice with whole serum from rainbow trout (*Oncorhynchus mykiss*), thereby eliminating the need for IgM purification. Hybridomas were screened using an indirect ELISA based on an unrelated antigen (e.g., maltose-binding protein) and trout sera containing antibodies against this antigen. This approach led to the identification of five stable hybridoma clones (1D4, 3D3, 3E3, 4B3, and 7F6). The resulting mAbs effectively recognized trout antibodies against *Infectious pancreatic necrosis virus, Lactococcus garvieae* bacterium and sheep red blood cells. Immunoblotting under reducing and non-reducing conditions confirmed the specificity of these mAbs for trout IgM. This method provides a practical and efficient alternative for producing functional monoclonal antibodies against fish IgM.

Key words: Monoclonal antibody, trout, IgM, ELISA, immunoblotting

Introduction

Immunoglobulins (Igs) are integral elements of the adaptive immune system in fish, with diverse roles in pathogen recognition and immune defense. In teleost fishes, three major immunoglobulin isotypes have been characterized: IgM, IgD, and IgT (Hikima et al, 2011). Among them, IgM exhibits substantial structural resemblance to mammalian IgM in terms of molecular size, glycosylation profile, and polymeric configuration (Coscia and Oreste, 1998). Functionally, IgM is the predominant isotype present in teleost serum and is central to systemic immune responses of fish. However, teleost IgM may also play role in mucosal protection (Parra et al, 2013).

IgT, a teleost-specific immunoglobulin isotype, is predominantly localized at mucosal surfaces and functionally resembles mammalian IgA, indicating an evolutionary adaptation for mucosal immunity (Mashoof et al., 2014; Ji et al., 2020). Studies utilizing polyclonal antibodies raised against IgT have demonstrated no detectable antigenic cross-reactivity between IgT and IgM in rainbow trout (Zhang et al., 2010).

In contrast to mammalian IgD, the heavy chain of teleost IgD exhibits a chimeric structure composed of a Cμ1 domain followed by multiple Cδ regions (Salinas et al., 2021). Given this structural overlap, antigenic cross-reactivity between IgM and IgD in teleosts is plausible. The precise immunological role of IgD in teleosts remains to be conclusively defined. Nonetheless, it has been postulated that IgD may serve as a surface receptor on B lymphocytes, participating in cellular activation, maturation, and differentiation (Edholm et al, 2010; Ji et al, 2020).

Production of monoclonal antibodies against fish immunoglobulins, particularly IgM, is a growing field with significant implications for aquaculture, fish health management and research. Anti-fish IgM monoclonal antibodies can facilitate immunodiagnostic assays and vaccine efficacy studies within the aquaculture sector. These antibodies are instrumental in identifying IgM-expressing cells, quantifying serum immunoglobulin levels, and characterizing immune responses against specific antigens.

Although novel methodologies such as phage display and recombinant antibody technologies are emerging, hybridoma technology remain the most widely utilized for mAb generation. This technique entails the immunization of a laboratory animal—typically mice—followed by fusion of B cells with immortalized myeloma cells to produce hybridomas. These hybridomas possess the dual capacity to secrete antibody and proliferate indefinitely. Subsequent screening and cloning facilitate the establishment of stable cell lines capable of sustained mAb production (Ausubel, 1992).

Conventional immunization protocols to produce anti-fish IgM mAbs, typically have been relied on the use of purified fish IgM as the immunogen (DeLuca et al, 1983; Thuvander et al, 1990; Sanchez et al, 1991; Sanchez et al, 1993; Estevez et al, 1994; Pettersen et al, 1995; Magnadottir et al, 1996; Vasely et al, 2006; Shin et al, 2006; Zhang et al, 2017; Yang et al, 2018; Huang et al, 2019; Jones et al, 2022; Oliver et al, 2023). However, the isolation of IgM from fish serum presents considerable technical challenges (Sanchez et al, 1991), especially in species such as those belonging to the Salmonidae family, wherein IgM concentrations are relatively low (Uchida et al., 2000). Given these constraints, the present investigation aimed to evaluate an alternative and simplified strategy for mAb production against rainbow trout IgM (as a model). Specifically, whole serum from rainbow trout was employed as the immunogen without prior purification of IgM. The proposed approach demonstrates promising applicability for the generation of anti-IgM monoclonal antibodies across various teleost species.

Materials and methods

Immunization of Balb/c mice with trout whole serum

Female Balb/c mice, six weeks of age, were immunized with trout whole serum emulsified in an equal volume of incomplete Freund's adjuvant (Sigma, USA). A total of six mice received 200 µl of the emulsion via intraperitoneal injection on days 0, 14, and 35. One week following the final immunization, blood samples were collected, and the sera were used to develop an indirect ELISA for hybridoma screening.

Immunization of rainbow trout with Maltose-Binding Protein (MBP)

Ten rainbow trout (average weight: 300 g) were housed in two glass aquaria (five fish per tank) maintained at 15–17°C. After acclimatization, five fish were immunized with MBP (Mahmoodi et al, 2015) emulsified in ISA 70 oil-based adjuvant (SEPPIC, France). Each fish received 100 µl of the emulsion containing 50 µg of MBP via intraperitoneal injection on days 0 and 14. The control group received PBS-adjuvant emulsion. Blood samples were collected on day 28, and the sera were used for indirect ELISA development.

Indirect ELISA for hybridoma screening

ELISA plates were coated with 0.1 µg of MBP per well and blocked with 5% skim milk in PBS containing 0.05% Tween 20 (PBST) for 3 hours at 37°C. After washing, 50 µl of serial dilutions (1:200 to 1:1600) of trout sera (from immunized and control fish) diluted in blocking buffer were added to the wells and incubated for 45 minutes at room temperature. Subsequently, 50 µl of mouse serum (1:2000 dilution) from a trout-serum-immunized mouse was added and incubated for another 45 minutes. After washing, 50 µl of peroxidase-conjugated anti-mouse IgG (Komabiotech, Korea; 1:4000 dilution) was added and incubated for 30 minutes. Plates were washed and developed using 50 µl of TMB-H□O□ substrate. After 10 minutes, the reaction was stopped, and absorbance was measured at 450 nm (Dynatec, Netherlands). A 1:200 dilution of trout sera was found to effectively differentiate immunized from control samples.

Production of monoclonal antibodies against trout IgM

To generate monoclonal antibodies against trout IgM, spleen cells from a trout-serum-immunized mouse were fused with Sp2/0 myeloma cells using polyethylene glycol (Roche, Germany). A total of 10^7 myeloma cells in logarithmic growth phase were fused with 10^8 spleen cells. The fused cells were cultured in 96-well plates containing feeder cells (harvested from the peritoneal cavity of a mouse) in RPMI medium completed with 10% fetal bovine serum and hypoxanthine-aminopterin-thymidine supplement (Sigma, USA) (Ausubel, 1992). Hybridoma supernatants were screened for anti-trout IgM antibodies using the previously described ELISA protocol, substituting hybridoma culture supernatants for mouse serum.

Evaluation of mAbs for detecting antigen-specific trout antibodies

To assess the utility of the mAbs in detecting antigen-specific rainbow trout antibodies, ELISA plates were coated with antigens from *Infectious pancreatic necrosis virus* (IPNV), *Lactococcus garvieae* bacterium and sheep red blood cells. Corresponding positive rainbow trout sera (prepared in-house) were used at a 1:100 dilution. The ELISA protocol was like the hybridoma screening assay, with the substitution of respective antigens for the MBP and the use of positive rainbow trout sera instead of MBP-immunized fish serum.

Immunoblotting to assess mAbs specificity

The specificity of the mAbs for trout IgM was evaluated by immunoblotting under reducing and non-reducing conditions. The whole trout serum was mixed with SDS-PAGE sample buffer containing β-mercaptoethanol (reducing) or without it (non-reducing) (Ausubel, 1992). Reduced samples were boiled for 10 minutes; non-reduced samples were incubated at room temperature. Proteins were separated by SDS-PAGE (10% and 6% gels for reducing and non-reducing conditions, respectively) and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in PBST, cut into strips, and incubated with each mAb for 1 hour. After washing, strips were incubated with peroxidase-conjugated anti-mouse IgG for 1 hour, washed again, and developed using 4-chloro-1-naphthol and H□O□.

Results

Production and screening of hybridoma cells

Following the fusion of splenocytes from immunized mice with Sp2/0 myeloma cells, approximately 150 hybridoma colonies were generated and screened for the production of monoclonal antibodies specific to rainbow trout antibody using an indirect ELISA. In the initial screening, eight hybridoma supernatants exhibited optical density (OD) values ranging from 0.8 to 1.8, indicating positive reactivity compared to the remaining clones. After three rounds of subcloning, five hybridoma clones—designated 1D4, 3D3, 3E3, 4B3, and 7F6—demonstrated stable and high-level antibody production, with OD values exceeding 1.7. One subclone from each of these five clones was selected for further analysis.

Evaluation of mAbs for detection of antigen-specific trout antibodies

To determine the specificity and utility of the generated mAbs for detection of rainbow trout serum antibodies, an indirect ELISA was conducted using antigens derived from *Infectious pancreatic necrosis virus* (IPNV), *Lactococcus g*arvieae bacterium and sheep red blood cells. Corresponding positive and negative rainbow trout sera were included for each antigen. The mAbs were employed as secondary antibodies prior to incubation with

a peroxidase-conjugated anti-mouse IgG. As illustrated in Figure 2, the mAbs consistently yielded higher optical density (OD) values with positive sera compared to negative controls, confirming their capacity to selectively recognize antigen-specific trout antibodies.

Specificity of mAbs for trout IgM assessed by immunoblotting

To determine the specificity of the selected monoclonal antibodies for trout IgM, immunoblotting was performed using rainbow trout whole serum under both reducing and non-reducing conditions. Under reducing conditions, all five mAbs recognized a protein band in rainbow trout serum with an approximate molecular weight of 75 kDa (Fig. 1A). Under non-reducing conditions, the antibodies reacted with serum proteins in the range of approximately 180 kDa, as well as with high-molecular-weight proteins that remained at the top of the nitrocellulose membrane, beyond the detectable range of the molecular weight marker (Fig. 1B).

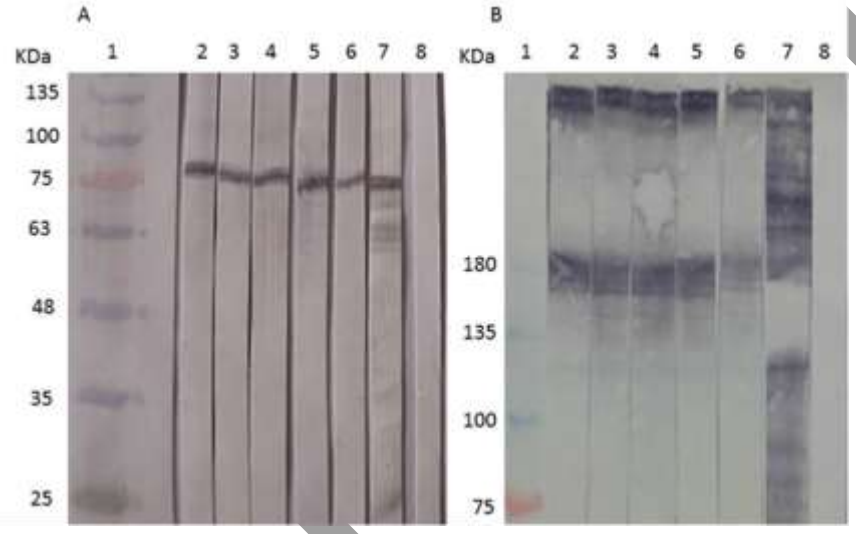

Fig 1. Immunoblot analysis under reducing and non-reducing conditions. (A) Reducing conditions: Strip 1 represents the molecular weight marker. Strips 2 through 6 show the reactivity of mAbs 1D4, 3D3, 3E3, 4B3, and 7F6 with a ~75 kDa protein present in the serum of rainbow trout. Strips 7 and 8 correspond to the positive control (serum from a mouse immunized with rainbow trout serum) and the negative control (RPMI medium supplemented with 10% fetal bovine serum), respectively. (B) Non-reducing conditions: Strip 1 indicates the molecular weight marker. Strips 2 through 6 demonstrate the binding of the same panel of mAbs to proteins of approximately 180 kDa and higher molecular weights in rainbow trout serum. Strips 7 and 8 represent the positive and negative controls, respectively.

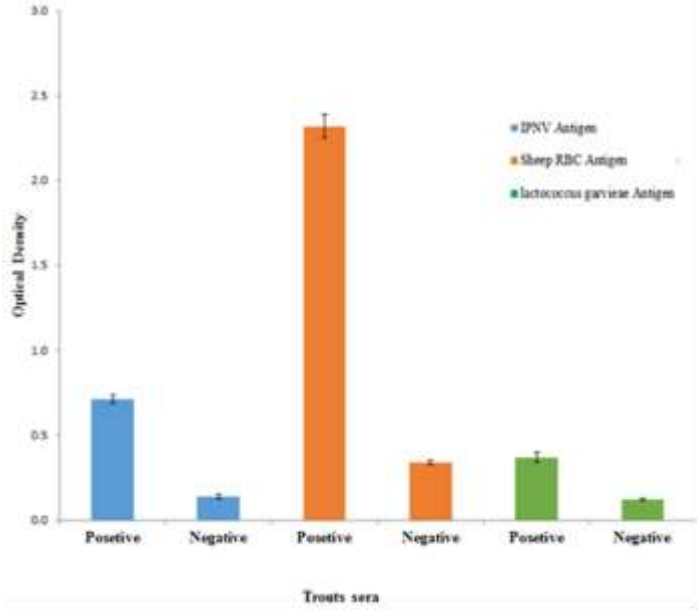

Fig 2. Evaluation of the applicability of the mAbs for detecting antigen-specific antibodies in positive and negative rainbow trout sera. This figure presents the mean optical density (OD) values obtained from the five mAbs in ELISA, targeting serum antibodies specific to antigens derived from *Infectious Pancreatic Necrosis Virus* (IPNV), *Lactococcus garvieae* bacterium, and sheep red blood cells (SRBCs). Each column represents the average OD response of all five mAbs, reflecting their collective ability to recognize antigen-specific immunoglobulins in rainbow trout sera.

Discussion

Effective disease control and prevention in aquaculture require the implementation of routine monitoring programs, complemented by eradication strategies or vaccination protocols. Within this context, serological assays play a critical role in both surveillance and diagnostic efforts (Morrison and Nowak, 2002). As in terrestrial animals, reliable serological testing in fish depends on the availability of species-specific anti-immunoglobulin antibodies, particularly monoclonal antibodies targeting IgM.

In recent years, considerable research efforts have been directed toward the development of monoclonal antibodies against IgM in a variety of freshwater and marine fish species. Conventionally, this process involves purifying IgM from fish serum and using it to immunize mice. However, the purification of fish IgM is notably time-consuming, labour-intensive and usually results in only partial purification (Sanchez et al, 1991). In order to overcome this challenge, researchers aiming to generate monoclonal antibodies against fish IgM have employed a range of purification techniques including ammonium sulfate precipitation (Al-Harbi et al, 2000), gel filtration (DeLuca et al, 1983; Magnadottir

et al, 1996; Vasely et al, 2006), ion exchange chromatography (Sanchez et al, 1993; Pettersen et al, 1995), and various affinity chromatography methods (Thuvander et al, 1990; Sanchez et al, 1991; Estevez et al 1994, Al-Harbi et al, 2000; Shin et al, 2006; Zhang et al, 2017; Yang et al, 2018; Huang et al, 2019; Jones et al, 2022; Oliver et al, 2023), as well as combinations thereof.

To circumvent the difficulties associated with IgM purification, some studies have adopted molecular approaches. For instance, the expression of a truncated IgM heavy chain sequence in *Escherichia coli* has enabled the production of a recombinant protein suitable for immunization (Jingzhuang et al, 2014, Rahnama et al, 2018). Additionally, synthetic peptides derived from conserved regions of the IgM heavy chain have been successfully used to generate monoclonal antibodies for species such as tilapia (Velázquez et al, 2021) and sea bass (Uchuwittayakul et al, 2025).

In the present study, whole serum from rainbow trout was employed for mouse immunization to facilitate the production of monoclonal antibodies targeting rainbow trout IgM. A review of the existing literature indicates that whole fish serum has not previously been utilized as an immunogen for the development of monoclonal antibodies against fish IgM. The only partial attempt was reported by Al-Harbi et al. (2000), who immunized mice using semi-purified tilapia IgM obtained via ammonium sulfate precipitation. However, during the hybridoma screening phase, they relied on affinity-purified IgM to ensure the selection of IgM-specific hybridomas.

The rationale for the present approach was based on two key considerations. First, fish serum is the primary source of IgM, with its concentration in rainbow trout estimated to be approximately 100 to 1000 times higher than that of IgD and IgT, respectively (Salinas et al., 2021). Second, IgM is the predominant immunoglobulin produced in response to systemic immunization in fish. Accordingly, following mouse immunization with whole serum, hybridoma screening can be effectively conducted using an ELISA designed with an unrelated antigen (e.g., MBP) and sera collected from fish immunized with that antigen. Using this strategy, five hybridomas (1D4, 3D3, 3E3, 4B3, and 7F6) were identified that produced monoclonal antibodies reactive with rainbow trout antibodies—presumably of the IgM isotype—bound to MBP in the screening assay. The ability of these monoclonal antibodies to differentiate between sera from non-immunized rainbow trout and those systemically immunized with various antigens (IPN virus, *Lactococcus garvieae* bacterium, and sheep red blood cells) was further confirmed by ELISA, demonstrating the capacity of the mAbs to detect trout antibodies bound to the antigens.

The specificity of these monoclonal antibodies for rainbow trout IgM was validated through immunoblotting. Under reducing conditions, all five antibodies successfully detected a protein in the trout serum with an estimated molecular weight of approximately 75 kilodaltons, which corresponds closely to the expected size of the IgM heavy chain

(Sanchez et al, 1993). Under non-reducing conditions, the antibodies recognized protein bands around 180 kDa and additional high-molecular-weight bands near the top of the electrophoresis lanes. The 180 kDa bands likely correspond to the IgM monomer, while the higher molecular weight bands are presumed to represent IgM tetramers (660–800 kDa) (Salinas et al., 2021), although precise molecular characterization was not possible due to the absence of suitable molecular weight markers. These findings are consistent with previous reports indicating that IgM exists in both monomeric and tetrameric forms in rainbow trout serum (Elcombe et al., 1985). Unlike IgM, serum IgT exists exclusively in a monomeric form with an approximate molecular weight of 180 kDa. In rainbow trout, secreted IgD is also present as two distinct monomeric variants of ~370 and ~400 kDa (Salinas et al., 2021), both of which exceed the upper limit of the molecular weight markers employed in this study. Therefore, based on the specific reactivity patterns observed in immunoblotting assays and successful performance of the mAbs in detecting systemic immune responses in rainbow trout against various antigens, it can be concluded that these antibodies are specific to trout IgM.

Considering the evidence presented by Zhang et al, (2010) that there is no cross-reactivity between IgT and IgM, it is reasonable to assume that the mAbs generated in the present study do not exhibit cross-reactivity with IgT. However, regarding IgD, it is noteworthy that the heavy chain of IgD contains the cμ1 domain, which is also present in IgM (Salinas et al., 2021). Therefore, a degree of cross-reactivity between IgD and IgM is theoretically plausible. Although the immunoblotting results in the current study did not reveal any detectable interaction between the produced mAbs and IgD, this absence of signal may be attributed to the relatively low concentration of IgD in serum compared to IgM (Salinas et al., 2021). Consequently, the possibility of cross-reactivity with IgD cannot be entirely ruled out and warrants further investigation using more sensitive detection methods or purified IgD preparations.

Collectively, the result of this study highlights the feasibility of using whole fish serum as an immunogen for monoclonal antibody production against IgM. Moreover, the incorporation of an unrelated antigen during hybridoma screening presents a practical approach for selecting antibodies capable of detecting antigen-bound IgM. This methodology may yield monoclonal antibodies with greater functional relevance than those selected solely based on reactivity with purified IgM.

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Conflict of Interest

The authors declare no conflict of interest.

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روشی ساده برای تولید آنتی بادی های مونوکلونال ضد IgM ماهی

چکیده

ایمونولوگوبولین M (IgM) ایزوتیپ آنتی بادی غالب در سرم ماهی‌های استخوانی است و نقش مرکزی در پاسخ‌های ایمنی سیستمیک ماهیان دارد. آنتی‌بادی‌های مونوکلونال (mAbs) برعلیه IgM ماهی‌ها ابزارهای ارزشمندی برای تحقیقات ایمنی‌شناسی و مدیریت بیماری‌ها در آبزی‌پروری می باشند. پروتکل‌های سنتی تولید این نوع mAb نیازمند IgM خالص هستند که تهیه آن به دلیل پیچیدگی روش های خالص سازی در گونه‌های ماهیان استخوانی با چالش همراه است. در این مطالعه، ما یک روش ساده برای تولید آنتی‌بادی‌های مونوکلونال ضد IgM با ایمن‌سازی موش‌ها با سرم کامل قزل‌آلای رنگین‌کمان (Oncorhynchus mykiss) ارائه می‌دهیم که به خالص سازی IgM نیاز ندارد. در این روش هیبریدوماها با استفاده از یک ELISA غیرمستقیم بر اساس یک آنتی‌ژن غیرمرتبط (مانند پروتئین متصل شونده به مالتوز) و سرم‌های قزل‌آلای حاوی آنتی بادی ضد این آنتی ژن غربالگری شدند. این شیوه منجر به شناسایی پنج کلون هیبریدومای پایدار (D4۱، D3۳، E3۳، B3۴ و F6۷) گردید. آنتی بادی های منوکلونال تولید شده به‌طور مؤثر آنتی‌بادی‌های قزل‌آلا بر علیه ویروس نکروز پانکراس عفونی، باکتری لاکتوکوکوس گارویه و گلبول های قرمز خون گوسفند را نیز شناسایی کردند. ایمونوبلاتینگ تحت شرایط احیا کننده و غیر احیا کننده ویژگی این آنتی بادی های منوکلونال را برای IgM ماهی قزل‌آلا تأیید کرد. بنابر این این روش یک شیوه جایگزین عملی و کارآمد برای تولید آنتی بادی های منوکلونال کاربردی بر ضد IgM ماهی فراهم می‌کند.

کلمات کلیدی: آنتی بادی منوکلونال، ماهی قزل آلا، IgM، ELISA، ایمونوبلاتینگ