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Short communication

Characterization of polyclonal antibodies against Japanese flounder IgM derived from recombinant IgM constant region proteins

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ABSTRACT

Cell and determinant markers are important in fish immunology and have vast applications in aquaculture but the availability of such markers are quite limited. Hence, there is a need to identify and also further improve existing markers in fish. Here, we developed effective polyclonal antibodies (pAbs) targeting specific parts of the Japanese flounder (*Paralichthys olivaceus*) IgM constant (C) region. Recombinant proteins from the CHμ2 and CHμ3 termed IgM fragment 1 (*r*IgM1) and from CHμ4 termed IgM fragment 2 (*r*IgM2) were expressed and used to construct mouse pAb-IgM1 and pAb-IgM2, respectively. pAb-IgM1 detected both the ~77 kDa and the ~72 kDa heavy chains detected while pAb-IgM2 marked only the ~77 kDa heavy chain of Japanese flounder. Both pAbs detected IgM heavy chain in immune-related tissues, heart and serum. pAb-IgM2, but not pAb-IgM1, revealed cross reactions with other fish species detecting pronounced multiple IgM bands suggesting that the CHμ4 is an important functional region in the teleost IgM molecules. Finally, the pAb-IgMs detected surface IgM+ (sIgM+) and cytoplasmic IgM+ (cIgM+) B cells in Japanese flounder kidney *in vivo*.

Cell and determinant markers for fish lymphocytes and leucocytes are very important in studying functional fish immunobiology, which is still at the beginning stage, and have wide ranging applications in aquaculture, yet the availability of such markers is so far extremely inadequate [1]. Hence, there is a tremendous need to identify and develop more, and also further improve existing fish cell markers.

Japanese flounder (*Paralichthys olivaceus*) is an important culture fish species. Marker monoclonal antibodies (mAbs) against its immunoglobulins (Igs), have been developed from purified serum Ig to characterize Igs *in vivo* and to mark Ig+ B lymphocyte populations in tissues [2–5]. These mAbs showed that the Japanese flounder serum Igs had 2 heavy chains at 72 kDa and 77 kDa, and 2 light chains at 26 kDa and 28 kDa. It likewise revealed distinct subpopulations of lymphocytes including surface immunoglobulin-positive (sIg+) and cytoplasmic immunoglobulin-positive (cIg+) cells. In addition, these same mAbs were utilized to isolate sIg+ cells by magnetic cell sorting, which allowed for identification of viral-induced genes from a homogenous population of sIg+ lymphocytes [6].

To further develop cell marker antibodies, it is important to identify and use antigens that can generate more sensitive and specific antibodies, and allow for a better understanding of binding reactions that are taking place. While, mAbs are accepted to be more specific and sensitive, polyclonal antibodies (pAbs) offer some advantage in that they are relatively cost-effective and easier to produce especially if the latter's specificity is increased with the use of the right form of antigens.

Here, we report the production of 2 mouse polyclonal anti-Japanese flounder IgM antibodies (pAb-IgM1 and pAb-IgM2) derived from recombinant Japanese flounder IgM constant region protein [7]. We show the sensitivity and specificity of the pAb-IgMs *in vivo* and demonstrate cross-reactivity of pAb-IgM2 in other fish species attributed to the specific conserved features of CHμ4 of the IgM constant region.

Available protein sequences of teleost fish IgMs constant region from Genbank were aligned using ClustalW. Species and corresponding accession numbers were: Japanese flounder, *P. olivaceus* (AB052744); Atlantic toothfish, *Dissostichus mawsoni* (ACA5188); Fugu, *Takifugu rubripes* (BAD26619); Rainbow trout, *Oncorhynchus mykiss* (AAW66972); Grass carp, *Ctenopharyngodon idella*

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(ABD76396) and Zebrafish, *Danio rerio* (AAT67447). Tilapia, *Oreochromis mossambicus* IgM (AY522596) was not included in the analysis as the sequence was partial and unconfirmed. Based on the alignment, two regions were selected for recombinant protein production; a region encompassing the IgM CHμ2 and CHμ3 domains (IgM1) and a region from the IgM CHμ4 domain containing a long conserved 40 aa portion (IgM2) (Fig. S1).

The IgM1 and IgM2 amplicons containing added NdeI and an EcoR1 sites were generated by PCR using specific primers (Table 1). IgM1 and IgM2, and the bacterial expression vector pET32 were then cut using NdeI and EcoR1 restriction enzymes, recovered using EASYTRAP ver. 2 (Takara, Japan) and ligated together using ligation high (TOYOBO, Japan) at 16 °C overnight. These were then transformed to competent *Escherichia coli* JM109 cells using the heat-shock method and cultured overnight at 37 °C on Luria-Bertani agar containing ampicillin (LB-AP) (100 μg ml^{-1}). Resulting bacterial colonies were then cultured in LB-AP overnight for plasmid extraction and purification using the alkaline-lysis method. Confirmation of positive expression vector constructs was done by automated sequencing using the ThermoSequenase kit (Amersham Biosciences, Piscataway, NJ) on an LC4200 automated DNA sequencer (Li-Cor, Lincoln, NE). Confirmed pET32-IgM1 and pET32-IgM2 constructs were each transformed to *E. coli* BL21 codon+ using the heat-shock method and culture-selected in LB-agar plates containing ampicillin (100 μg ml^{-1}) and chloramphenicol (30 μg ml^{-1}) (LB-AP/CP) overnight. Resulting bacterial colonies were then used for protein expression and purification.

To check and select clones expressing high amounts of the 2 recombinant IgM protein fragments (*r*IgM1 and *r*IgM2), an initial small-scale experiment was performed. Briefly, selected clones were cultured overnight at 3 ml LB-AP/CP at 37 °C. From these, 200 μl was taken and inoculated in fresh 2 ml LB-AP/CP broth at 37 °C until OD_{600} of about 0.6. These were then induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. An empty pET32 vector and non-induced cells were used as controls. Resulting cultures were centrifuged and the cell pellets were resuspended in 300 μl phosphate buffered saline (PBS). From this suspension, 20 μl was mixed with 2× SDS buffer and boiled for 10 min. After which 20 μl was run in a 15% SDS-PAGE for about 1.5 h at 30 milliAmp. Coomassie brilliant blue staining and Western blotting using an anti-His antibody (Promega, Madison, WI) were used to visualize expressed protein.

Large-scale expression of *r*IgM1 and *r*IgM2 was carried out. Briefly, a 2 ml overnight LB-AP/CP culture of selected clones was placed in 300 ml LB-AP/CP and then cultured again overnight at 37 °C. Cultures were centrifuged and then resuspended in phosphate buffer saline (PBS). After which, freeze-thaw method and then sonication (20 s at amplitude 20) were performed to lyse the cells. Sonicated samples were centrifuged, and the pellet resuspended in PBS, then filtered in 0.45 μM filter (Millipore). *r*IgM1 and *r*IgM2 proteins were then purified from inclusion bodies by metal affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen K.K Tokyo, Japan). Briefly, inclusion bodies were solubilized in denaturing buffer (8.0 M Urea, 100 nM NaH_2PO_4, 10 mM Tris-HCL, 20 mM imidazole, pH 8.2) for 12 h and centrifuged at 6000 rpm for 30 min at 10 °C. Supernatant was applied to the Ni-NTA beads in propylene column, which were resuspended in wash buffer (50 mM Na_2HaPO_4, 300 mM NaCl, pH 8.2). Washing was conducted with wash buffer containing 30 mM and 100 mM imidazole. Elution was performed with wash buffer containing 250 mM imidazole. Eluents were then subjected to filter sterilization using 0.22 μm Millex™ Syringe Driven Filter Unit (Millipore) and then dialyzed overnight in 3 L 1× PBS. Purified recombinant proteins were visualized by SDS-PAGE and coomassie brilliant blue (CBB) staining and quantified using the bicinchoninic acid method in the BCA™ Protein assay kit (Pierce, Rockford, IL, USA).

Purified *r*IgM1 (738 μg ml^{-1}) and *r*IgM2 (2250 μg ml^{-1}) were injected intraperitoneally (IP) into each of 5 BALB/c strain. Intraperitoneal injections were administered at 2-weekly intervals at the following volumes; 0.1 ml, 0.15 ml, 0.2 ml and 0.2 ml. The mice were bled and the pooled serum was designated as pAb-IgM1 and pAb-IgM2, respectively.

Western blot was done following Lin et al. (2005) [8]. Briefly, samples were mixed with equal amounts of 2× SDS loading buffer and boiled for 10 min. After which, these (20 μl) were loaded in a 15% SDS-PAGE gel. A Precision Plus Protein Standard (Bio-Rad) was used as weight markers. Western blot transfer was done in an ATTO clear blot membrane at 110 mA for 1 h. 1st antibody binding was done using pAb-IgMs in Tris buffered saline with Tween 20 (TBST) at 1:2500 dilution for 1 h. 2nd antibody binding used anti-mouse IgG conjugated with alkaline phosphatase (Promega, Madison, WI, USA) diluted in TBST at 1:10,000 dilution also for 1 h. Visualization of the reaction was carried out using a 5-bromo-4-chloro-3-indotyl phosphate/nitro blue tetrazolium alkaline phosphatase (BCIP/NBT) substrate (Sigma–Aldrich, St. Louis, MO, USA).

Sensitivity and tissue distribution analysis of the pAb-IgMs was carried out by Western blotting of Japanese flounder tissues including heart, head kidney, tail kidney, liver, muscle, spleen and serum. Briefly, tissues were dissected out/extracted from 3 individual fish having about 10 cm total length. Solid tissues were lyzed by 1/3× PBS with sonication. Serum was separated from blood cells by centrifugation at 6000 rpm for 15 min. Total protein concentration was measured as described above and standardized at 350 μg ml^{-1} (fish 1), 250 μg ml^{-1} (fish 2) and 150 μg ml^{-1} (fish 3). On the other hand, specificity of the pAb-IgMs was conducted by Western staining whole tissue lysate of zebrafish (*D. rerio*) and serum of carp (*Cyprinus carpio*) and tilapia (*Tilapia niloticus*) at 5× dilution. Two fish samples per species were collected.

Immunohistochemical assay was done following Takano et al. (2007) [9]. Briefly, paraffin embedded-microtome-sectioned or fresh smear samples of apparently healthy Japanese flounder kidney were formalin fixed in APS coated slides. These were then serially washed with xylene and decreasing concentration of ethanol and then by 1× PBS. These were incubated with hydrogen peroxide (H_2O_2) for 30 min, then with pAb-IgMs overnight inside a moist staining tray. After washing with 1× PBS, the 2nd antibody (Histofine: MAX-PO-MULTI) was introduced for 1 h and then washed with 1× PBS. The substrate, simple stain AEC counterstained with hematoxylin was used for visualization. Resulting slides were mounted in glycerol and the cells were photographed using a microscope-mounted digital camera.

Table 1
Primers used in this study.

Species	Primer name	Primer sequence (5′–3′)
Japanese flounder (*Paralichthys olivaceus*)	IgM1F	TTTTCATATGGAACTGAAAGTGTCTGCCTTCT
	IgM1R	TTTTGAATTCTTAATGATGGTGATGATGGTGTGATGTCAACAGGAACGATG
	IgM2F	TTTTCATATGGAATGGAGCCAGGGGGATAAAG
	IgM2R	TTTTGAATTCTTAATGATGGTGATGATGGTGGCAGCTGTACATCACACCCT

We were able to produce two recombinant Japanese flounder IgM proteins expressed from two constant region fragments, *r*IgM1 and *r*IgM2 showing weights of ~23 kDa and ~13 kDa, respectively, whether as inclusion bodies or in Ni-NTA His-tag/dialysis purified form (Fig. S2). The *r*IgM1 and *r*IgM2 proteins were then used to make the mouse pAb-IgM1 and pAb-IgM2, respectively and succeeding western blots revealed that the pAb-IgM antisera produced were clearly detecting their respective *r*IgM1 and *r*IgM2 proteins at various concentrations (Fig. S3).

Western analysis under denaturing conditions of tissues from 3 individual Japanese flounder samples at 3 different protein concentrations (350 μg ml^{-1}, 250 μg ml^{-1} and 150 μg ml^{-1}) showed that pAb-IgMs can detect a ~75 kDa IgM heavy chain (Fig. 1). Moreover, pAb-IgM1 marked a secondary band that is ~70 kDa but not pAb-*r*IgM2. Both pAb-IgMs can detect multiple bands in serum. The western blots also consequently showed that Japanese flounder IgM protein was equally expressed in heart, kidney and spleen but not in liver and muscle. In serum, IgM was detected to be in several different sizes.

Western blots to detect cross reactions in other fish species revealed that pAb-IgM1 is specific to Japanese flounder IgM with only an apparent low cross-reaction in carp serum detecting a ~50 kDa band (Fig. 2). In contrast, pAb-IgM2 showed minor reaction with one sample of zebrafish and strong cross-reactivity in carp and tilapia. The zebrafish sample showed a band of ~45 kDa, the two carp samples showed two bands (~75 kDa and ~50 kDa) and the tilapia samples showed multiple bands of various sizes and intensity.

Immunohistochemical staining of head kidney revealed that pAb-IgM1 and pAb-IgM2 were able to detect IgM in normal kidney cells *in vivo* (Fig. 3). Both antibodies detected 2 kinds, cells labelled only on its periphery and cells labelled fully.

In this study, we showed that recombinant proteins (*r*IgM1 and *r*IgM2) were highly expressed by the bacterial expression system pET32-BL21 codon+ and the purified proteins did not show any noticeable bacterial proteins. Moreover, the mouse polyclonal antibodies produced from these proteins were quite effective in detecting the fish IgM heavy chains *in vivo*. This strongly suggests that the pET32-BL21 codon+ bacterial expression system, the His-tag/dialysis purification and the mouse polyclonal production system used in this study are more than adequate systems to produce cell markers and determinants.

The Japanese flounder IgM has been shown by a purified serum Ig-derived mAb to be composed of 2 heavy (H) chains having masses of about 77 kDa and 72 kDa, respectively [4]. Our results revealed a ~75 band detected by both pAb-IgMs, which could very well be the 77 kDa because it is difficult to estimate exact sizes in the gel. The other band detected (~70 kDa) by pAb-IgM2 would then be the 72 kDa H chain. This indicates that the pAb-IgMs possess the ability to discriminate its target molecule *in vivo*. Functionally, the detection of pAb-IgM1 of the second band but not pAb-IgM2 suggests that a post-translational structural difference between the 77 kDa and 72 kDa heavy chains of Japanese flounder IgMs exist and it lies in their CHμ2 and CHμ3 regions and could therefore be functionally important.

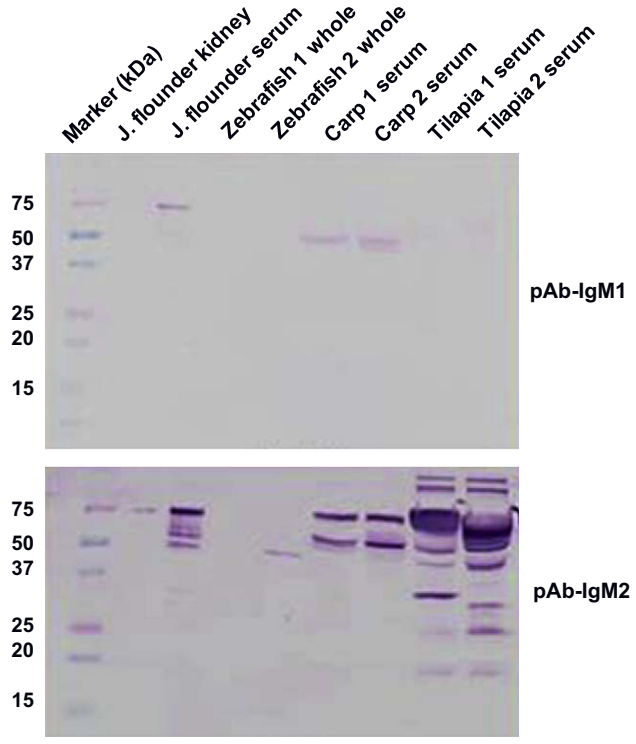

Fig. 2. Specificity of pAb-IgM1 and pAb-IgM2 as shown by western blot analysis of Japanese flounder, zebrafish, carp and tilapia tissues and serum. Set-up under denaturing condition at 15% SDS-PAGE gel with pAb-IgM dilution at 1:2500.

As expected, IgM heavy chains were present in immune-related tissues such as the kidney and spleen, and to a lesser extent in muscle and liver. Interestingly, however, the IgM heavy chain concentration in heart was relatively equal to that of kidney and spleen. This could perhaps be as a result of blood contamination although if this is the case, higher amounts of IgM heavy chain should be detected in muscle and liver. Hence, it is also possible that IgM could have an important yet unknown function in the heart.

The presence of multiple IgM bands in the serum was unexpected as such pattern has not been observed previously in

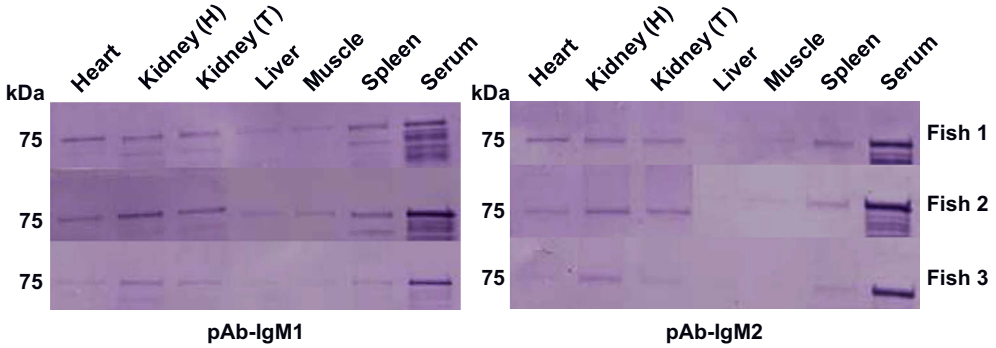

Fig. 1. Western blot analysis of the constitutive expression of Japanese flounder IgM as detected by pAb-IgM1 and pAb-IgM2 in 3 fish samples under denatured conditions. pAb-IgM dilution is 1:2500. Fish 1–350 μg ml^{-1} total protein; Fish 2–250 μg ml^{-1} total protein; Fish 3–150 μg ml^{-1} total protein.

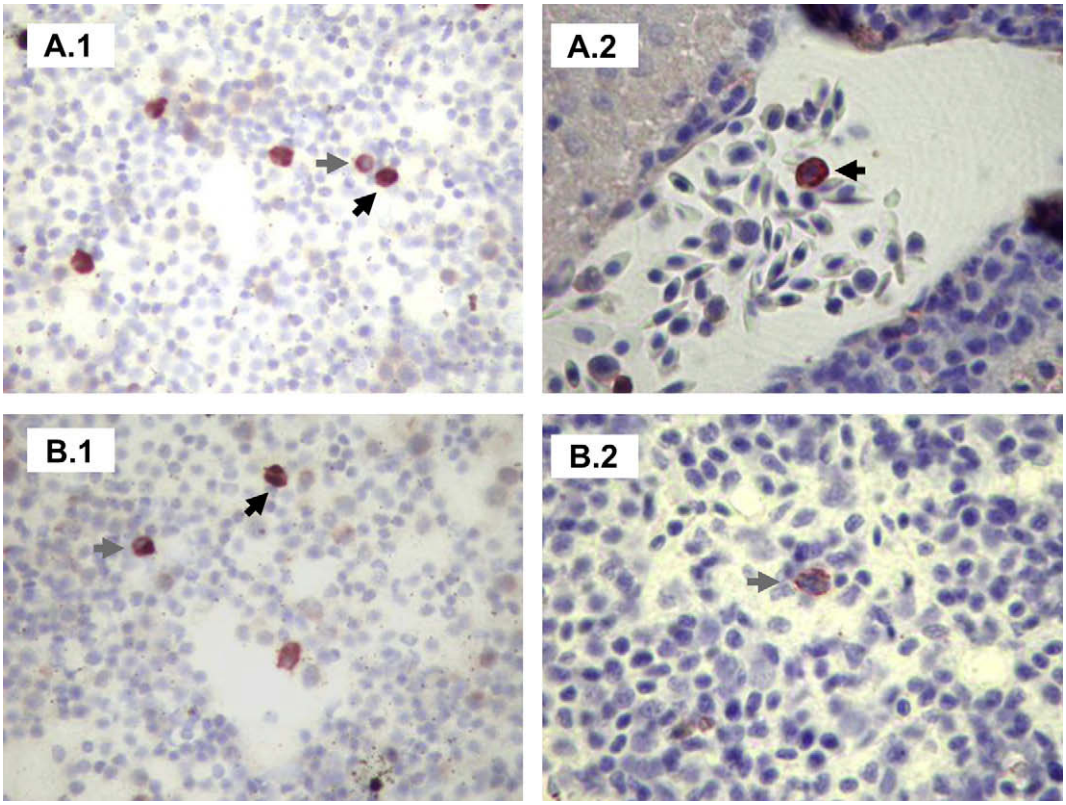

Fig. 3. Immunohistochemical analysis of Japanese flounder kidney cells using pAb-IgMs. pAb-IgM1-labelled kidney smear fresh mount (A.1) and paraffin mount (A.2); pAb-IgM2-labelled kidney smear fresh mount (B.1) and paraffin mount (B.2). Surface immunoglobulin+ (black arrow) and cytoplasmic immunoglobulin+ (gray arrow) are indicated.

Japanese flounder using mAbs [2–4]. It is likely to be a product of cross reactions of the pAbs with the numerous secreted proteins found in the serum. Curiously, however, such pattern is not found in tissues, which may suggest that these are "redox" forms [10]. Secreted teleost fish IgM have been shown to be of various "redox" types thought to be a result of differing disulfide bonding between adjacent heavy chains plus the presence of non-covalent bonding used by teleost fish to generate antibody diversity for important immune functions [10].

Results of the cross-reaction assay showed that pAb-IgM1 is specific to Japanese flounder while pAb-IgM2 can produce other bands in teleost fish species (Fig. 2). While there are IgM heavy chain bands in carp and tilapia equal in size to Japanese flounder suggesting that these are IgM heavy chains, the nature of the other bands (lower and higher) produced by pAb-IgM2 is unclear. As these are marking relatively well, we could only speculate that these other bands are multiple "redox" forms [10]. The very strong reactivity in tilapia, in particular, is puzzling and could not have been caused by protein load since pure serum was loaded for all fish serum samples. Tilapia IgM CHμ4, covered by pAb-IgM1, to our knowledge, has not been sequenced, although the large 40 aa conserved region encompassing CHμ4 as seen in other species (Fig. S1) dictates that the tilapia also contains the concensus sequence. Since this long conserved region is not seen in pAb-IgM1, it is therefore very possible that this region is responsible for the cross reactions seen with pAb-IgM2. Taken together, our data suggests that the CHμ4 has indeed a structural importance in teleost fish IgMs.

The staining of Japanese flounder kidney cells is similar to the surface Ig+ and cytoplasmic Ig+ cells described previously in Japanese flounder using Ig serum-derived mAb [2,3,5]. This suggests that in this study, the cells labelled in the periphery were surface IgM+ cells while those fully labelled were cytoplasmic IgM+ cells. This further demonstrates that the marked cells were IgM+ B cells and that the labeling was likely not due to cross reactions or F receptor-bound IgMs. Moreover, this is similar to the finding in Atlantic halibut (*Hippoglossus hippoglossus*), a close relative to Japanese flounder, where its serum IgM pAb was shown to immuno-histochemically label clusters of IgM + cells in spleen and kidney [11]. There is, however, a need to fully confirm IgM-marked Japanese flounder cells by staining isolated leucocytes using immunofluorescence. Furthermore, future studies should be undertaken in Japanese flounder on investigating cell population in tissues because of the availability of antibodies for flow cytometry.

In conclusion, our results suggest that the pAbs we developed were comparable to monoclonal antibodies for Japanese flounder published before and were useful in elucidating not only Japanese flounder but also teleost fish IgM biology. As such, they represent a cost-effective antibody resource that can be used in immunological studies and immunocompetence monitoring in Japanese flounder, a commercially important aquaculture species. Furthermore, we present a methodology that is effective in producing sensitive and specific polyclonal antibodies. The recombinant protein antigens produced may well be used for development of a more specific monoclonal antibody in the future.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2009.03.008.

References

[1] Randelli E, Buonocore F, Scapigliati G. Cell markers and determinants in fish immunology. Fish Shellfish Immunol 2008;25:326–40.
[2] Tokuda Y, Toyohara H, Ikemoto M, Kina T, Sakaguchi M. Distribution of immunoglobulin-positive cells in the spleen and kidney of Japanese flounder *Paralichthys olivaceus*. Fish Sci 2000;66:1082–6.
[3] Tokuda Y, Toyohara H, Kina T, Sakaguchi M. Characterization of distinct subpopulations of Japanese flounder lymphocytes with monoclonal antibody against serum immunoglobulin. Fish Sci 1999;65:347–52.
[4] Jang HN, Woo JK, Cho YH, Kyong SB, Choi SH. Characterization of monoclonal antibodies against heavy and light chains of flounder (*Paralichthys olivaceus*) immunoglobulin. J Biochem Mol Biol 2004;37:314–9.
[5] Li Q, Zhan W, Xing J, Sheng X. Production, characterization and applicability of monoclonal antibodies to immunoglobulin of Japanese flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol 2007;23:982–90.
[6] Aoki T, Hirono I, Kim MG, Katagiri T, Tokuda Y, Toyohara H, et al. Identification of viral induced genes in Ig+ leucocytes of Japanese flounder *Paralichthys olivaceus*, by differential hybridization with subtracted and un-subtracted cDNA probes. Fish Shellfish Immunol 2000;10:623–30.
[7] Srisapoome P, Ohira T, Hirono I, Aoki T. Genes of the constant regions of functional immunoglobulin heavy chain of Japanese flounder, *Paralichthys olivaceus*. Immunogenetics 2004;56:292–300.
[8] Lin OE, Ohira T, Hirono I, Saito-Taki T, Aoki T. Immnoanalysis of antiviral Mx protein expression in Japanese flounder (*Paralichthys olivaceus*) cells. Dev Comp Immunol 2005;29:443–55.
[9] Takano T, Kondo H, Hirono I, Endo M, Saito-Taki T, Aoki T. Molecular cloning and characterization of Toll-like receptor 9 in Japanese flounder, *Paralichthys olivaceus*. Mol Immunol 2007;44:1845–53.
[10] Kaatari S, Evans D, Klemer J. Varied redox forms of teleost IgM: an alternative to isotypic diversity? Immunol Rev 1998;166:133–42.
[11] Grove S, Tryland M, Press CM, Reitan LJ. Serum immunoglobulin M in Atlantic halibut (*Hippoglossus hippoglossus*): characterization of the molecule and its reactivity. Fish Shellfish Immunol 2006;20:97–112.