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Cloning and expressional analysis of secretory and membrane-bound IgM in rock bream (*Oplegnathus fasciatus*) under megalocytivirus infection and vaccination

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ABSTRACT

In this study, for better understanding the humoral immunity of rock bream (*Oplegnathus fasciatus*), 2 transcripts of immunoglobulin M (IgM) heavy chain gene including membrane bound (m-IgM) and secretory (s-IgM) forms were sequenced and analyzed their tissue distribution and differential expression in rock bream under rock bream iridovirus (RBIV) infection and vaccination since RBIV has caused mass mortality in rock bream aquaculture in Korea. Consequently, s-IgM cDNA was 1902 bp in length encoding a leader region, a variable region, four constant regions (CH1, CH2, CH3, CH4) and a C-terminal region while m-IgM cDNA was 1689 bp in length encoding shorter three constant regions (CH1, CH2, CH3) and two transmembrane regions. The predicted s-IgM and m-IgM represent a high structural similarity to other species including human. In tissue distribution analysis in healthy fish, the highest expression of s-IgM was observed in head kidney followed by body kidney, spleen, and mid gut whereas m-IgM expression was the highest in blood followed by head kidney and spleen. *In vitro*, s-IgM expression was up-regulated by LPS in head kidney and spleen cells at 24 h with no change of m-IgM expression. *In vivo* upon vaccination, s-IgM expression was up-regulated in liver and blood but not in head kidney while m-IgM expression was only up-regulated in head kidney. After challenge with RBIV, s-IgM expression level was higher in vaccinated fish than in unvaccinated fish and m-IgM expression was up-regulated in head kidney of vaccinated group. In conclusion, differential expression of m-IgM and s-IgM may indicate their differential functions to produce the most effective IgM during adaptive immune response. Although it is not able to assess specific IgM at protein level due to a lack of antibody against rock bream IgM, the present study on s-IgM and m-IgM gene expressions upon infection and vaccination will be useful in developing efficient vaccines in the future.

1. Introduction

Immunoglobulins are major effective molecules in humoral immunity of vertebrates, and secreted by B lymphocytes to neutralize antigens and activate the complement cascade and other innate immune responses by opsonization [1]. Immunoglobulin(Ig)s are composed of two heavy chains and two light chains, in mammals, and divided into five isotypes of IgM, IgG, IgA, IgD and IgE based on immunoglobulin heavy chains (IGHs) [2]. Meanwhile, in teleost, three isotypes of IGHs including IgM [3], IgD [4–6], IgZ or IgT [7,8] have been reported so far. Each isotypes of IGHs in fish mediates different immune responses to pathogens [9]. IgM was the first reported immunoglobulin in fish [10] and is the main component of systemic immunity in fish, but also presents in mucus, whereas IgT/Z is known to be specialized to mucosal immune responses [11]. Although the exact role of IgD has not yet been fully elucidated in fish immunity, studies in catfish and humans indicate that IgD might have a role in the interface of inflammation and immunity [12].

IgM is the first antibody produced in immune response and provides a crucial first line of defense for the immune system [13,14]. Structurally, IgM makes up tetramers in teleost, in contrast to pentameric IgM in mammals and elasmobranch fishes [15,16]. While IgD and IgZ have shown a structural divergence among different species [17,18], IgM monomer is evolutionarily stable with four constant Ig heavy chain domains of CH1 to CH4.

IgM molecules exist as two forms as one is membrane bound form (m-IgM) and another one is secretory form (s-IgM) which is produced by plasma cells and secreted into body fluids. M-IgM molecule is

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expressed on the B cell membrane, recognizes antigens as an antigen specific receptor and then causes antigen processing by transmitting antigen binding signal into intracellular signal that regulate B cell growth and differentiation into plasma cell [19,20]. Once B cells recognized the antigen, they suffer hypermutation for affinity maturation of Ig with the help of follicular T helper cells and then the B cells expressing the right m-Ig on their surface are chosen for clonal expansion and differentiation into plasma cells. At this time, the expression of m-IgM increases. After affinity maturation, B cells become plasma cells or memory B cells and increase the expression of s-IgM or m-IgM, respectively. At the same time, stimulation such as pathogen associated molecular pattern (PAMP)s makes B cells to differentiate into plasma cells without follicular affinity maturation and produce s-IgM [20]. S-IgM has a key function in protecting against a range of viral, bacterial, fungal and parasitic infections. It binds to pathogens, and enhances pathogen neutralization and agglutination [21].

In fish, expression of IgM gene has been studied after vaccination or pathogenic infections. For example, in rainbow trout, IgM gene was highly expressed on day 10 after immunization and IgM-positive cells were found to be involved in antiviral responses after infection with viral hemorrhagic septicemia virus [22,23]. Also antibody concentration was significantly increased in channel catfish (*Ictalurus punctatus*) about 2 weeks after infection with *Edwardsiella ictaluri* [24]. In mandarin fish (*Siniperca chuuatsi*), IgM mRNA was highly expressed in 3–4 weeks after *Flavobacterium columnare* infection [9]. IgM mRNA expression significantly increased at 21 day post-immunization with *Yersinia ruckeri* infection in rainbow trout [25]. In blunt snout bream (*Megalobrama amblycephala*), IgM mRNA expression attained the peak at day 7 [26]. However, most studies detected IgM gene expression without major concerns about differential expression of m-IgM and s-IgM. Moreover, IgM gene expression after viral infection or vaccination was not extensively studied in fish.

In Korea, rock bream (*Oplegmathus faciatus*), one of the most expensive marine cultured fin fishes, is very popular for sashimi, but suffering supply shortages due to a high mortality caused by megalocytivirus, i.e. rock bream iridovirus (RBIV) infection. The only reliable way for preventing RBIV infection is vaccination. It is known that several fish species including red sea bream, the malabar grouper (*Epinephelus malabaricus*), mandarin fish (*Siniperca chuuatsi*) and turbot (*Scophthalmus maximus*) were well protected from red seabream iridovirus disease after immunization with inactivated whole vaccines [27–31]. However, protection of fish species belonging to the genus *Oplegnathus* by vaccination is not proved yet and these species are known to have high susceptibility to megalocytivirus. Rock bream is particularly susceptible to infection of RBIV, resulting in mass mortality. To develop effective vaccine or treatment, it will be important to understand humoral immune system in rock bream. Indeed, vaccine efficacy against an infectious disease can be confirmed by the presence of the neutralizing antibodies raised against a pathogen in infectious diseases [32]. Thus, IgM gene expression level could be a critical marker to screen efficient vaccines.

In this study, IgM of rock bream were cloned by rapid-amplification of cDNA ends (RACE)-PCR. After obtaining full-length cDNA sequences of s-IgM and m-IgM, gene and protein structures, and expressional characteristics in different tissues of healthy rock bream were analyzed. Furthermore, the transcriptional changes of IgM gene following stimulation with RBIV infection and vaccination were investigated to understand the humoral immunity induction against the vaccine by using quantitative PCR (Q-PCR).

2. Materials and methods

2.1. Fish and sampling

Apparently healthy rock bream (body weight 20 ± 1 g) was obtained from an aquaculture farm in Geoje Island in Korea. Before experiment, fish were acclimatized in 5 ton aquarium at 23 ± 0.5 °C for 2 weeks and fed once a day with satiation. After acclimation, RBIV infection was checked by PCR using primer pair designed to amplify conserved region of major capsid protein (MCP) gene in spleen [33]. After confirming that the fish was not infected by RBIV, fish was anesthetized using 3 mg/ml of 2-phenoxyethanol. Tissue samples were aseptically collected, added into TRIzol reagent (Invitrogen, USA), and then stored at −70 °C until RNA isolation. Tissue distribution of s-IgM and m-IgM expression was determined in 14 different tissues including gill, liver, spleen, heart, head kidney, body kidney, stomach, midgut, eye, skin, muscle, brain, fin and blood. All samples were aseptically collected into TRIzol reagent (Invitrogen, USA), frozen immediately in liquid nitrogen and stored at −70 °C until RNA isolation.

2.2. Preparation of virus and vaccine

The virus was prepared in rock bream embryo cells experimentally infected with megalocytivirus sachun-1 (IVS-1) strain isolated from rock bream with a high mortality in 2000 [33]. The concentration of viral particles was estimated by Q-PCR method using the primers of qMCP-F (GGC GAC TAC CTC ATT AAT GT) and qMCP-R (CCA CCA GGT CGT TAA ATG A) to amplify MCP gene. Q-PCR was performed using LightCycler 96 (Roche) in a 20 μl reaction volume containing 10 μl 2 X SYBR Green I Master (Takara), 0.5 pM of each primer and 1 μl of template DNA. The amplification conditions were as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 10 s, 52 °C for 15 s and 72 °C for 15 s. As a standard, recombinant plasmid pMD20-T vector containing 141 bp of the MCP gene was amplified and purified from transformed *E. coli* (DH-5α). Serial 10-fold dilutions of the control plasmid were used to establish a standard curve ($1.0x10^8$ to $1.0x10^1$ copies/ml). The standard curve was generated using the means of triplicate determinations.

The vaccine was produced by inactivating IVS-1 strain prepared in PI-PMF cell line [34]. The propagation of IVS-1 in PI-PMF was consistent and the highest concentration was reached at $10^{10.1}$ copies/ml. The virus in PI-PMF cell culture supernatant was inactivated with treatment of 0.1% formalin (37% formaldehyde, Sigma-Aldrich) for 7 days at 4 °C. The undiluted vaccine was highly concentrated and viral copy number was 1×10^{10} copies/ml. To prepare lower concentration, it was 10 times diluted in PBS and named supernatant of cultured megalocytivirus vaccine (SCMV, 1.0×10^9 copies/ml).

2.3. Cloning of IgM cDNA

Total RNA extracted from kidney of the rock bream after injecting IVS-1 was reversely transcribed using The RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) by following the manufacture's protocol. The partial cDNA sequences of IgM were obtained from next generation sequencing (NGS) of spleen RNA library and designed specific primer set to amplify partial cDNA of IgM based on Contig sequence. The amplified partial cDNA sequence was confirmed by sequencing the PCR product. The PCR reaction was performed under following conditions: pre-denaturing (94 °C for 5 min); 32 cycles of denaturation (94 °C for 30 s), annealing (60 °C for 30 s), and extension (72 °C for 30 s). The PCR products were electrophoresed through a 1.5% agarose gel and purified with a commercial gel extraction kit (GeneAll, Korea). The purified PCR products were then subcloned into a pMD20-T Vector (TaKaRa, Otsu, Japan) and sequenced with primers of M13F and M13R.

The full-length IgM cDNA sequences were assembled with the products of RACE. The RACE PCR (The FirstChoice® RLM-RACE Kit, Thermo Scientific, USA) was performed with gene specific primers designed according to the obtained partial cDNA sequence and the adaptor primer in the following PCR reaction: in the case of 3′ RACE PCR, pre-denaturing (94 °C for 5 min); 32 cycles of denaturation (94 °C for 30 s), annealing (59.5 °C for 30 s), and extension (72 °C for 25 s) and

Table 1
Primers used to clone rock bream IgM gene and to perform real-time qPCR.

Name	Sequence(5′ to 3′)	Objects
IgM F	GGCAGGTGTTGATGGTCAGA	Partial cDNA
IgM R	GATGCCTCGAAGCCAATGTG	
5′RACE Outer	GCTGATGGCGATGAATGAACACTG	5′ RACE PCR
5′RACE Inner	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	
Out-IgM R	ATGTCCACATTGGCTTCGAG	
In-IgM R	CGAGGCATCCGTCAGAAATT	
3′RACE Outer	GCGAGCACAGAATTAATACGACT	3′ RACE PCR
3′RACE Inner	CGCGGATCCGAATTAATACGACTCACTATAGG	
Out-sIgM F	GACTGAGCGTCCTTCAGTGT	
Out-mIgM F	GCCCATTCAGCCTTCCAC	
In-sIgM F	TTGGTGTCTTGGCTTGCTGA	
In-mIgM F	TATGACGAATGGAGCAAGGG	
s-IgM F	AGACAAGGTGACCCTGACTT	Real-time qPCR
s-IgM R	GCAGAGTAGGTTCCATGGTT	
m-IgM F	ACCTACCAATGGAACAGTGC	Real-time qPCR
m-IgM R	CAGTGGTTCCAATGGTGAAC	
EF-1alpha F	CAGGGAGAAGATGACCCAGA	Real-time qPCR
EF-1alpha R	CATAGATGGGCACTGTGTGG	
MCP F	GGCGACTACCTCATTAATGT	Real-time qPCR
MCP R	CCACCAGGTCGTTAAATGA	
M13F	GTTTTCCCAGTCACGAC	Sequencing
M13R	CAGGAAACAGCTATGAC	

then a final elongation step (72 °C for 10 min). In the case of 5′ RACE PCR, pre-denaturing (94 °C for 5 min); 30 cycles of denaturation (94 °C for 30 s), annealing (61 °C for 30 s), and extension (72 °C for 100 s) and then a final elongation step (72 °C for 10 min). The primers are listed in Table 1.

2.4. Sequence analysis and protein structure analysis

The nucleotide sequences were compared to the GenBank/EMBL databases with a searching program BLAST from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genbank) and translated into amino acid sequences with NTI Advance software (Invitrogen, USA). Multiple alignments were performed by ClustalW using MEGA6 program. The variable region and transmembrane domain were predicted with IMGT/DomainGapAlign Program (http://www. imgt.org) and HMMTOP (http://www.enzim.hu), respectively. A 3D model of the IgM heavy chain was predicted using SWISS-MODEL (http://swissmodel.expasy.org) [35,36]. The molecular weight was calculated on Expasy ProtParam (http://web.expasy.org/protparam).

2.5. SDS-PAGE analysis

The blood was sampled from the tail vein of rock bream. After centrifugation at 3000 × *g* for 15 min at 4 °C, serum were collected and stored in −70 °C until used. To purify immunoglobulin, protein A column (GE cat no. 17-5280-02) was equilibrated with binding buffer (0.02 M sodium phosphate, pH 7.0) and added serum to the column. Impurities and unbound material were washed with the binding buffer until no protein is detected in the eluent (determined by UV absorbance at 280 nm). And then the sample was eluted with elution buffer (0.1 M citric acid, pH 4). Purified IgM was immediately neutralized to pH 7.4 with neutralization buffer (1 M Tris-HCl, pH 9.0), and was stored at −80 °C until used.

Serum and purified immunoglobulin were analyzed by SDS-PAGE on 12% polyacrylamide gel. SDS-PAGE was performed with Bio-Rad systems according to the instruction given by the manufacturer (Mini-PROTEAN® Tetra Cell, Bio-Rad, USA). The gel was visualized by staining with Coomassie brilliant blue (Coomasie Blue G-250, Bio-Rad, USA).

2.6. RNA isolation and cDNA synthesis

Total RNA was extracted with TRIzol (Invitrogen, USA) following manufacturer's instructions. Briefly, after adding chloroform and centrifuging, an upper aqueous phase containing RNA was separated, precipitated the total RNA using equal volume of isopropanol, washed with 75% ethanol, and dissolved in diethyl pyrocarbonate (DEPC) treated water after air dry. RNA concentrations were measured by UV-spectrophotometer and their integrity was checked with 1.5% agarose gel electrophoresis. First-strand cDNA was synthesized from total RNA using The RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's protocol. Briefly, the 3 μg of total RNA in 12 μl DEPC treated water was incubated with 1 μl of oligo $(dT)_{18}$ primer (100 μM, Thermo) at 65 °C for 5 min. Then, 0.5 μl of RevertAid reverse transcriptase (100 U/ml, Thermo), 4 μl of 5x first strand buffer (Thermo) containing 250 mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$ and 50 mM DTT, 0.5 μl of RiboLock RNase Inhibitor (20 U/ml; Thermo) and 2 μl of 10 mM dinucleoside triphosphate (dNTP) mix (Thermo) were added and the mixture incubated at 42 °C for 2 h. The reaction was terminated by heating to 70 °C for 10 min and added 380 μl of TE buffer to make up the final volume to 400 μl.

2.7. Q-PCR analysis

Gene expression was analyzed by Q-PCR using the LC96 real-time thermocycler (Roche) by the method modified from Hong et al. [37]. The Q-PCR reaction was performed in a 20 μl reaction containing 10 μl of SYBR Green Real time PCR Master Mix (TaKaRa, Japan), 0.4 mM of each forward and reverse primers and 4 μl of cDNA using the following protocol: 60 s at 95 °C; the template was amplified for 40 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. Q-PCR was performed in duplicate for each sample, along with a serial dilution of references and transcript level was calculated using the integrated software as described previously [37]. Gene-specific primers are listed in Table 1. Primers for s-IgM and m-IgM were designed to selectively amplify either membrane-bound or secreted transcripts by locating in the transmembrane exon or in the 3′ end sequence specific for the secreted form. Serially diluted references were used for absolute quantification analysis. In some cases, an efficiency was obtained using serial dilutions of references and used for quantification. Data normalization was performed using a house keeping gene, i.e. elongation factor (EF)-1α. EF-1 α is one of the reference genes, which is most

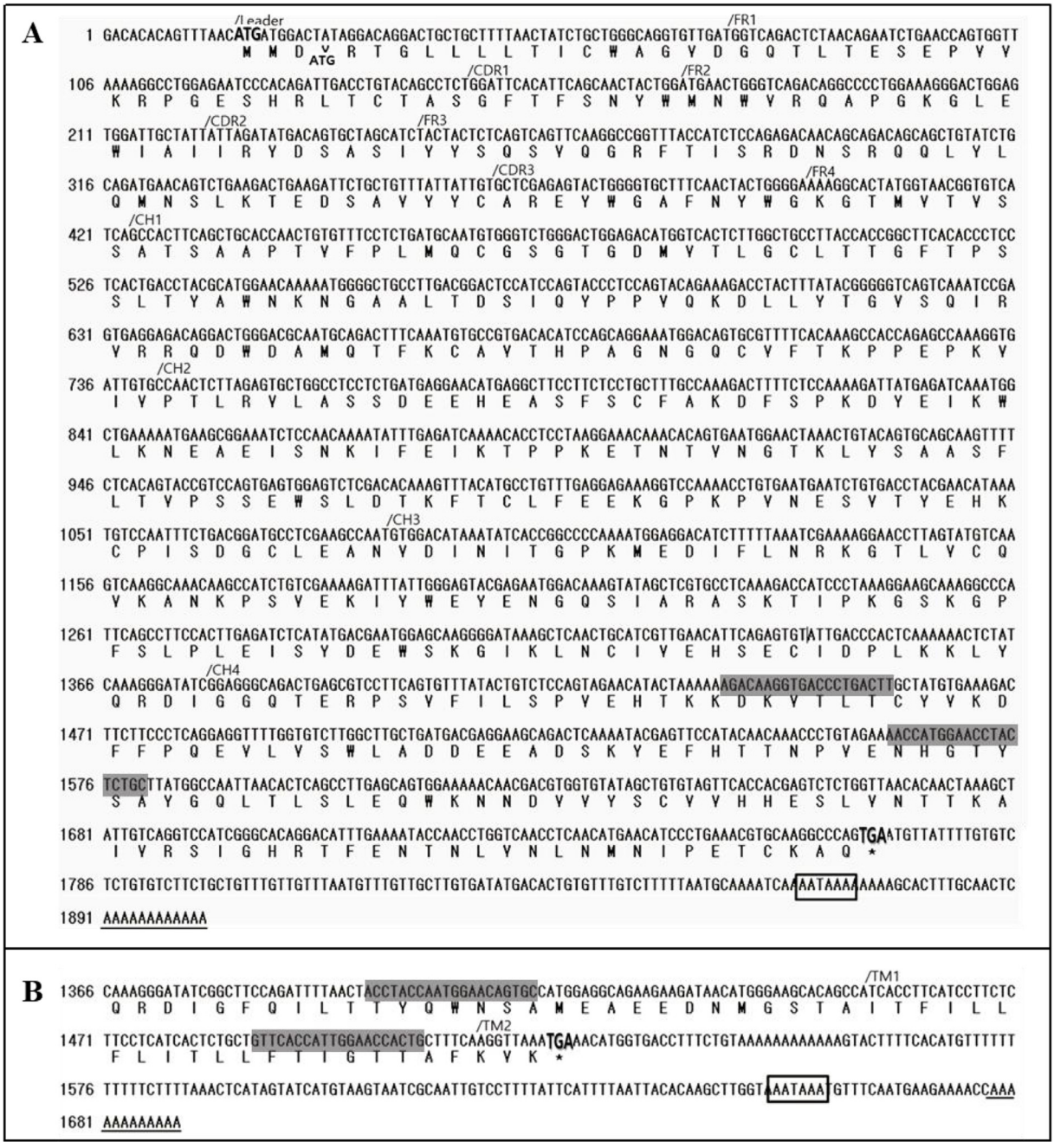


Fig. 1. Nucleotide and translated amino acid sequences of rock bream s-IgM (A) and m-IgM (B). The IgM heavy chain cDNA sequences and the deduced amino acid sequences of rock bream. (A) Secretory form. The variable region (FR1, 2, 3, 4 and CDR 1, 2, 3) was predicted with IMGT/Gap (http://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi); (B) The transmembrane domain of membrane-bound IgM (TM 1, 2) was predicted with SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui), and TMHMM (http://www.enzim.hu/hmmtop). The start and stop codons are indicated in bold and poly adenylation signal (AATAAA) are boxed and the poly A sites were underlined. Q-PCR primer locations are shadowed.

commonly used to reduce possible error generated in a quantification of gene and to normalize Q-PCR [38]. After normalization, fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point.

2.8. Tissue distribution of immunoglobulin gene expression in healthy fish

Eight healthy rock bream were anesthetized, sacrificed by cutting the spinal cord and aseptically taken 14 tissues. Tissues were immediately frozen in liquid nitrogen and stored at −70 °C. Tissues were homogenized in Qiazol (Qiagen) using a TissueLyzer (Qiagen) following the manufacturer's instructions. The Q-PCR was performed as above. The relative expression of s-IgM and m-IgM was calculated as arbitrary units after normalized against the expression level of EF-1α. One arbitrary unit equals to the expression level of s-IgM and m-IgM in brain, respectively, since brain has expressed the lowest levels of each gene.

2.9. IgM gene expression in spleen and head kidney cells after PAMPs stimulation and RBIV infection in vitro

The s-IgM and m-IgM gene expression levels were analyzed in head kidney and spleen cells because the organs are major lymphoid organs in fish and consist of different cell types, playing the different roles in immune response. Head kidney and spleen were aseptically taken from

4 fish and freshly prepared as primary cell cultures. Briefly, the organs were passed through a sterile mesh (BD, USA) in L15 medium (Sigma) containing penicillin (100 U/ml, Gibco) and streptomycin (100 μg/ml, Gibco). The cells were washed by centrifugation at 2000*g* for 5 min at 4 °C and adjusted to $2x10^6$ cells/ml. One ml of the cells was added onto a well of 24 well culture plate, and stimulated with lipopolysaccharide (LPS; *Escherichia coli* strain 055:B5, 1 μg/ml; Sigma Aldrich), polyI:C (1 μg/ml; Sigma Aldrich) and different doses of IVS-1 (0.1, 1, $10x10^5$ copies/ml) for 4 h, 8 h and 24 h at 25 °C. LPS was chosen as a T helper independent antigen which do not induce m-IgM expression and polyI:C was chosen as a non-antigenic stimulant to see if non-specific stimulation could induce IgM gene expression. The concentration chosen for LPS and polyI:C was known to be optimal for immune gene expression studies based on previous studies. The Q-PCR were performed as described above. Q-PCR quantification of expression was expressed as fold change relative to the time matched negative controls.

2.10. IgM gene expression by RBIV vaccination and challenge in vivo

One hundred forty four rock bream were divided randomly into two groups (72 fish/group) and i.p. injected with 100 μl of PBS as negative control or SCMV (10^9 copies/ml) using 1 ml disposable syringes. Eight fish in a group was sacrificed at 1, 3, 7 and 14 days post-vaccination, taken head kidney, liver and blood, and frozen in liquid nitrogen, immediately. At day 21 fish was challenged with 100 μl of IVS-1 (10^4 copies/ml), sacrificed at 1, 3 and 7 days post-infection and aseptically taken head kidney, liver and blood. Total RNA was isolated by Qiazol (Qiagen) after homogenizing in TissueLyzer (Qiagen) following the manufacturer's instructions. Q-PCR was performed as described above. The relative expressions of s-IgM and m-IgM were calculated as fold change relative to the time-matched negative controls after normalized against the expression level of EF-1α.

2.11. Statistical analysis

The data obtained from Q-PCR analysis were expressed as means ± standard error (SE), and subjected to a one-way of variance (ANOVA) followed by LSD post hoc test or paired *t*-test to determine differences among the treatments. Differences were considered to be statistically significant at $P < 0.05$. Statistical analysis was performed using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL).

3. Result

3.1. Characterization of rock bream IgM cDNA sequences

The s-IgM and m-IgM transcripts were cloned and found that heavy chain of s-IgM cDNA was 1902 bp in length including the open reading frame (ORF), 5′ untranslated region (UTR) and 3′UTR of 1749 bp, 15 bp and 137 bp, respectively. The ORF encoded 583 amino acid residues and the relative molecular weight was 65.6 kDa. According to analysis with the program of IMGT/Domain GapAlign, s-IgM of rock bream was divided into a leader region, a variable region and four constant regions of CH1, CH2, CH3 and CH4 (Fig. 1A). The heavy chain of m-IgM cDNA was 1689 bp in length and the ORF, 5′UTR and 3′UTR were 1506 bp, 15 bp and 168 bp, respectively, encoding 502 amino acid residues of 56.2 kDa in ORF with the same leader region, variable region as s-IgM, three constant regions (CH1, CH2 and CH3) and two transmembrane regions of TM1 and TM2 (Fig. 1B).

3.2. Multiple alignments and phylogenetic analysis

The alignments of s-IgM and m-IgM are shown in Fig. 2. S-IgM and m-IgM shared the same amino acid sequence from the variable region (FR1, 2, 3, 4 and CDR 1, 2, 3) to CH3 region but s-IgM and m-IgM possess CH4 or TM regions after CH3, respectively. To determine the evolutionary position of rock bream IgM, a phylogenetic tree was generated and analyzed based on the amino acid alignment of IgM, IgD and IgZ orthologs from different organisms. The tree comprised two clearly distinct branches for fish and higher vertebrates. Rock bream s-IgM and m-IgM were closely related to other fish IgM and placed within IgM of Perciformes, in close proximity to *Siniperca chuatsi* IgM. In addition, rock bream IgM was clearly separated from IgD and IgZ of Perciformes (Fig. 3).

3.3. Tertiary structure modeling and SDS-PAGE analysis

The secretory form of IgM heavy chain is composed of a variable domain VH, constant domain CH1, CH2, CH3, CH4, as shown in the tertiary structure modelling (Fig. 4). The tertiary structure of rock bream IgM shares a great similarity to human IgM, especially the functional region of complementarity-determining regions (CDRs) loops that bind to the antigen (Fig. 4). To detect IgM at the protein level, Ig was purified from rock bream serum using protein A agarose beads. In SDS-PAGE analysis, 2 bands were clearly visualized with the sizes of around 66 kDa and 25 kDa, indicating heavy chain and light chain of Ig, respectively. The size of heavy chain was identical to the size of IgM heavy chain protein (molecular weight of 65.6 kDa) predicted from nucleotide sequences, indicating it is the heavy chain of IgM which is the most abundant Ig in the serum of teleost (Fig. 4C).

3.4. Tissue distribution of s-IgM and m-IgM gene expression

Using the primers located in transmembrane exons and secreted isoforms, tissue distributions of s-IgM and m-IgM gene expressions were analyzed in 14 tissues of normal rock bream and showed differential expressions depending on tissues. Both of s-IgM and m-IgM gene expression levels were the lowest in brain as the relative expressions to EF-1α were 0.002301 and 0.002307, respectively, while those were the highest in head kidney and blood, respectively. The expression levels of s-IgM and m-IgM were especially higher in immune organs such as spleen, head kidney and body kidney than in other tissues. Comparing s-IgM and m-IgM gene expression, s-IgM was more highly expressed in liver and gill than m-IgM, while m-IgM was more highly expressed in blood than s-IgM (Fig. 5).

3.5. IgM gene expression in head kidney and spleen cells in vitro

Freshly prepared primary cultures of rock bream head kidney and spleen cells were stimulated with LPS or polyI:C for 4, 8, or 24 h to analyze the s-IgM and m-IgM gene expression. In both of head kidney and spleen, s-IgM gene expression was gradually increased and significantly up-regulated by LPS at 24 h while m-IgM gene expression level was not significantly altered until 24 h (Fig. 6). Meanwhile, s-IgM and m-IgM gene expressions were not significantly altered by polyI:C in head kidney and spleen cells. To analyze the s-IgM and m-IgM gene expression under RBIV infection, head kidney and spleen cells were stimulated with various concentrations of IVS-1 (0.1, 1, $10x10^5$ copies/ml) for 4, 8, or 24 h. In head kidney, s-IgM and m-IgM gene expression were significantly up-regulated by $0.1x10^5$ copies/ml of IVS-1 at 4 h, and down-regulated by $0.1x10^5$ and $1x10^5$ copies/ml of IVS-1 at 24 h. By $10x10^5$ copies/ml of IVS-1, s-IgM and m-IgM gene expression seemed to be gradually up-regulated although not significant. In spleen, s-IgM gene expression was down-regulated at 4 h and 8 h and was up-regulated at 24 h by all of concentrations of IVS-1. While m-IgM was up-regulated by $0.1x10^5$ copies/ml of IVS-1 at 4 h then gradually decreased to normal levels. By $0.1x10^5$ copies/ml and $1x10^5$ copies/ml of IVS-1, m-IgM gene was up-regulated at 8 h and then decreased at 24 h, but there was no significant difference.

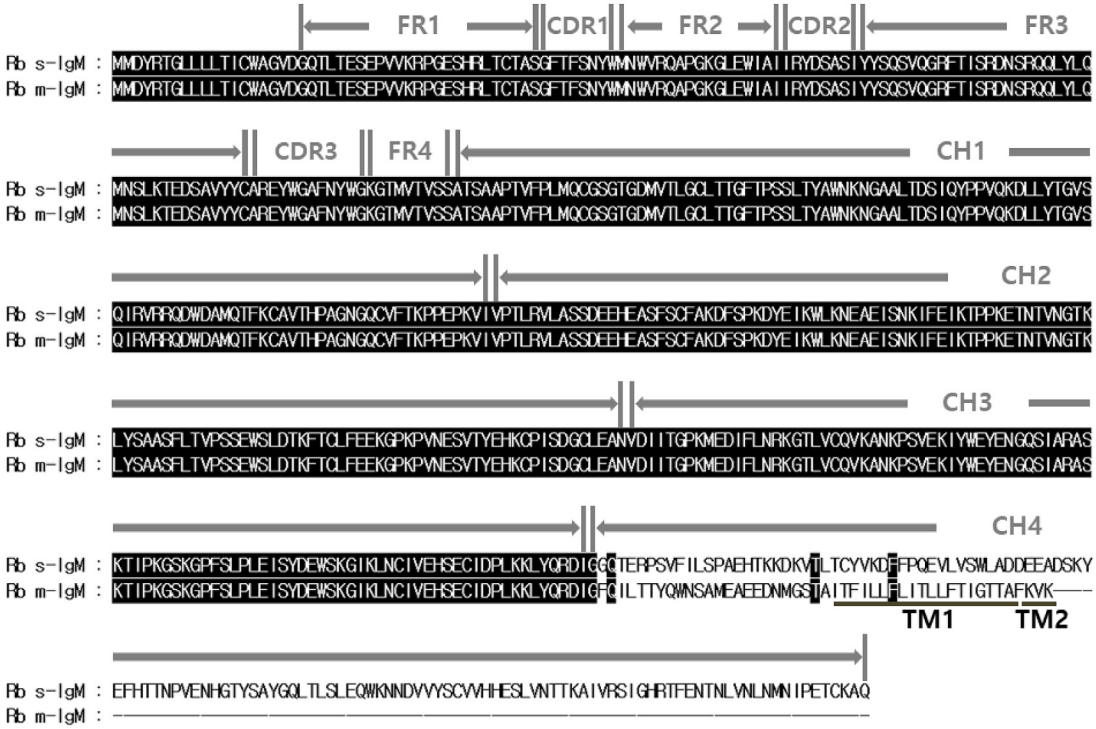


Fig. 2. Multiple alignments of s-IgM and m-IgM in rock bream. Dashes (−) indicate gaps and black shadow indicates identical residues in the aligned amino acid sequences.

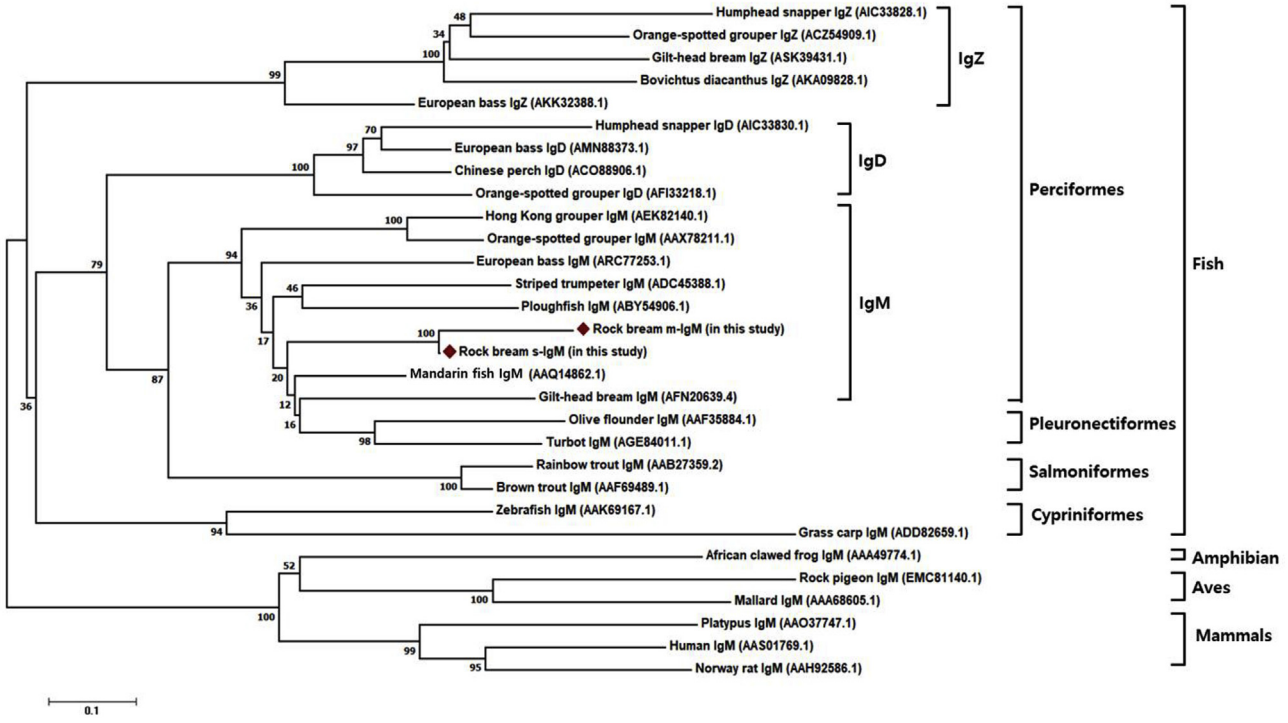


Fig. 3. Phylogenetic trees of fish IgMs. The tree was constructed using multiple alignments of amino acid sequences and the neighbour-joining method within the MEGA6.06 program. The percentage bootstrap values are shown at the branch nodes. Node values represent percent bootstrap confidence derived from 1000 replicates.

3.6. Immunoglobulin gene expression in fish vaccinated with a formalin inactivated RBIV

By vaccination with SCMV (1×10^9 copies/ml), s-IgM and m-IgM gene expressions were differentially regulated in head kidney, liver and blood (Fig. 7 A). The s-IgM expression level in head kidney was not dramatically changed by vaccination while up-regulated at day 3 and 14 in liver and at day 1 and 14 in blood. The m-IgM expression was

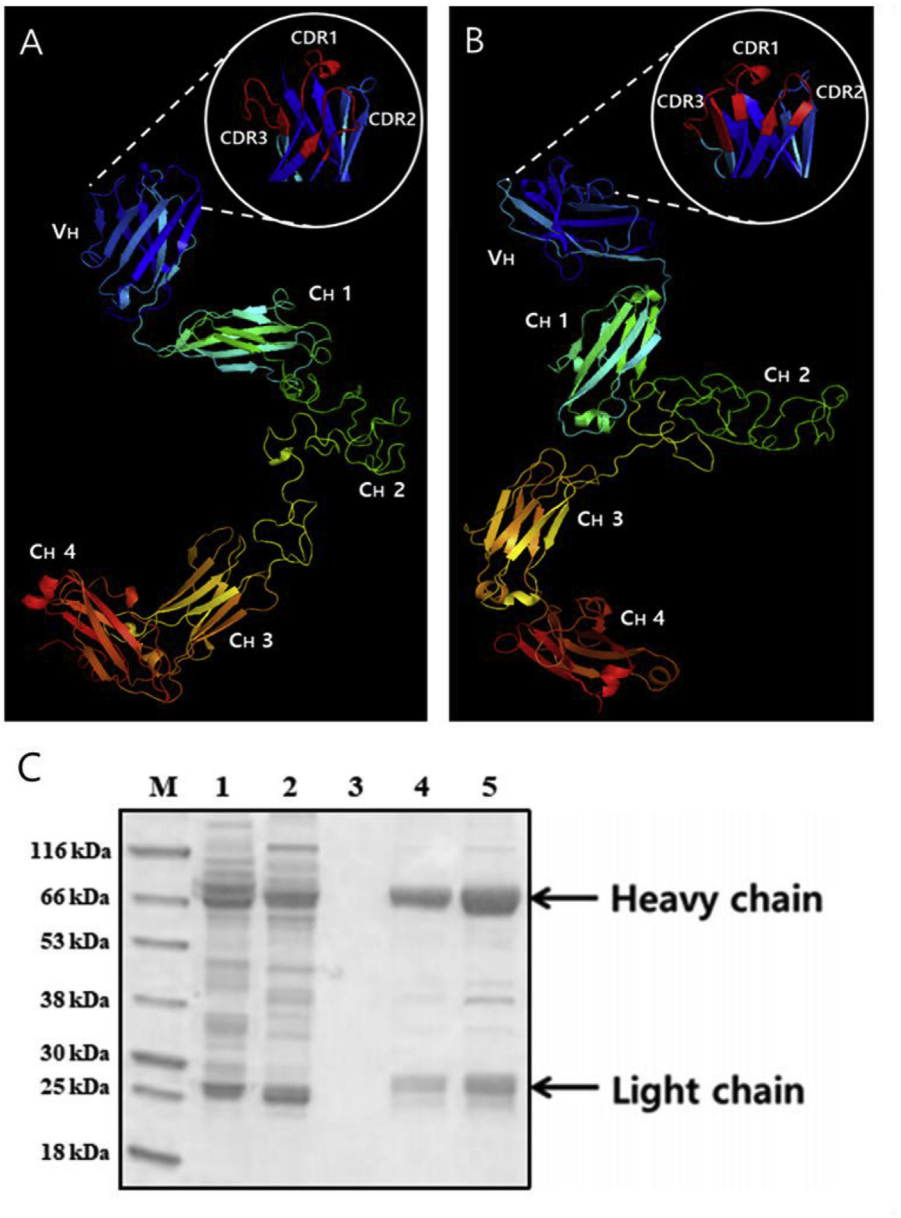


Fig. 4. Tertiary structure modelling of rock bream IgM heavy chain (A) and human IgM heavy chain (B) and SDS-PAGE analysis (C). The IgM structures of rock bream and human are shown as 'VH-CH1-CH2-CH3-CH4'. The enlargement of the variable domain shows a ribbon representation of the β-sheet framework and CDR loops. The model was predicted using SWISS-MODEL (http://swissmodel.expasy.org/). SDS-PAGE analysis of purified Immunoglobulin from vaccinated rock bream serum (C). Lane M, marker; lane 1 and 2, serum of rock bream; line 3, blank; lane 4 and 5, purified immunoglobulin using protein A column.

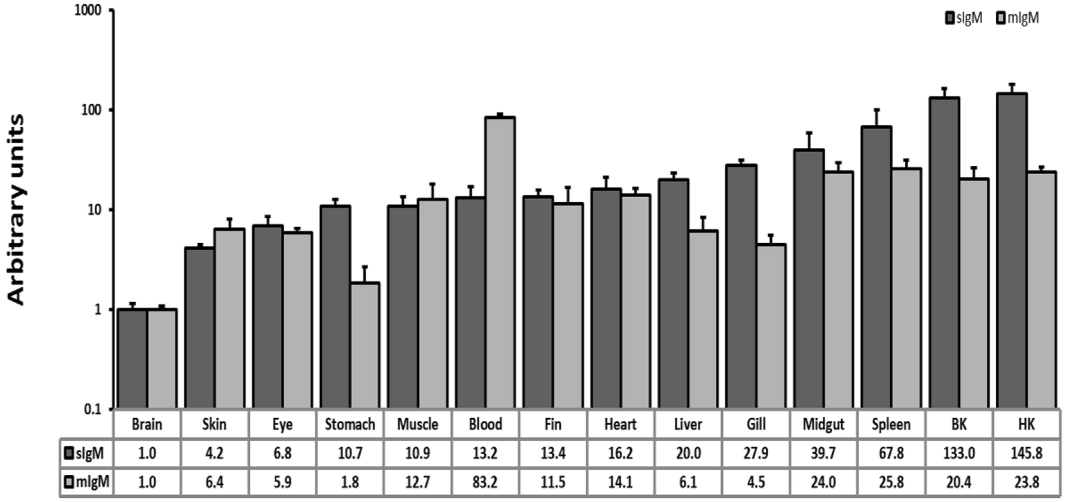
significantly up-regulated at day 14 post-vaccination in head kidney while it was not changed in liver and blood.

3.7. Immunoglobulin gene expression in fish infected with RBIV

To analyze the s-IgM and m-IgM gene expression under RBIV infection, unvaccinated rock bream and vaccinated rock bream were infected with IVS-1 (10^4 copies/ml) (Fig. 7 B). In head kidney and liver, the s-IgM expression level was up-regulated at day 7 post-challenge in vaccinated group while there was no change in unvaccinated group. The m-IgM expression was up-regulated only in head kidney of vaccinated group. In blood, the s-IgM expression level was significantly up-regulated from day 1–7 compared to the unvaccinated group. In vaccinated group, s-IgM and m-IgM expression level were significantly higher at day 3 post-challenge than at other days.

4. Discussion

To analyze the IgM gene expression under RBIV vaccination in rock bream, we have cloned transcripts of secretory and membrane-bound IgM and analyzed their expression and tertiary structure of s-IgM to compare with mammalian IgM structure. In addition, the heavy chain protein was visualized as a band at the same size of the predicted heavy chain of rock bream IgM by SDS-PAGE analysis of the purified Ig from rock bream serum. The reason why there were only 2 major bands of a heavy chain and light chain in the SDS-PAGE analysis though there are at least 3 isotypes of IGH in teleost might be that IgM is the most prevalent immunoglobulin isotype in the serum of teleost fish [39]. The predicted sizes of IgD and IgZ in Perciformes are around 110 kDa (*Siniperca chuatsi* ACO88906.1 and *Lutjanus sanguineus* AIC33830.1) and 60.08 kDa (*Epinephelus coioides* ACZ54909.1), respectively. Although rock bream IgD and IgZ are not reported yet, since Perciformes share a



	Brain	Skin	Eye	Stomach	Muscle	Blood	Fin	Heart	Liver	Gill	Midgut	Spleen	BK	HK
sIgM	1.0	4.2	6.8	10.7	10.9	13.2	13.4	16.2	20.0	27.9	39.7	67.8	133.0	145.8
mIgM	1.0	6.4	5.9	1.8	12.7	83.2	11.5	14.1	6.1	4.5	24.0	25.8	20.4	23.8

Fig. 5. Constitutive expression of s-IgM and m-IgM genes in healthy rock bream tissues. The expression levels were determined by real-time RT-PCR in 14 tissues from ten fish. The expression level was quantified using a serial dilution of references and normalized against the expression level of EF-1α. The relative expression level is shown as fold change; the expression level of s-IgM in brain, which was the lowest and defined as 1 was expressed as fold change. The results represent the mean ± SEM of ten fish. HK, Head kidney; BK, Body kidney.

high homology in Ig genes, rock bream IgD and IgZ heavy chains are expected to be much bigger or slightly smaller than IgM heavy chain.

The predicted protein structure of rock bream IgM heavy chain was similar to IgMs reported in other species including human, suggesting that the IgM function might be conserved from teleost to mammals. The rock bream IgM heavy chain secretory form was comprised of a variable region (V) and four constant regions (CH1 ~ CH4) and the membrane form was comprised of a variable region, three constant regions (CH1 ~ CH3) and transmembrane regions (TM1, 2). This is in agreement with previous studies on other fish species such as olive flounder, Atlantic salmon, and channel catfish [40–42]. In this study, rock bream IgM and human IgM share similarities in the molecular weights of 65.6 kDa and 64.3 kDa, respectively, and the functional regions like CDR 1, 2 and 3 loops in tertiary structure analysis. Previous studies also reported that both teleost and mammalian IgM have similar CDR, variable and constant domains [43].

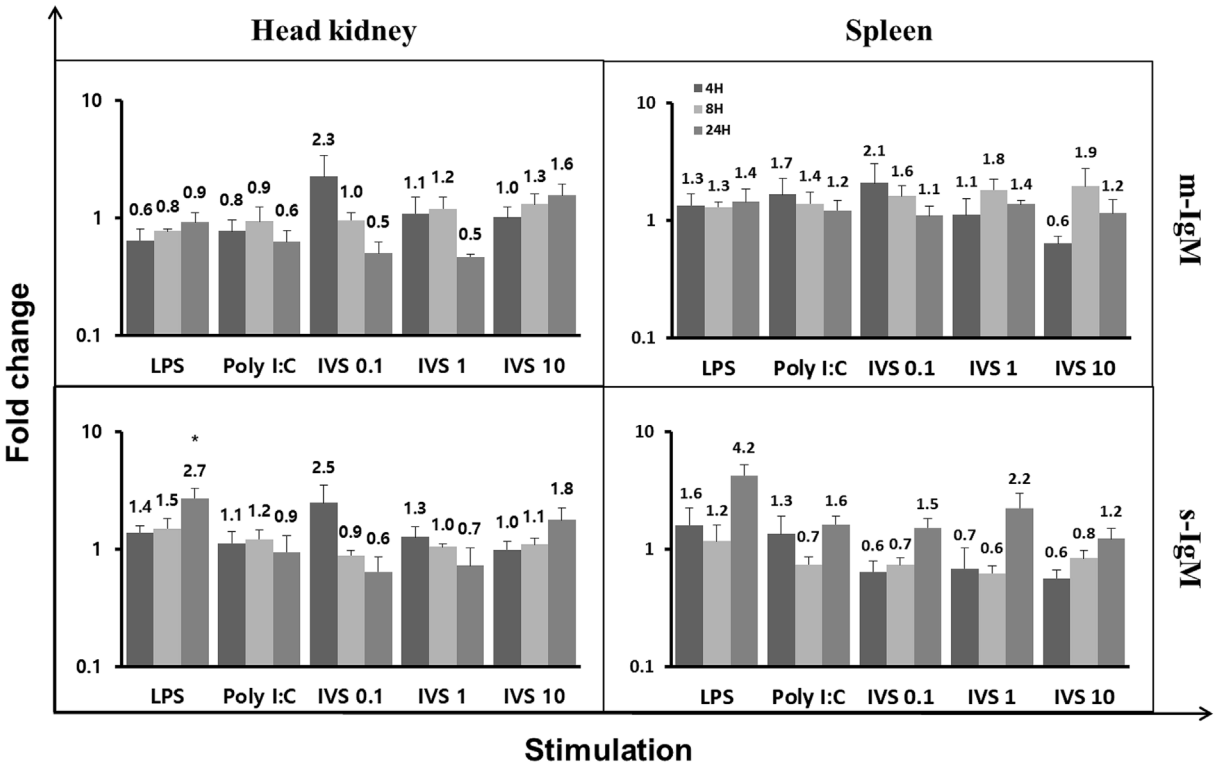


Fig. 6. Expression of s-IgM and m-IgM in head kidney and spleen cells stimulated with LPS (1 μg/ml), polyI:C (1 μg/ml) and various doses of RBIV (0.1, 1, 10x10^5 copies/ml) for 4, 8, 24 h at 25°C. The qPCR data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control sample at each time point. Negative controls were unstimulated samples. The results represent the mean ± SEM of 4 fish. The mean fold changes are shown above the bars. The asterisks (*) above the mean value are marked when the p values of paired *t*-test between stimulated samples and their time-matched controls are below 0.05.

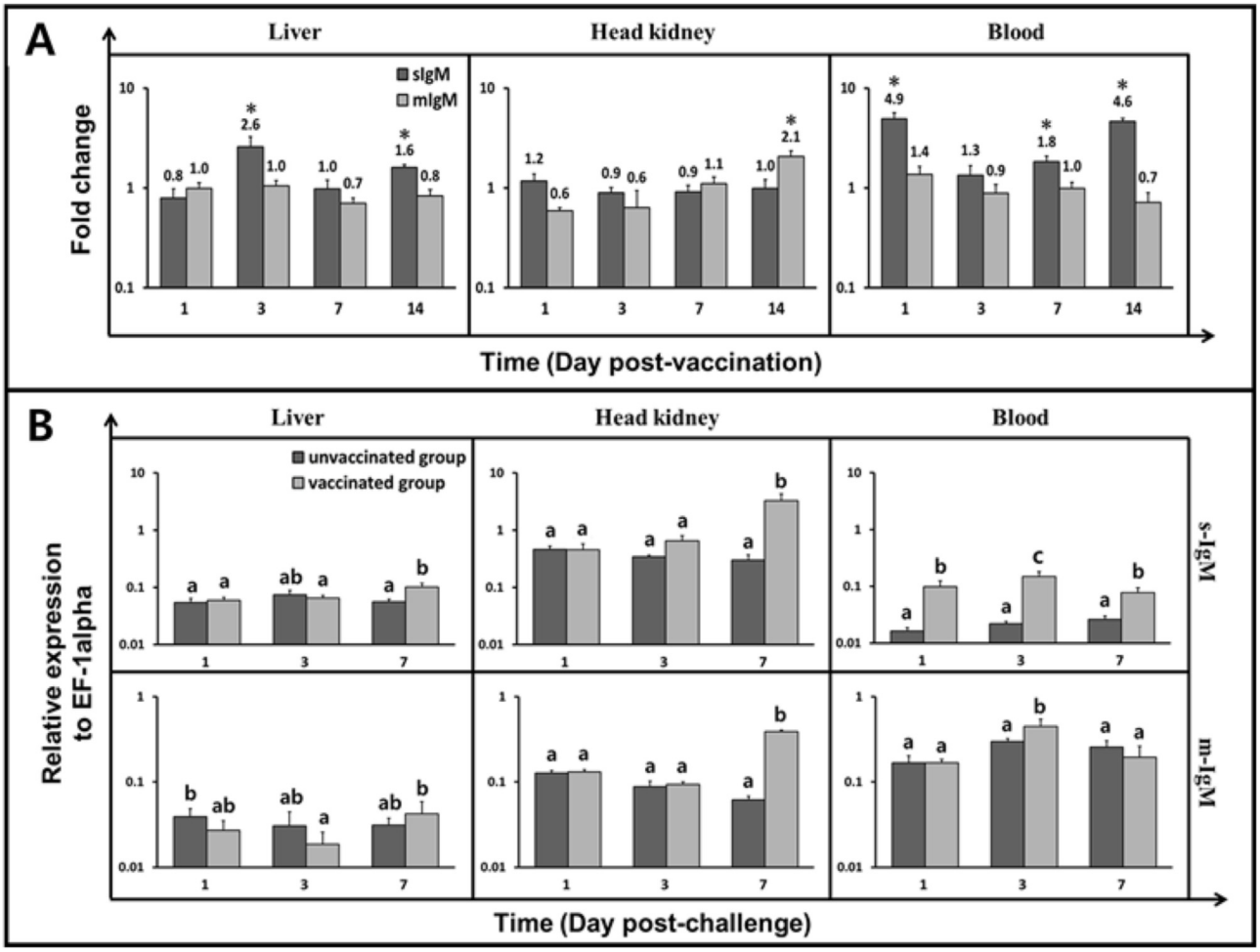


Fig. 7. Expression of s-IgM and m-IgM genes in liver, head kidney and blood of rock bream vaccinated with SCMV (1×10^9 copies/ml) (A) and artificially challenged with RBIV ($1x10^4$ copies/ml) (B) by i.p. injection. (A) The data are normalised relative to the expression of EF-1α and fold change was calculated by dividing the ratio to EF-1α by negative control fish injected with PBS at each time point. The results represent the mean ± SEM of 8 fish. The asterisks (*) above the mean value are marked when the p values of ANOVA LSD post hoc test between stimulated samples and their time-matched controls are below 0.05. (B) The mRNA expression levels of sIgM and mIgM genes relative to the expression of EF-1α were calculated for each subpopulation. The results represent the mean ± SEM of 8 fish. Bars without shared letters are significantly different from each other ($P < 0.05$).

Tissue distribution analysis of s-IgM and m-IgM using Q-PCR showed that s-IgM expression was higher in spleen and kidney than in other tissues. This is similar to a previous study that the highest level of IgM gene expression was observed in kidney followed by spleen and gill in European eel, *Anguilla anguilla* [44]. The higher expression of s-IgM in those tissues can be explained by the abundance of lymphocytes in those tissues since head kidney and spleen are the major lymphoid organs and the main sites for the differentiation of B cells in fish [45]. These organs are also known to initiate adaptive immune responses by activating lymphocytes [46]. Indeed, kidney is surrounded by lymphatic tissues containing lymph nodes that are rich in lymphocytes. Transcript profile of m-IgM was similar to s-IgM, as the higher expression was observed in main systemic immune organs like spleen and kidney but the highest expression was in blood. This may be due to that naïve B cells expressing m-IgM circulate through the blood and migrate to the lymphoid tissues.

The differential expression of m- and s-IgM of rock bream also have been investigated upon various stimulations *in vitro* and *in vivo*. *In vitro*, LPS significantly up-regulated s-IgM expression at 24 h without modulation of m-IgM expression in both organs while polyI:C did not affect IgM expression. This might be because polyI:C is less antigenic while LPS is a thymus independent antigen which induces B cells to produce immunoglobulins without T helper (Th)2 cell activity. The m-IgM expression is essential during adaptive immune response for the production of high-affinity immunoglobulins and development of memory B cell through Th2 cell and follicular dendritic cell activity [47]. There might be a deficiency of follicular dendritic cells responsible for affinity maturation and memory development, which are rare and fragile *in vitro* [48,49].

In vivo, the s-IgM mRNA level was not significantly affected by vaccination in head kidney but m-IgM expression was up-regulated at day 14. In blood and liver, vaccination and viral infection changed only s-IgM expression without a change in m-IgM expression. The expression of m-IgM is essential during the proliferation of B cells for somatic hyper mutation and affinity maturation of m-IgM at the embryonic center during adaptive immune response. It can be thought that the maturation process of the Ig did not occur in blood and liver during the vaccination and viral infection. In channel catfish, clusters of cells containing melano-macrophages were found in kidney and spleen and those cells might represent primitive germinal centers for Ig maturation [52]. And m-IgM expression is essential during adaptive immune responses to produce high affinity immunoglobulins. In a previous study, the expression of s-IgM in *M. amblycephala* was significantly decreased at 4 h, then up-regulated and reached the peak at day 7 in mesonephros and spleen after *Aeromonas hydrophila* infection [26].

Meanwhile, in blood and liver, s-IgM expression was up-regulated at

day 1 or day 3 after vaccination, respectively, indicating a production of s-IgM in plasma cells without affinity maturation. It has been reported that antigen-recognized B cells are driven into plasma cells that produce low-affinity Ig by cognate T cell or without T helper cell (Th) at the early stage of immune response [50]. Although we could not confirm the specificity of s-IgM raised in the blood during the initial vaccination reaction, it is known that virus particles have a repetitive/non-random structure which is tend to preferentially generate a T cell independent (TI) antigen response. TI antigens can induce a specific IgM response within 48 h for very early protection against pathogens [53].

A significant up-regulation of s-IgM expression in the blood was observed again at day 7 and 14 after vaccination in this study. This delayed up-regulation of s-IgM might be caused by affinity maturation process in plasma cells to produce a high affinity antibody through Th2 activation. Considering the significant up-regulation in s-IgM and m-IgM expression at day 7 post-challenge in vaccinated group, the up-regulation of the m-IgM expression at day 14 after vaccination can be a process of affinity maturation and differentiation to memory B cell. Thus, it can be thought that a certain period after antigenic stimulation is required at the early stage of immune response for the antigen processing and presentation, being more likely to be an adaptation. During this period, low expression of the antibody is mobilized to recompense for the shortage of the specific antibody [51].

In conclusion, the full-length mRNA of rock bream s-IgM and m-IgM heavy chain have been cloned and characterized in this study. The differential expressions of s-IgM and m-IgM were found in different tissues and against RBIV vaccination and infection in head kidney, liver and blood. Characterization of immunoglobulins will help to understand the mechanism of the adaptive immune system in fish. Also, knowledge on s-IgM and m-IgM gene expressions upon infection and vaccination will be useful in developing efficient vaccines as markers for evaluating a process of antibody production. Indeed, future study will be needed to analyze a specific antibody against a target antigen at protein level after vaccination.

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