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IgM and IgD heavy chains of yellow catfish (*Pelteobagrus fulvidraco*): Molecular cloning, characterization and expression analysis in response to bacterial infection

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ABSTRACT

Three different immunoglobulin (Ig) isotypes, namely IgM, IgD, and IgT/IgZ have been described in most teleost, among which IgM and IgT are considered crucial in systematic and mucosal immunity, respectively. However, some teleost have no IgT/IgZ and it is unclear how other Ig isotypes interact to perform immune-protective roles in both systematic and mucosal sites. In this study, the complete cDNA sequences of IgM and IgD heavy chains were cloned and analyzed from yellow catfish (*Pelteobagrus fulvidraco*). The full-length cDNA of *Pf*-IgM and *Pf*-IgD heavy chains contained an open reading frame (ORF) of 1710 and 2991 bp encoding a predicted protein of 570 and 997 amino acids, respectively. Tissue-specific expression analysis indicated that both IgM and IgD were highly expressed in kidney and spleen, and higher expression levels were found at zygote and 13th day post hatching during early development. Multiple sequence alignment and phylogenetic analysis showed IgM and IgD of yellow catfish are closely related to other fish of Siluriformes. Moreover, we also constructed the infection model of yellow catfish with bacteria (*Flavobacterium columnare* G_4) for the first time to study the function of *Pf*-IgM and *Pf*-IgD heavy chain genes in immune response. Quantitative real-time PCR (qRT-PCR) showed that significantly up-regulated expression of *Pf*-IgM was not only detected in liver and spleen, but also in mucosal tissues including skin and intestine, while *Pf*-IgD was just significantly increased in liver and spleen, which might suggest the main immune-protecting roles of IgM in mucosal tissues of yellow catfish.

1. Introduction

Adaptive immunity first arose in jawless vertebrates, but these species lack immunoglobulins or T cell antigen receptors and rely on variable lymphocyte receptors for antigen recognition [1,2]. Immunoglobulins (Igs), which are produced by B lymphocytes, are the pivotal factors of adaptive immune system [3]. Igs emerge in jawed vertebrates [1], and are considered to neutralize the corresponding antigens and bind on the mucosal pathogens in a process of immune exclusion [4]. In mammals, Igs are classified into five isotypes, namely IgM, IgD, IgG, IgA and IgE [5], while four isotypes, IgM, IgD, IgY and IgA were defined in avians [6,7]. Thus far, four Ig heavy chain classes, IgM, IgY, IgA and IgD, have been reported in reptiles [8]. IgA is similar to Xenopus IgX and bird IgA [9]. In anuran amphibians, five isotypes have so far been reported, namely, IgM, IgD, IgY, IgX and IgF. Research shows that IgY as a functional homologue is similar to mammalian IgG [10] and IgX has been identified as an analogue of mammalian IgA [11]. While, IgF is similar to IgY of amphibian, because its heavy chain also contains two constant domains, with the difference that IgF harbors a separately encoded genetic hinge [12]. Interestingly, in teleost, only three different Ig isotypes were discovered, IgM [13], IgD [14–16], and IgT/Z [17,18]. Importantly, IgM is considered to mainly play a central role in systemic responses, while IgM also showed high mRNA and protein level in mucosal tissues and could be significantly induced after pathogenic infection [19,20]. In contrast, the function of IgD remains unknown although secreted IgD has been discovered in the

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coating of a small percentage of microbiota at the gill mucosa surface in teleost [21]. IgT, as a special Ig of teleost fish, was considered to play a crucial role in fish mucosal immunity and its function is similar to mammalian IgA [19–21].

Yellow catfish (*Pelteobagrus fulvidraco*, *Pf*) is one of commercially important fish species with excellent meat quality in southern China [22,23]. In recent years, with the development of yellow catfish culture industry, several types of bacterial diseases (including ascites disease, ulcerative syndrome, enteric septicemia and crack-head disease) have caused serious economic losses to its aquaculture industry every year [24–26]. *Flavobacterium columnare* (*F. columnare*) G_4, a gram-negative bacterium, is the causative agent of columnaris disease. *F. columnare* G_4 always caused necrosis of fish fin, skin and gill [27] and resulted in the second annual economic losses in commercial catfish industry [28]. There have been several studies about the innate immune response of yellow catfish in response to bacterial infection, including toll-like receptors and complement components [29–31]. However, little is known about its adaptive immune response to bacterial infection, especially the expression patterns of yellow catfish Igs in systemic and mucosal tissues. Interestingly, the genomic sequences have shown that channel catfish (*Ietalurus Punetaus*) lack IgT/IgZ in their gene locus [32]. Moreover, significantly up-regulated expression of IgM heavy chain gene were detected in both intestine and gill of channel catfish after parasitic infection, which suggested that IgM maybe play a crucial role in mucosal immunity of fish which lack IgT/IgZ [33]. Therefore, considering the similar evolutionary status, we hypothesize that IgM may be perform the immune-protective roles in mucosal and systemic immune tissues of yellow catfish during bacterial infection.

In this study, based on transcriptomic data, full-length sequences of IgM and IgD heavy chain genes from yellow catfish were cloned using rapid amplification of cDNA ends (RACE). Then the expression profiles of *Pf*-IgM and *Pf*-IgD during early developmental stages and tissue-specific distribution were investigated using real-time quantitative PCR (qPCR). We also evaluated the morphological changes of skin and gill in *P. fulvidraco* after infection with *F. columnare* G_4. Finally, the expression patterns of *Pf*-IgM and *Pf*-IgD in different tissues were checked in response to serious bacterial infection with *F. columnare* G_4. This is the first study to explore the immune functions of both IgM and IgD in mucosal tissues of yellow catfish, which will also help us to understand the role of Igs in the disease defense of yellow catfish.

2. Materials and methods

2.1. Fish husbandry and sampling

Juvenile (average body weight: 8.0 ± 1.0 g) and adult (average body weight: 120.0 ± 20.0 g) yellow catfish were obtained from Hannan research base of Wuhan Zhengda Aquatic Products Co. Ltd (Hubei, China). Fish were maintained in tanks with recirculation water and continuous aeration to maintain the dissolved oxygen level near saturation. During the two-week acclimation period, fish were fed with a commercial diet (35% crude protein and 7.5% crude lipid) twice per day. Water temperature was controlled at 25 ± 1 °C and a photoperiod of 12 h light/12 h dark was maintained throughout the trail.

To investigate the expressions of *Pf*-Igs in different tissues, 6 fish were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222) and then tissues including blood, skin, muscle, spleen, liver, stomach, gut, head kidney, trunk kidney, heart, gill, brain and fin ray were collected. To investigate the expression of *Pf*-Igs in early developmental period, ovulation and spermiation of adult yellow catfish were induced by intraperitoneal injection of HCG and LHRH-A (des Gly10 (D-Ala6) LHRH ethylamide) according to methods described by Pan et al. (2008) [34]. Activated sperms were added to eggs, mixed well for fertilization and fertilized eggs were incubated at 28 ± 1 °C. Then samples at early developmental period including ovum, zygote, 2 cells, blastula, gastrula, neurula, sarcomere, organogensis, otoconia, hatching, and larvae of 2, 5, 8, 11 and 13 days post hatching (dph) were collected. All collected samples were immediately frozen in liquid nitrogen, and then stored at −80 °C for further analysis.

2.2. Flavobacterium columnare G_4 infection

F. columnare G_4 strain was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Hubei, China). G_4 strain was firstly recovered at 28 °C for 24 h on a Shieh medium plate, then a single colony were picked and inoculated into Shieh broth at 28 °C in a shaker incubator at 100 rpm to get final OD_{540} at 0.6 [35]. The colony forming units per milliliter (CFU/mL) in the final culture were counted under a microscope.

In experimental group, 150 juvenile individuals were distributed into three tanks and immersed with a dose of 100 ml F. *columnare* suspension (1×10^8 CFU/ml) in 10 L aeration water for 3 h. Sham-infection (control group) followed an identical procedure using MS broth inoculum without bacteria for another three tanks of fish (50 fish per tank). Fish in experimental group and control group were netted out and then returned to previous tanks with same water conditions and reared for up to 28 days.

After bacterial infection, every six fish were randomly selected for sampling at 0 h, 3 h, 6 h, 12 h, 1 d, 2 d, 3 d, 4 d, 7 d, 10 d, 14 d, 21 d, and 28 d, respectively. Fish were firstly anesthetized with 100 mg/L tricaine methanesulfonate (MS-222), and then skin and gill were dissected and fixed in 4% neutral buffered paraformaldehyde for 24 h to do histological analysis. Other tissues (skin, gill, hindgut, head kidney, spleen and liver) for RNA extraction were rapidly excised, frozen in liquid nitrogen, and stored at −80 °C before analysis.

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted from yellow catfish samples using Trizol Reagent (Invitrogen Life Technologies, USA) according to manufacturer's protocol. The integrity and purity of RNA were checked with 1% agarose gel electrophoresis and Nanodrop 2000 spectrophotometer (Thermo scientific, USA), respectively. In order to remove the potential genomic DNA, the RNA samples were treated with DNase, then approximately 1 μg of total RNA was reverse-transcribed into cDNA using a 5 × All-In-One MasterMix with AccuRT Genomic DNA Removal Kit (Abm, Canada) following the manufacturers' instructions.

2.4. Cloning the full-length cDNA of Pf-IgM and Pf-IgD heavy chains

Based on partial fragments of *Pf*-Igs sequences identified from *P. fulvidraco* transcriptome database, gene-specific primers were designed by Primer Premier 5.0 to amplify the internal region of *Pf*-Igs heavy chains. The PCR program was set as follows: initial incubation at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min, the final extension was conducted at 72 °C for 10 min. The PCR reaction was performed with a total volume of 20 μl: 10 μl Premix Taq DNA polymerase (TaKaRa, Dalian, China), 2 μl cDNA, 0.5 μl (10 mM) of each primer and 7 μl ddH_2O. Target products were isolated using a Gel Extraction Kit (Sangon Biotech, China), cloned into pGEMT-Easy vector (Promega, USA) and then sequenced (TSINGKE, China). Then smart 5′-RACE and 3′-RACE were performed using SMARTer RACE cDNA Amplification Kit (Clontech, USA) following manufacturer's instructions to obtain the 3′ and 5′ ends of the cDNA sequences. All primers used in the present study are listed in Table 1.

2.5. Sequence analysis

Open reading frames were identified from whole gene sequences using the ORFfinder of NCBI (https://www.ncbi.nlm.nih.gov/projects/gorf/). Sequences of nucleotide and deduced amino acid were edited using the Editseq tool within DNAstar software. Then positions of the

Table 1
Primer sequences and their designated application in this study.

Name	Sequence (5′–3′)	Application
Clo-IgM-F	GTTCTCTACATCTCTACTGCTC	Conserved region cloning
Clo-IgM-R	AAGACATAGAAGACGGGG	
Clo-IgD-F	CAGTGAATCCTGGAACAAGTG	
Clo-IgD-R	CCCCTTTATACCAGTCTTGCT	
IgM5′-Race1	GAGTTCCTTTTCCCCAGTAGTCG	RACE-PCR
IgM5′-Race2	TAAAGGTCAGTCCGTCGGCAGA	
IgD5′-Race1	TCAGTTTCAACAGAACAGGAGGC	
IgD5′-Race2	GTTCCAGGATTCACTGTATTTCTTG	
IgM3′-Race1	GCAAGAAGGAAACCCCCAAAGT	
IgM3′-Race2	TCACCTGTGGGGTTTACCATGAGT	
IgD3′-Race1	CTCTGGTCAAAGTGAAGACTGCTGAATAC	
IgD3′-Race2	ATTGTTAGCAGCAGTGACAGTATTGTTCTG	
5′CDS	(T)25VN	
3′CDS	AAGCAGTGGTATCAACGCAGAGTAC(T)30VN	
UPM	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT	
NUP	AAGCAGTGGTATCAACGCAGAGT	
IgM-F	AGTTTACTTGCTCCCTCC	qRT-PCR
IgM-R	GAAAACAGAGCCACTATCC	
IgD-F	GAAACCTCACCTCGTATC	
IgD-R	TTGTCCTTTCTCGCTGT	
18S—F	CTGCCGGTGGTCTTCTTCCA	
18S-R	ATTCAGCGGGTCGTCTCGTC	

signal peptide and characteristic domains of protein sequence were predicted by Simple Modular Architecture Research Tool (SMART) online website (http://smart.embl-heidelberg.de/). Sequences of Igs genes in different species were obtained from GenBank databases and then multiple alignments of amino acid sequences were performed by the online software Multalin (http://multalin.toulouse.inra.fr/multalin/). The transmembrane domain was marked based on the results of TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Based on amino acid sequences of Igs in different species acquired from NCBI, the phylogenetic trees of Igs were constructed by adopting the Neighbor-Joining (NJ) method, using MEGA 6.0 software.

2.6. Quantitative real-time PCR

Specific primer pairs were designed for *Pf-Igs* using Primer Premier 5, whose amplification efficiencies were controlled at 100 ± 5%. Quantitative real-time PCR was conducted on 7500 Real-time PCR system (Applied Biosystems, USA) using the EvaGreen 2 × qPCR Master mix (ABM, Canada). All samples were performed in triplicate wells and the cycling conditions were 30 s at 95 °C, 1 s at 95 °C and 10 s at 58 °C for 40 cycles. In addition, the specificity of each primer pair was verified by dissociation curve to ensure only one specific-sized single amplicon was amplified. The relative quantification of the *Pf*-Igs genes was determined via normalized against housekeeping gene (18S). Then relative abundance of *Pf*-Igs was calculated by using the $2^{-\triangle\triangle Ct}$ method.

2.7. Histological assays

Fixed tissues were extracted from 4% neutral buffered paraformaldehyde after 24 h, dehydrated in a graded ethanol series, cleaned in xylene and then paraffin-embedded. Sections of 5 μm were cut with a rotary microtome (HM 325 Manual Microtome, MICROM International GmbH, Waldorf, Germany) and then stained with hematoxylin and eosin (H. & E.). The stained sections of skin and gill were detected for morphological changes under microscope (Olympus, BX53, Japan) using the Axiovision software.

2.8. Statistics

All data were expressed as mean ± standard deviation of the mean (SD). And all test data were analyzed by using SPSS 16.0 software (SPSS Inc., USA). Differences with $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Molecular characterization of Pf-Igs

The complete cDNA sequences of *P. fulvidraco* Igs' heavy chains were obtained by the 5′ and 3′ rapid amplification of cDNA ends (RACE) method and submitted to NCBI. The full-length cDNA of *Pf*-IgM heavy chain was 1968 bp, containing an open reading frame (ORF) of 1710 bp which encoded a predicted protein of 570 amino acids. Heavy chain of *Pf*-IgM was constituted by a signal peptide (contains 18 amino acids), one VH and four Ig-like constant domains (CH1, CH2, CH3, CH4). VH could be further divided into three complementarity determining regions (CDR1-3) and four framework regions (FR1-4) (Fig. 1a). The full-length cDNA of IgD heavy chain was 3359 bp, and encoded a predicted protein of 997 amino acids (2991 bp). *Pf*-IgD heavy chain was composed of a signal peptide (contains 18 amino acids), one VH, seven Ig-like constant domains (CH1, CH2, CH3, CH4, CH5, CH6, CH7) and a transmembrane region (TM) (Fig. 1b).

3.2. Multiple sequence alignment and phylogenetic analysis of Pf-Igs

Conserved cysteine residues were labeled, where disulfide bonds could be formed, as shown in Fig. 2. Multiple protein sequence alignment indicated that yellow catfish IgM presented 54% similarity with *Ietalurus Punetaus*, 44% with *Paralichthys olivaceus*, 43% with *Ctenopharyngodon idella*, 42% with *Danio rerio* and 40% with *Epinephelus malabaricus*. And the amino acid sequence of *Pf*-IgD showed 51%, 43%, 39%, 36%, 40% identities with *Ietalurus Punetaus*, *Paralichthys olivaceus*, *Ctenopharyngodon idella*, *Danio rerio* and *Epinephelus malabaricus*, respectively (Table 2). To further analyze the relationship of *Pf*-Igs with Igs of other animals, phylogenetic trees were constructed using neighbor-Joining (NJ) method (Fig. 3). The phylogenetic analysis suggested that *Pf*-IgM was grouped with IgM of *Ietalurus Punetaus* and *Silurus meridionalis*, and *Pf*-IgD represented the highest similarity with *Ietalurus Punetaus*.

3.3. Tissue-specific expression of Pf-Igs

The expression levels of *Pf*-Igs were detected in all examined tissues

Fig. 1. The nucleotide and deduced amino acid sequences of the heavy chains of IgM (a) and IgD (b) in *P. fulvidraco*. The numbers on the left is used to show the nucleotide positions. The signal peptide, variable region and constant region are shown. N-linked glycosylation sites are underlined. The transmembrane is shaded in dark grey. The stop codon is represented with an asterisk (*).

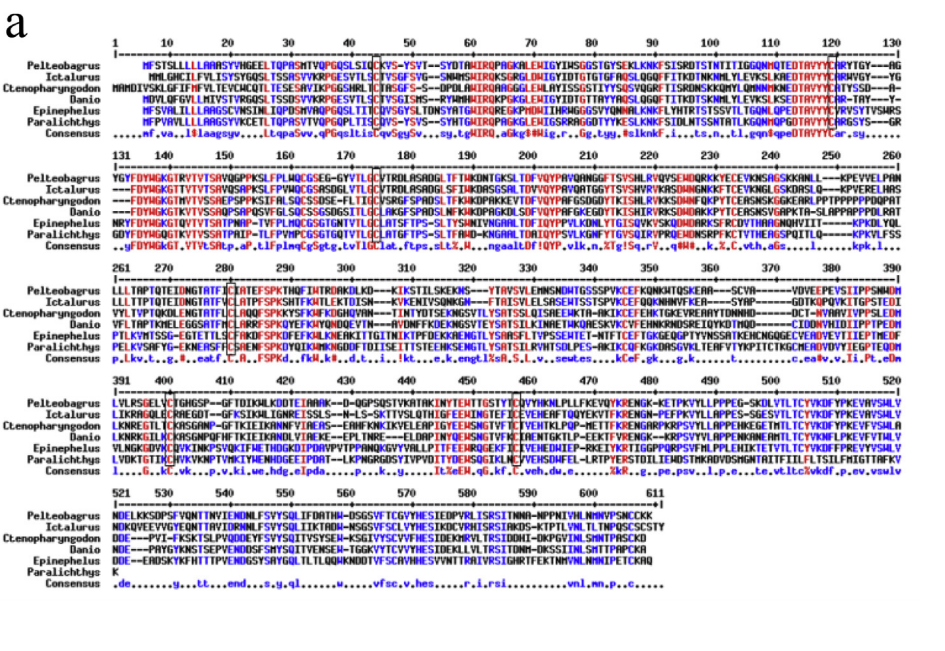
including blood, skin, muscle, spleen, liver, stomach, gut, head kidney, trunk kidney, heart, gill, brain and fin ray (Fig. 4). The results showed that the highest expression level of IgM was detected in the spleen, followed by trunk kidney, head kidney, gill and gut, and the lowest expression was found in muscle. The *Pf*-IgD showed relatively higher expression in head kidney, trunk kidney, spleen and blood, but much lower expression in muscle and stomach.

3.4. *Pf-Igs expression in early developmental stages*

The expression levels of *Pf*-Igs during early developmental period were analyzed by qRT-PCR (Fig. 5). It is interesting to note that *P. fulvidraco* immunoglobulins showed the identical trend, which raise firstly, then descend, but finally goes up again. Significant increase was observed during periods from ovum to zygote and from 5 dph to 13 dph. The lowest expression of both IgM and IgD were found in the otoconia period.

3.5. *Hematoxylin-eosin staining of skin and gill*

Morphological changes were evaluated in yellow catfish skin and gill after infection with *F. columnare* G_4 (Fig. 6). The skin and gill of fish



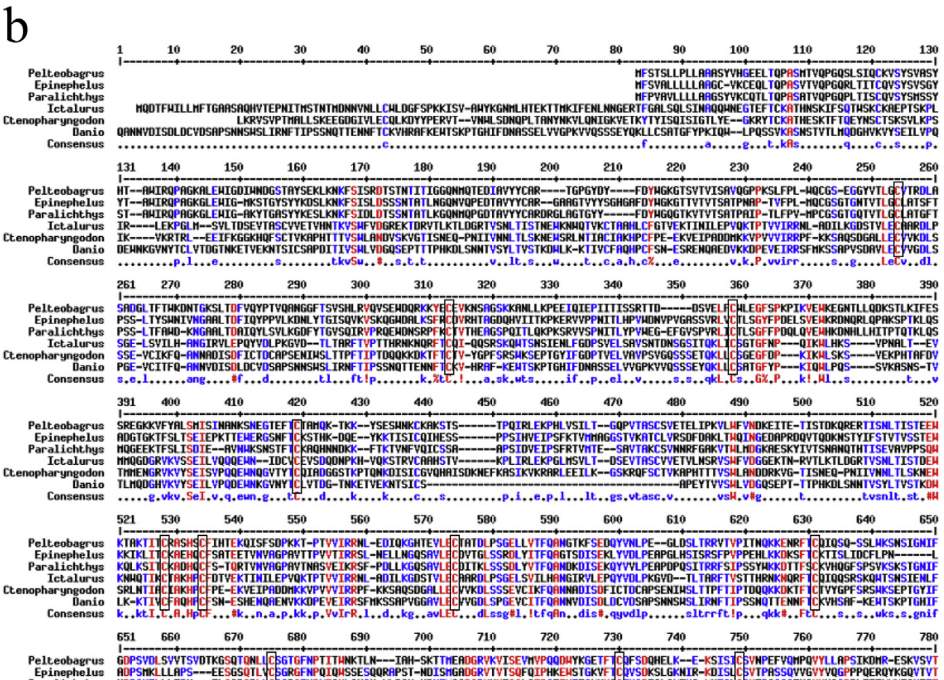


Fig. 2. Multiple sequence alignment of deduced amino acid sequences of *Pf*-IgM (a) and *Pf*-IgD (b) with the corresponding sequences of other species, respectively. Cysteine residues are marked with black frame. The GeneBank accession numbers of the sequences used in the alignment are showed as follows: *Epinephelus coioides*-IgM (AFI33217.1); *Paralichthys olivaceus*-IgM (BAC99314.1); *Ictalurus punctatus*-IgM (A45804); *Danio rerio*-IgM (AAK69167.1); *Ctenopharyngodon idella*-IgM (ABD76396.1); *Epinephelus coioides*-IgD (AFI33218.1); *Paralichthys olivaceus*-IgD (BAB41204.1); *Ictalurus punctatus*-IgD (ADF56020.1); *Danio rerio*-IgD (XP 021330467.1); *Ctenopharyngodon idella*-IgD (ADK66818.1).

Table 2
Identification of Igs from *P. fulvidraco* and other fish.

Species	Identities with *Pf*-IgM	Identities with *Pf*-IgD
Ietalurus Punetaus	54%	51%
Paralichthys olivaceus	44%	43%
Ctenopharyngodon idella	43%	39%
Danio rerio	42%	36%
Epinephelus malabaricus	40%	40%

in the control group were normal, while lesions of skin and gill were found in experimental group. Epithelial layer of infected fish was significantly thinner than that in normal fish, due to the exfoliation of epithelial cells after bacterial infection. Significant changes were also found in gill after infection. The thickening of lamellae and obliteration of many interlamellar spaces were observed at 1 day post infection (dpi), but this change gradually diminished at 4 dpi, 7 dpi and 14 dpi. Nevertheless, hyperplasia of epithelial cells was found in gill again at 28 dpi when compared to control group.

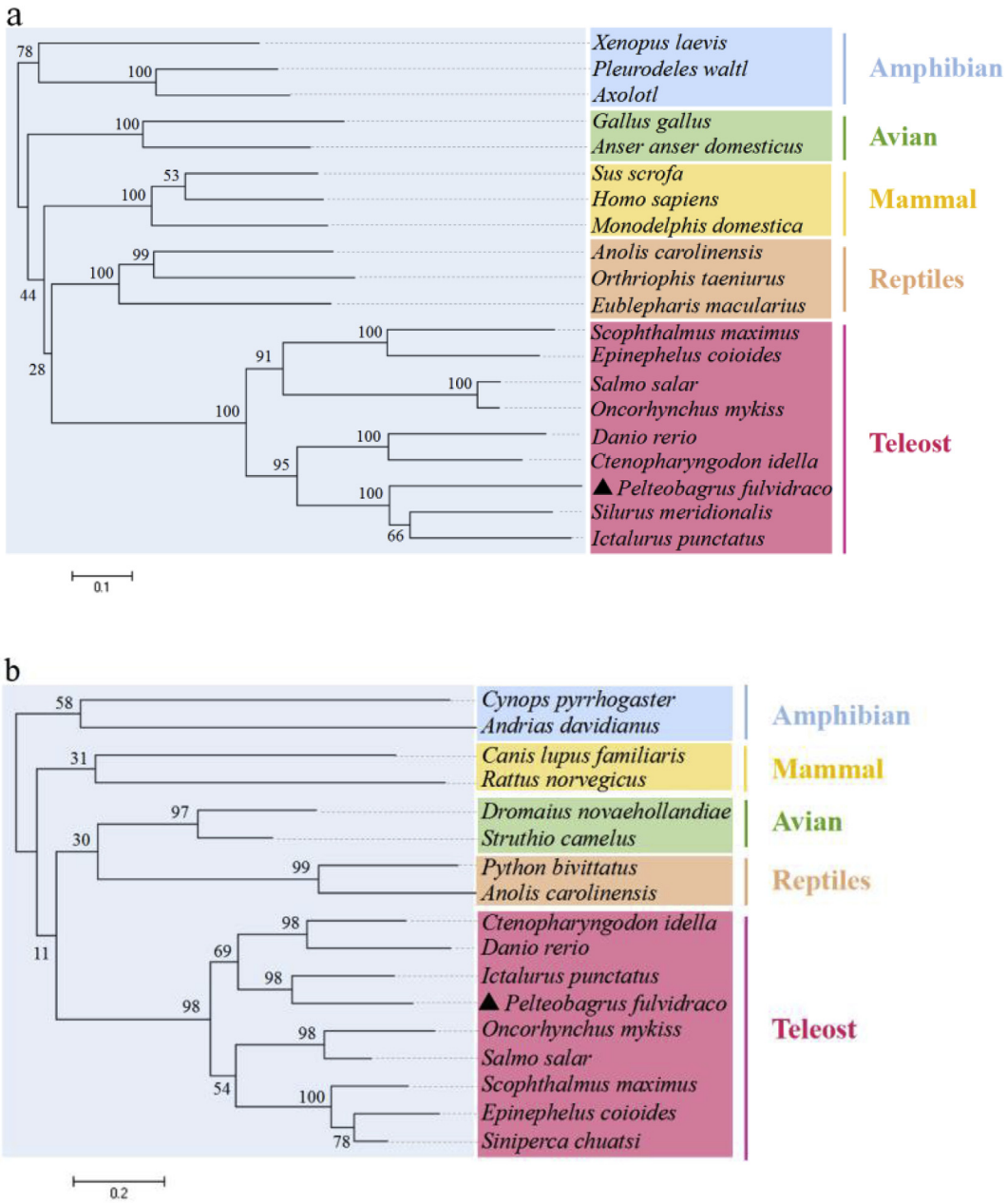


Fig. 3. Phylogenetic analyses of *Pf*-IgM (a) and *Pf*-IgD (b) with other vertebrates using neighboring-joining (NJ) methods. Bootstrap values are calculated from 1000 replicates. Igs of *P. fulvidraco* are indicated with ▲. GenBank accession numbers: *Xenopus laevis*-IgM, AAH84123.1; *Pleurodeles waltl*-IgM, CAE02685.1; *Axolotl*-IgM, A46532; *Gallus gallus*-IgM, P01875.2; *Anser anser domesticus*-IgM, AFM77858.2; *Sus scrofa*-IgM, AAC48775.1; *Homo sapiens*-IgM, AAS01769.1; *Monodelphis domestica*-IgM, AAD24482.1; *Anolis carolinensis* -IgM, ABV66128.1; *Orthriophis taeniurus*-IgM, AFR33841.1; *Eublepharis macularius*-IgM, ABY74509.1; *Scophthalmus maximus*-IgM, AGE84011.1; *Epinephelus coioides*-IgM, AFI33217.1; *Salmo salar*-IgM, AAB24064.1; *Oncorhynchus mykiss*-IgM, AAB03838.1; *Danio rerio*-IgM, AAK69167.1; *Ctenopharyngodon idella*-IgM, ABD76396.1; *Silurus meridionalis*-IgM, AJL46903.1; *Ictalurus punctatus*-IgM, A45804; *Cynops pyrrhogaster*-IgD, BAV35207.1; *Andrias davidianus*-IgD, AIW06017.1; *Canis lupus familiaris*-IgD, ABB89467.1; *Rattus norvegicus*-IgD, AAO19643.1; *Dromaius novaehollandiae*-IgD, APB61242.1; *Struthio camelus*-IgD, AKM28416.1; *Python bivittatus*-IgD, AFR33768.1; *Anolis carolinensis*-IgD, ABV66130.1; *Ctenopharyngodon idella*-IgD, ADK66818.1; *Danio rerio*-IgD, XP 021330467.1; *Ictalurus punctatus*-IgD, ADF56020.1; *Oncorhynchus mykiss*-IgD, AAW66976.1; *Salmo salar*-IgD, AAD43527.1; *Scophthalmus maximus*-IgD, AFQ38975.1; *Epinephelus coioides*-IgD, AFI33218.1; *Siniperca chuatsi*-IgD, ACO88906.1.

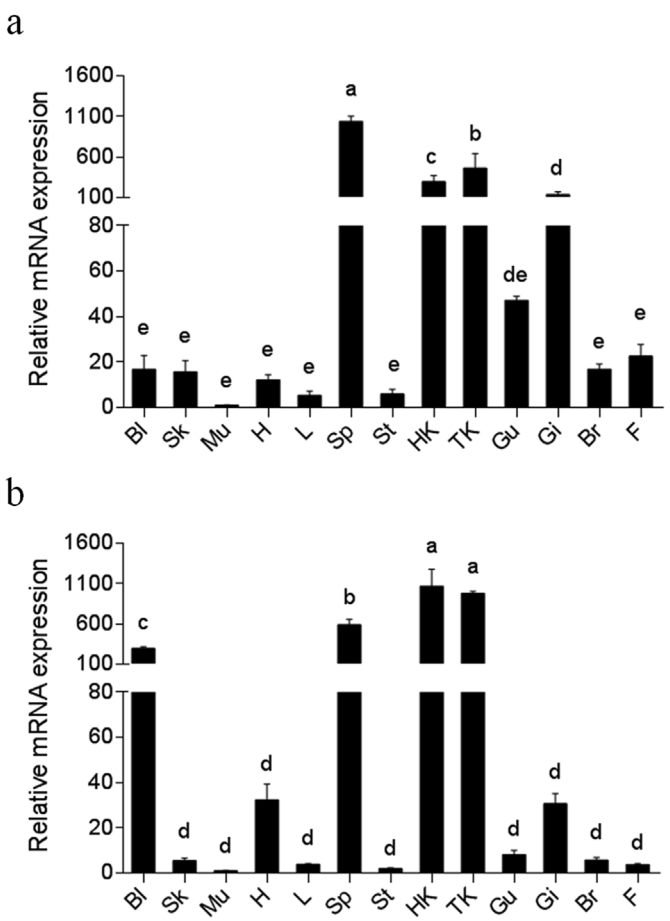


Fig. 4. The distribution patterns of *Pf*-IgM (a) and *Pf*-IgD (b) mRNA in different tissues of *P. fulvidraco*. Relative expression of *Pf*-IgM and *Pf*-IgD mRNA were detected in 13 tissues (n = 6). Bl: blood; Sk: skin; Mu: muscle; H: heart. L: liver; Sp: spleen; St: stomach; HK: head kidney; TK: trunk kidney; Gu: gut; Gi: gill; Br: brain; F: fin ray. **Different letters above bars represented significant difference at the levels of $P < 0.05$, and same letters above bars indicated no significant difference.**

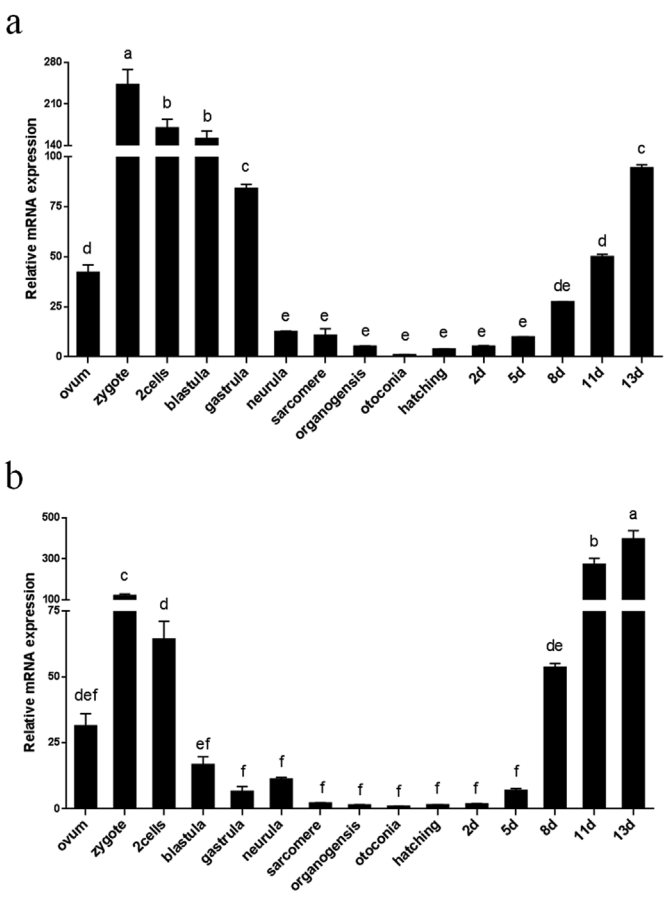


Fig. 5. The expresssion patterns of *Pf*-IgM (a) and *Pf*-IgD (b) mRNA in embryonic developmental stages of *P. fulvidraco*. Relative expression of *Pf*-IgM and *Pf*-IgD mRNA were detected in different embryonic developmental stages (n = 6). **Different letters above bars represented significant difference at the levels of $P < 0.05$, and same letters above bars indicated no significant difference.**

3.6. Expression of Pf-Igs post F. columnare G_4 infection

Expression levels of *Pf*-Igs in skin, gill, hindgut, liver, head kidney and spleen were examined after immersion with *F. columnare* G_4 (Fig. 7). In mucosal tissues including skin and hindgut, the expression of IgM was significantly up-regulated after *F. columnare* G_4 infection. Although the expression of IgD also showed significant differences in these tissues from 3 h post infection (hpi) to 28 dpi when compared with control group, however, the increased fold of IgD much lower than that of IgM. IgM expression was significantly increased at over 4 fold from 12 hpi to 14 dpi and peaked (~15.91-fold) at 4 dpi in skin. In hindgut, IgM showed over 5 fold higher expression at 4 dpi, 14 dpi and 28 dpi and peaked at 14 dpi (~13.47-fold). Another mucosal tissue, gill, showed less sensitive expression of IgM compared to other two mucosal tissues and only showed about 2 fold increased at 3 dpi and 4 dpi, compared to control.

In classical immune tissues including head kidney, spleen and liver, the expression levels of both IgM and IgD were significantly increased after *F. columnare* infection. Both IgM and IgD expression peaked in liver at 28 dpi (~14.25-fold and ~20.53-fold, respectively), although significant increased expression of IgD (~8.34-fold) has been found at 3 dpi. In spleen, the significant increased expression of IgM and IgD were mainly found from 3 dpi to 10 dpi and both peaked at 10 dpi (~5.44-fold and ~11-fold, respectively). In head kidney, IgM expression significantly increased with increasing infecting period and the highest value was detected at 28 dpi (~3.97-fold). However, IgD showed a trend to firstly increase and then decrease after infection and the highest expression levels of IgD was found at 4 dpi (~2.56-fold).

4. Discussion

Teleost fish are the most primitive bony vertebrates that contain immunoglobulins [20]. To date, three different Ig isotypes have been reported in teleost, i.e. IgM, IgD and, IgT/IgZ. Critically, IgT/IgZ acts as the most ancient reported immunoglobulin specialized in mucosal immunity, while IgM mainly functions in plasma of teleost in response to infection or vaccination [20]. Moreover, teleost IgT/IgZ could coat a multitude of bacteria on the mucosal surface [19,21], which is similar to mammalian IgA [36,37]. However, functions of IgD are always being debated. Especially in IgT/Z-absent teleost, which kinds of Ig take the role of IgT/Z in mucosal immunology is still not clearly so far. Currently, only two types of immunoglobulins IgM and IgD have been described in catfish. It's reported that catfish IgM, like teleost IgM in general, is a structural and functional homologue of mammalian IgM [13,32]. Interestingly, previous studies have shown that the expression of IgM and IgD heavy chain genes in liver, spleen and intestine of catfish was up-regulated shortly after vaccination with live Ich theronts by intraperitoneal injection [33,38]. However, the information is still limited that which kind of immunoglobulins in IgT/Z-absent teleost plays a key role in mucosal defense during bacterial infection. In the present study cloned the sequence of IgM and IgD heavy chain genes in *P. fulvidraco* and conducted multiple sequence analysis with reported

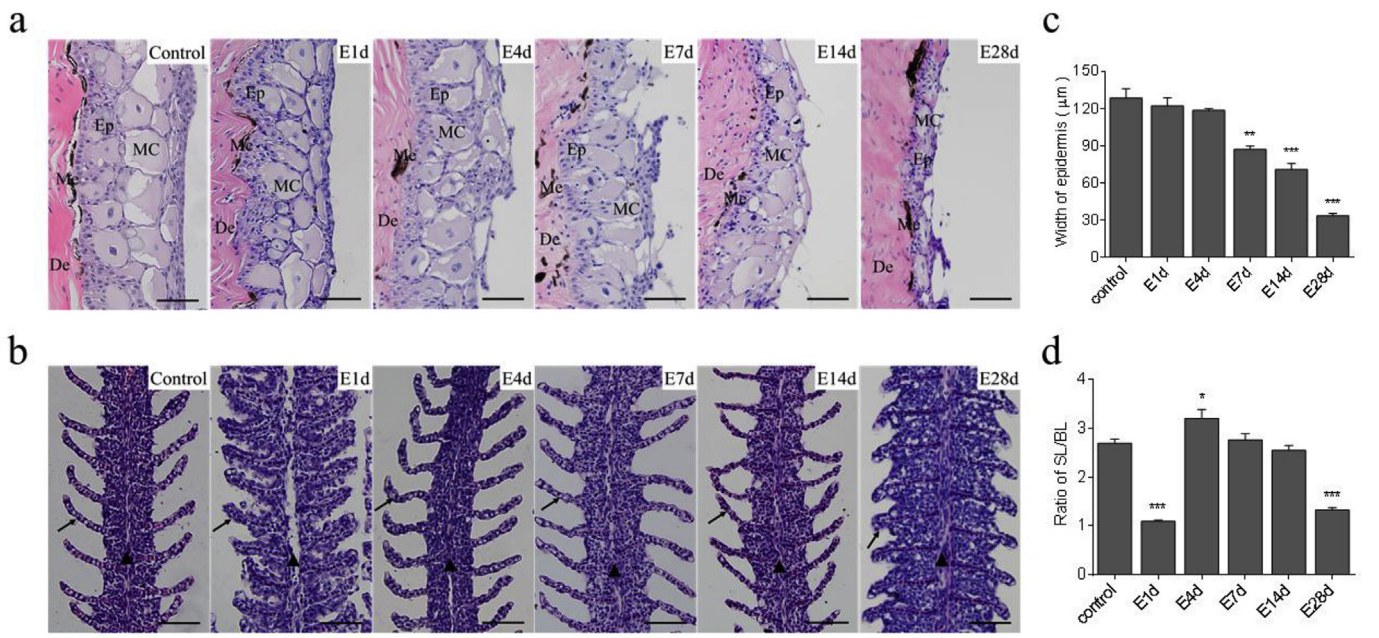


Fig. 6. Morphological changes in skin and gill of *P. fulvidraco*. Hematoxylin & eosin staining of yellow catfish skin (a) and gill (b) from 0, 1, 4, 7, 14 and 28 days infected fish, respectively. Epidermis (Ep); mucus cells (MC); Melanophores (Me); dermis (De); primary lamellae (PL) (filled arrowhead); secondary lamellae (SL) (long filled arrow). (c) The width of skin epidermis in control and infected fish (n = 6 per group). (d) The length ratio of gill SL vs basal lamina (BL) of fish in control and infected group (n = 6 per group). Scal bars, 50 μm $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (unpaired Student's t-test). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

IgM and IgD heavy chain sequences in other teleost fish. Then the expressions of these two immunoglobulins during *F. columnare* G_4 infection via immersion were examined, which will help to illustrate the separate function of IgM and IgD in mucosal and systemic immunity of *P. fulvidraco*.

Sequence analysis showed that both IgM and IgD heavy chain genes of *P. fulvidraco* shared a high degree of sequence similarity and phylogenetic relationship with the Igs heavy chains of *I. punctatus*, which might be due to the fact that they both belong to Siluriformes. In the constructed phylogenetic tree with NJ method, Igs heavy chains of *P. fulvidraco* and *I. punctatus* could be grouped together with fish of Cyprinidae, which might reveal the nearer relationship between *P. fulvidraco* and Cyprinidae fish. Gene structural analysis showed that IgM heavy chain of *P. fulvidraco* consisted of a signal peptide, a variable domain (VH), four Ig-like constant domains (CH1, CH2, CH3, and CH4), and a C-terminus. However, IgD heavy chain gene of *P. fulvidraco* contained a signal peptide constituted by 18 amino acids, followed by one VH, seven constant domains (CH1, CH2, CH3, CH4, CH5, CH6 and CH7), and a transmembrane region. Meanwhile, qRT-PCR was conducted to illustrate the expression patterns of *Pf*-IgM and *Pf*-IgD during ontogeny and relative expression levels in different tissues. Results showed that *Pf*-Igs were expressed in high quantity during zygote stage, low quantity during otoconia stage, while significantly increased during larva stage. The high levels of Igs in fertilized eggs have also been observed in other fish species [39], which could be due to maternal inheritance. In our study, expression levels of both IgM and IgD were significantly increased since 2 dph and much higher expressions were detected on the 13 dph, which were similar to previous results in rohu [40]. These results may indicate that larvae will have certain immune-competence at 2 weeks post hatching. Tissue-specific expression analysis indicated that *Pf*-Igs showed high expression levels in lymphoid organs, due to the abundant B cells content in these classical immune organs. Notably, relative high expression levels of IgM in gut and gill might signify its key role in mucosal responses, which was consistent with the findings in mandarin fish [41]. However, relative high expression of IgD was examined in blood, which was probably due to the large number of circulating B cells and also suggest the importance of *Pf*-IgD in systemic immunity.

Bacterial infection model of yellow catfish with ***F. columnare*** G_4 via immersion was then constructed to evaluate the immune functions of *Pf*-IgM and *Pf*-IgD in systemic and mucosal tissues during bacterial infection. Firstly, the morphological changes of yellow catfish mucosal tissues were examined by hematoxylin/eosin staining, and significant histo-pathological changes of skin and gill were found after bacterial infection, similar to earlier studies [42]. Skin epidermis was much thinner due to detachment after infection, while gill lamellae got thicken and many interlamellar spaces became obliterated, indicating the success infection of *F. columnare* on yellow catfish. Recent studies have reported the up-regulation of Igs mRNA expressions in several fish species during pathogenic infection [43–48]. In mandarin fish, IgM transcript significantly increased to 12-fold in gill while IgZ expression increased to 6.57-fold after *Flavobacterium columnare* stimulation [41]. Even under normal state, the expression of IgM was also higher than IgZ/IgT in mucosal organs of many fish species. For example, IgM transcripts were more abundant (up to 10 times) than IgT in mucosal tissues, including skin, gill and hind gut of Atlantic salmon [49], and the protein content of IgM was also significantly higher than IgT in gut and skin mucus of rainbow trout [19,20]. In gilthead sea bream, the up-regulation of IgM was more prominent in the intestine at 10 dpc (six fold), but the expression of IgT was almost unchanged by *Photobacterium damselae* subsp. *Piscicida* bathing [50]. These results indicated that the role of IgM in mucosal immunity should not be discarded. One study in turbot also showed that both the expressions of s-IgM and m-IgM in gut, skin and gill showed significant increase at 28 days post bath-vaccination with *Vibrio anguillarum* [43]. Several studies also compared the expression of IgM and IgD in mucosal tissues during bacterial infection. In ayu, IgM mRNA level was significantly increased in gill at 10 and 20 days post immersion with *Vibrio anguillarum*, while no significant changes of IgD expression was found in gill [51]. Studies in rohu indicated that IgD expression was down-regulated in skin and intestine, and was ~1.5 fold at 24 h post infection in gill then it gradually decreased with the advancement of time after infection with

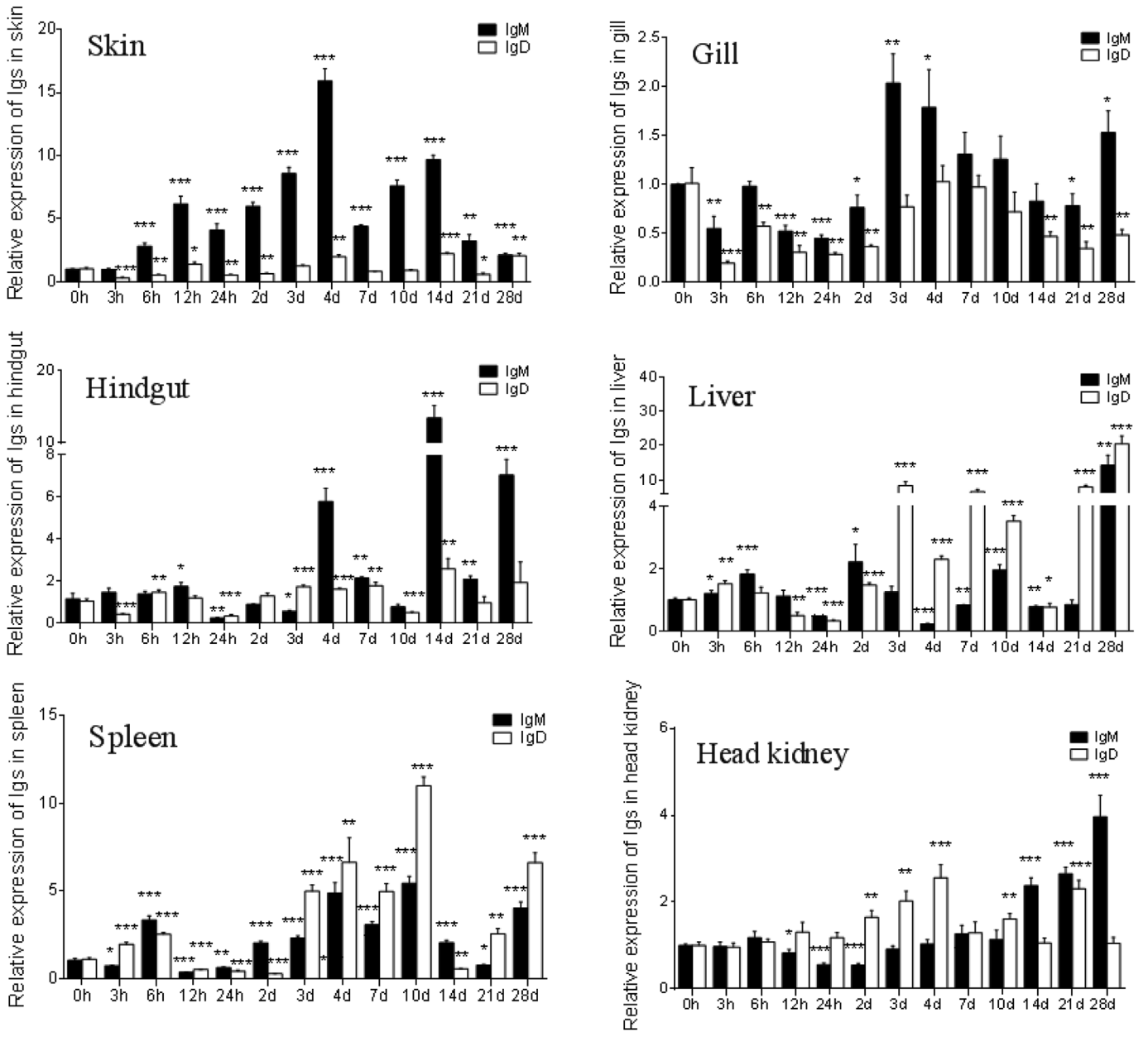


Fig. 7. The dynamic changes of *Pf*-Igs mRNA transcripts expression in different tissues (skin, gill, hindgut, liver, spleen, head kidney) were determined by qRT-PCR after bath immersion with *F. columnare* G_4 (1.0×10^6 CFU/ml for 3 h). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired Student's t-test). Data are representative of six independent experiments (mean ± SD).

Aeromonas hydrophila [40]. These results suggested that IgD might not perform the main roles in mucosal tissues of fish. Our results showed elevated expression of IgM in hindgut at 14 dpi (~13.47-fold) and in skin at 4 dpi (~15.91-fold) during infection with *F. columnare* G_4 in yellow catfish, while the expression of IgD did not show any significant increase in these tissues, which suggested that IgM might play the predominant role in mucosal immunity.

The expression of Igs in classical immune organs including kidney, spleen and liver were also checked in different fish species. Several studies indicated the up-regulation of IgD in these tissues after bacterial infection, for example, the expression of IgD was significantly up-regulated in kidney and liver after bacterial stimulation in rohu [40]. Similar results have also been found in blunt snout bream, as the pre-nominent increase of IgD expression was detected in liver, kidney, and spleen after *Aeromonas hydrophila* injection [45]. In Nile tilapia, IgD expression occurred predominately in spleen and kidney following *Streptococcus agalactiae* infection and reached its peak at 48 h and 72 h in the head kidney and spleen, respectively [52]. In *Catla catla*, IgD mRNA was expressed significantly more strongly in spleen (~25-fold) after administration of inactivated rhabdovirus [53]. More studies have also reported the up-regulation of IgM in kidney, spleen and liver after bacterial infection. In turbot, the transcriptions of both s-IgM and m-IgM in liver, spleen and kidney were significantly up-regulated post infection with *Vibrio anguillarum* via both injection and immersion [43]. In ayu, the IgM mRNA level was also significantly upregulated in trunk kidney after injection and immersion with *Vibrio anguillarum* [51]. In rohu, the expression level of IgM peaked in head kidney at 30 days post-infection with *Argulus siamensis* (3.57-fold) [54]. Our results showed similar trend of *Pf*-Igs during infection with *F. columnare* G_4 to above mentioned results. In this study, the expressions of *Pf*-IgD and *Pf*-IgM were significantly increased in liver, head kidney and spleen during *F. columnare* infection. However, the time points of *Pf*-Igs up-regulation

did not coincide. The up-regulation of *Pf*-IgD mRNA in liver, head kidney and spleen were mainly found at early time-points, while dramatic increased expression of *Pf*-IgM mainly occurred at 28 dpi in liver and head kidney. The expression patterns of IgM and IgD in spleen were similar. These results might suggest that IgM and IgD might work together to perform immune functions in immune organs including kidney, spleen and liver and further studies are needed to illustrate their interacting roles.

In conclusion, we characterized the molecular structure features of IgM and IgD heavy chain genes from *Pelteobagrus fulvidraco* at the same time for the first time. Moreover, the expression of *Pf*-IgM and *Pf*-IgD were detected during ontogeny and in different tissues. To explore the function of a model of bacterial infection with *F. columnare* G_4 in fish that closely mimics natural infection was established. Morphological analysis in yellow catfish skin and gill tissues showed changed after infection. Significantly higher expressions of *Pf*-IgD and *Pf*-IgM were detected in liver and spleen while skin and intestine showed significantly increased *Pf*-IgM expression in response to infection with *F. columnare* G_4. This observation opens the possibility that IgM may have had an alternative function in mucosal immunity of yellow catfish. Further investigations are needed to explore the immune responses of IgM and IgD especially in mucosal tissues under other infectious conditions (viruses and parasites).

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