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Partial characterization and tissue-specific expression profile of immunoglobulin M heavy chain gene in rohu, *Labeo rohita* (Hamilton, 1822)

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ABSTRACT

The present study was aimed to characterize the immunoglobulin M (IgM) heavy chain gene in rohu, *Labeo rohita,* and its tissue-specific expression. A 451 bp segment of IgM heavy chain gene of rohu spanning the variable region was amplified and sequenced. The partial nucleotide sequence of the rohu IgM heavy chain gene showed 91, 90 and 87% homology with IgM of *Cyprinus carpio, Danio rerio* and *Ctenopharyngodon idella* respectively. The deduced amino acid sequence of the partial rohu IgM heavy chain gene was 85, 82 and 79% identical to that of *C. carpio*, *C. idella* and *D. rerio*, respectively. Quantitative real-time PCR analysis of the IgM heavy chain gene expression in various tissues revealed that the highest expression was in the lymphoid tissues, namely kidney and spleen followed by heart, intestine, gill, liver and muscle. It was also observed that the IgM heavy chain gene expression increased with age, i.e. 6-month-old fish showed higher levels than 4-month-old and 2-month-old fishes.

Key words: Freshwater fish, IgM, Immunoglobulin, *Labeo rohita*

So far, 6 isotypes of immunoglobulins (Igs), the important mediators of humoral immune system, have been reported in teleost fish, namely, IgM, IgD, IgZ, IgT, IgM-IgD chimera and IgM-IgZ chimera (Wilson *et al.* 1997, Hordvik, 2002, Hirono *et al*. 2003, Danilova *et al*. 2005, Hansen *et al*. 2005, Savan *et al*. 2005). IgM is the major isotype of fish Igs. In bony fishes, immunoglobulin occurs as a tetramer (Lobb 1985, Kaattari *et al.* 1998). Intensive culture of Indian major carps (*Labeo rohita, Catla catla* and *Cirrhinus mrigala*) has led to outbreaks of several diseases. Annual losses due to diseases in carp aquaculture in Andhra Pradesh alone were estimated to be ₹ 40 million (Shankar *et al*. 2000). Better understanding of immune system and accurate and rapid identification of pathogens are keys to effective management of diseases in rohu. In rohu the study of IgM is mainly on preliminary analysis of serum IgM, its isolation and immunological characterization (Rathore *et al*. 2008, Suresh Babu *et al*. 2008, Bag *et al*. 2009). The Ig of rohu is presumed to be IgM based on molecular weight of whole molecule and light and heavy chains. But for a small sequence of 78 bp of rohu IgM heavy chain gene, sequence information of rohu IgM is lacking. Work on other teleost fishes showed that kidney, spleen and intestines are the major lymphoid organs, information on the distribution of lymphoid tissue in rohu and its maturation is not available. In this background, the objective of the present investigation was to generate sequence information of IgM heavy chain gene of rohu and to elucidate tissue-specific expression of the gene.

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MATERIALS AND METHODS

Animals: The rohu fingerlings aged about 2 months (6–10 g body weight), 4 months (40–50 g) and 6 months (100–120 g) were procured from a commercial fish farm in Gujarat and maintained in the wet lab of the Central Institute of Fisheries Education, Mumbai. The fishes were maintained in a 500 litre fiberglass tank with continuous aeration and 20% water exchange daily. The water temperature varied between 25° and 30°C throughout the experiment. The fish were fed once a day with commercial fish feed pellets.

Isolation of total RNA and reverse transcription: The fishes were anaesthetized using clove oil (100 µl in 2 litre of water), and the kidney tissue was collected aseptically and processed immediately for total RNA isolation. Total RNA was isolated from 30 mg of kidney tissue using RNeasy mini kit following the manufacturer's protocol. The total RNA obtained was quantified using Nanodrop 2000 and cDNA was synthesized using cDNA synthesis kit following the manufacturer's protocol. The reverse transcription reaction was carried out in 20 µl volume containing 4 µl of RNA

(equivalent to 4 µg) and 1 µl oligo-dT primer, 4 µl reaction buffer (5X), 1 µl riboLock RNase inhibitor (20 u/µl), 2 µl dNTP mix (10 mM) and 2 µl M-MuLV reverse transcriptase (20 u/µl). The solution was mixed thoroughly, spun and incubated at 37°C for 1 h. The reaction was terminated by heating at 70°C for 5 min. The resulting cDNA was used immediately for PCR and the remaining cDNA was stored at –20°C.

Polymerase chain reaction (PCR): A set of forward and degenerate reverse primers were designed for the amplification of IgM variable heavy chain gene of rohu based on the conserved IgM gene sequence from *Cyprinus carpio* (Accession no. AB194134), *L. rohita* (Accession no. EU552488, 78 bp) and *Ctenopharyngodon idella* (Accession no. DQ417927). The primer sequences designed were IgMF1 5'- ACTGGGTTTTATCTTCATGTTCGCT and IgMR1 5'- ACCTCTTGMCAHGCAGCCGA. ß-actin primers previously reported for common carp (Mohanty and Sahoo 2008) were used for amplifying the house keeping gene. A 25 µl PCR mix consisting of 12.5 µl 2x master mix (Taq polymerase 0.05 units/µl, 4 mM $MgCl_2$ and dNTPs 0.4 mM each), 25 picomoles of forward (IgM F1) and reverse (IgM R1) primers and 1 µl of template (cDNA) was amplified using a thermal profile of 95°C for 4 min followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min in a thermal cycler. The PCR products were analyzed in 2% agarose gel stained with ethidium bromide.

Cloning and sequencing of the IgM heavy chain fragment: The PCR product in the agarose gel was eluted using a kit following the manufacturer's protocol. The purified PCR fragments were ligated into pTZ57R/T vector using InsTAclone PCR cloning kit following the manufacture's protocol and transformed into DH5á strain of *Escherichia coli* competent cells. The plasmid DNA was isolated using purification spin kit and sequenced using M13F and M13R primers in ABI 3730xl DNA analyzer.

Sequence analysis: The sequence was analyzed using BLAST and multiple sequence alignment with other related IgM sequences of fish was done using ClustalW program and DNASTAR software.

Expression analysis of IgM heavy chain gene in different tissues: Tissues collected from 3 healthy fish (body weight, 40 g) were used to study the normal expression profile of IgM. Tissues such as kidney, spleen, liver, gill, intestine, muscle and heart, collected from individual fish were processed immediately for RNA extraction. Uniform quantity of RNA was reverse transcribed into cDNA as described above. IgM-specific primers were used for the PCR amplification. ß-actin was used as house-keeping gene. The reaction mixture consisted of 12.5 µl of 2X master mix, 10.5 µl of nuclease-free water, forward and reverse primer 0.5 µl each (25 pM) and 1 µl of cDNA. The PCR was carried out on a thermal cycler with the cycling condition of 95°C for 4 min followed by 28 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 1 min and a final extension at 72°C for 10 min. The amplified products were analyzed on 2% agarose gel.

Similarly, quantitative real-time PCR was carried out using cDNA prepared from all the tissues. The PCR reaction mixture consisted of 7.5 µl of SYBR Green qPCR master mix, primers (IgM F1 and IgM R1), 0.25 µl (25 pM) each, 1 µl of cDNA and 6 µl of nuclease free water in triplicates. Real-time PCR was carried out on an ABI 7500 Real time PCR system. The thermal cycling profile used for PCR amplification was 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 60°C for 1 min. The threshold cycle (Ct) value was determined using the automatic setting on the ABI 7500 real-time PCR system. The difference in the Ct value between IgM heavy chain gene and the corresponding internal control, ß-actin gene, ΔCt ($Ct_{IgM} - Ct_{actin}$), were calculated. The relative expression level of target gene to ß-actin was derived using the equation $2^{-\Delta Ct}$ (Su *et al*. 2008).

Expression analysis of IgM heavy chain gene in kidney of different age groups of rohu: Three different age groups of apparently healthy rohu, viz. 2-month-old (6–10 g body weight), 4-month-old (40–50 g) and 6-month-old (100–120 g) were selected to study the IgM expression with relation to age. IgM heavy chain gene expression was determined in the kidney tissue collected and pooled from three animals of each age group.

Statistical analysis: All real-time PCR assays were performed in triplicate for each sample and the mean±SE value was calculated. The mean values were compared by one-way ANOVA followed by Duncan's multiple range test to determine significant difference at 5% ($P < 0.05$) level using SPSS 16.0 software (SPSS Inc.).

RESULTS AND DISCUSSION

PCR amplification and sequencing of IgM heavy chain gene fragment: In this study, the cDNA sequence encoding IgM heavy chain gene in rohu was amplified from total RNA isolated from kidney. Kidney tissue was earlier reported to be the major site of expression of IgM in fishes such as common carp (Savan *et al*. 2005), European eel (Feng *et al*. 2009), Japanese flounder (Hirono *et al*. 2003) and large yellow croaker (Tian *et al*. 2009). RT-PCR amplification of rohu kidney RNA showed 488 amplicon on agaorose gel as product (Fig. 1) and got it sequenced. The chromatogram has a clear read of up to 451 bp and this partial sequence up on BLAST revealed maximum homology (91%) with *C. carpio* Ig gene sequence, followed by *D. rerio* (90%), *C. idella* (87%), and *Ictalurus punctatus* (78%). In BLAST homology search the rohu partial IgM heavy chain gene sequence showed high homologies with the sequences from fishes belonging to Family Cyprinidae. This corresponds with the reports where high degree of conservation was observed among members of this family (Ishikawa *et al*. 2004). Multiple sequence alignment of rohu IgM heavy chain gene

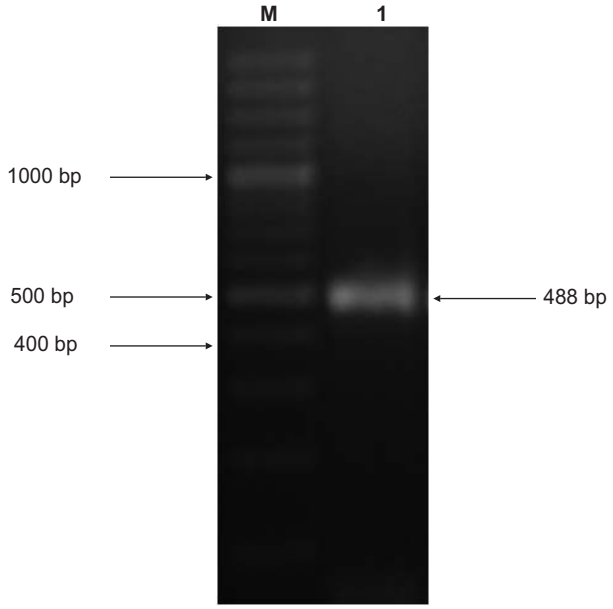

Fig. 1. Rohu partial IgM heavy chain gene (RT-PCR product) amplified with IgM F1 and IgM R1 primers. M: 100 bp molecular weight marker; Lane 1. RT-PCR product (488 bp).

with that of *C. idella* (DQ417927), *C. carpio* (AB194134), and *D. rerio* (AF273883) is shown in Fig. 2. The sequence of rohu IgM was submitted to the GenBank (Accession No. HM581639).

The multiple sequence alignment of the deduced aminoacid sequence of *L. rohita* with that of *C. carpio, C. idella* and *D. rerio* showed 85, 82 and 79% identity respectively (Fig. 3).

Similar homologies with members of family Cyprinidae were seen for the deduced amino acid sequence (Nakao *et al*. 1998, Savan *et al*. 2005, Cheng *et al*. 2006). Alignment of rohu IgM heavy chain with Ig variable regions available in PDB database revealed that the rohu IgM sequence is of the variable region spanning the FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4 motifs. The aminoacid sequence was analyzed in comparison with AHo numbering scheme (Honegger and Pluckthun 2001). The cys23 and cys106 which form the intra-domain disulphide bond is conserved in rohu as in most other species secreting Ig. The highly conserved motif 'YYCAR' in FR3 and the tryptophan residue at position 43 of heavy chain (Kabat *et al*. 1991, Danilova *et al*. 2000) was also found to be conserved in rohu.

Expression analysis of IgM heavy chain gene in different

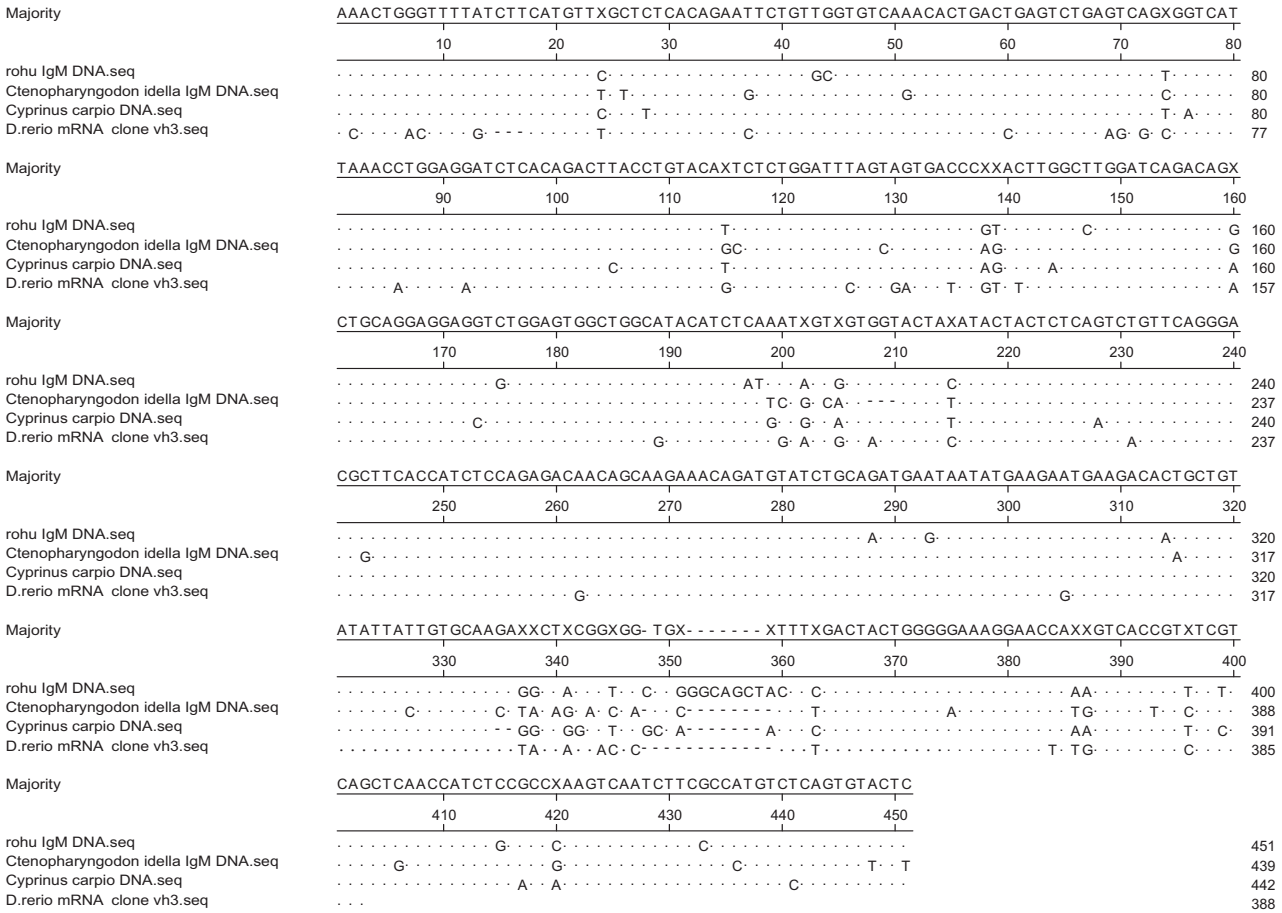

Fig. 2. Multiple sequence alignment of partial IgM heavy chain gene of rohu with *Ctenopharyngodon idella* (DQ417927), *Cyprinus carpio* (AB194134) and *Danio rerio* (AF273883). Consensus sequences are denoted by dots and gaps by dashes.

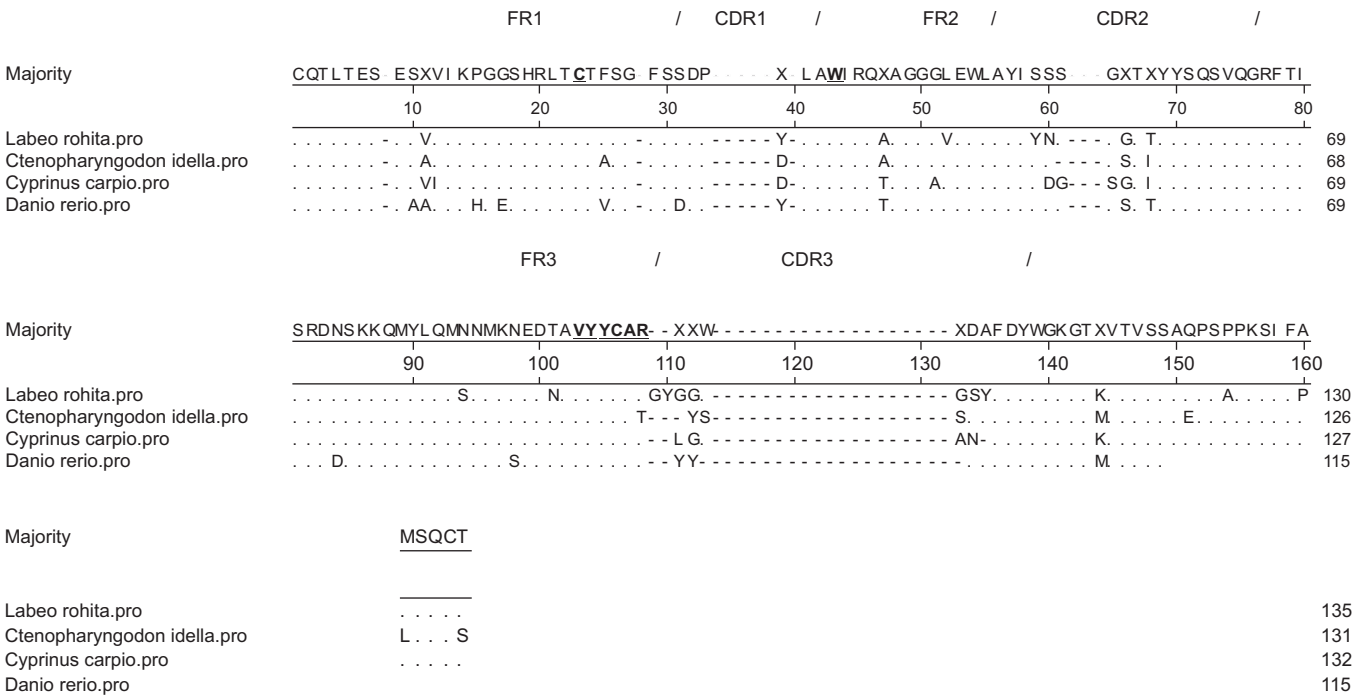

Fig. 3. Multiple alignment of the deduced rohu IgM heavy chain amino acid sequence with that of other species as in figure 2. Consensus aminoacids are denoted by dots and gaps introduced manually to match AHo numbering are denoted by dashes. The highly conserved amino acids are in bold and underlined.

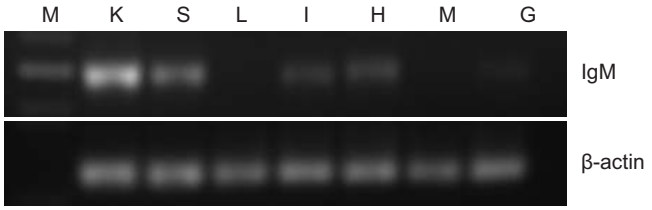

Fig. 4. Expression of IgM heavy chain gene and ß-actin gene in different tissues of rohu. M: 100 bp Molecular weight marker; K, kidney; S, spleen; L, liver; I, intestine; H, heart; M, muscle; G,gill.

tissues of rohu: In semi-quantitative PCR, higher expression of IgM heavy chain mRNA was observed in kidney, followed by spleen, heart and intestine. Difference in expression levels between liver and muscle could not be distinguished by gel electrophoresis. However, gill showed relatively higher levels of expression when compared to liver and muscle (Fig. 4).

Real-time PCR assay was used to confirm the level of IgM heavy chain gene expression observed in different tissues. The expression of IgM heavy chain gene was found to be relatively higher in kidney, followed by spleen, heart, intestine, gill, liver and muscle (Fig. 5). Among these tissues, kidney exhibited the highest expression and muscle showed the lowest.

These results indicated that the lymphoid tissues such as kidney, spleen and intestine are the major sites of B-lymphocyte maturation in rohu, as in other teleosts such as fugu (Saha *et al*. 2004), large yellow croaker (Tian *et al*. 2009), Japanese flounder (Hirono *et al*. 2003) and European

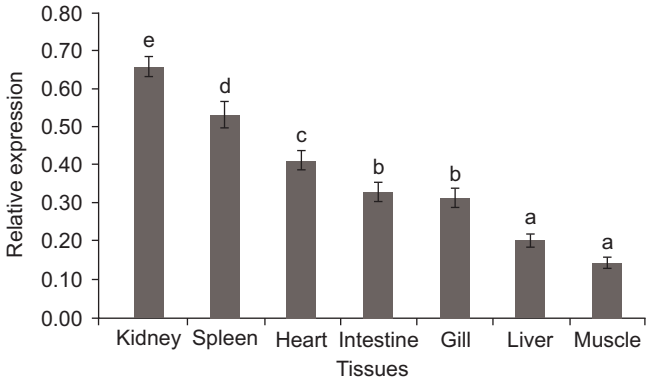

Fig. 5. Relative IgM heavy chain gene expression in different tissues of rohu. Data are presented as mean±SE. Significant differences between tissues are indicated by different letters (a, b, c, d, e) over the bars.

eel (Feng *et al*. 2009). However a comparatively higher level of IgM expression was observed in heart tissue of rohu than in Japanese flounder (Hirono *et al*. 2003) and European eel (Feng *et al*. 2009).

Expression analysis of IgM heavy chain gene in kidney of different age groups of rohu: The IgM heavy chain gene in kidney of 6-month-old fish showed relatively higher expression followed by 4-month-old and 2-month-old fishes in semi-quantitative PCR as well as in real-time PCR (Figs 6, 7).

The increase in expression of IgM heavy chain gene with the age of the fish as observed in this study was also reported

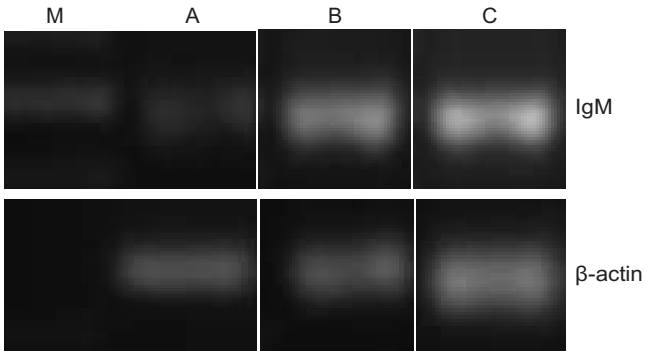

Fig. 6. Expression of IgM heavy chain gene and ß-actin gene in kidney of different age groups of rohu. M, 100 bp molecular weight marker; A, 2-month-old fish; B,4-month-old fish; C, 6-month-old fish.

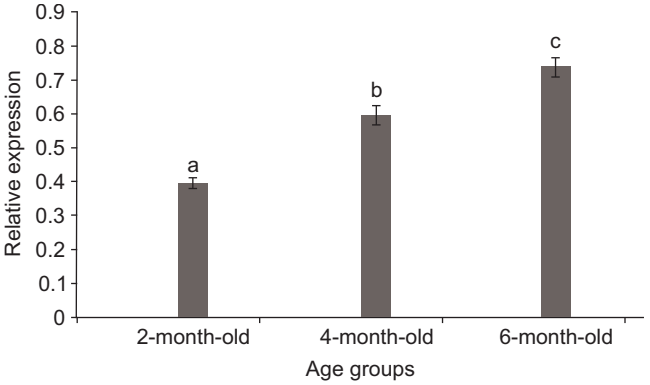

Fig. 7. Relative IgM heavy chain gene expression in kidney of different age groups of rohu. Data are presented as mean±SE. Significant differences between different age groups are indicated by different letters (a, b, c) over the bars.

in common carp where the natural antibodies increased with age (Kachamakova *et al.* 2006). This could be either due to increase in exposure to antigens with increase in age or due to the increased maturity of the immune system or both.

The present study confirmed that the sequence of IgM heavy chain and the sites of its expression are highly conserved within the Family Cyprinidae. Also the IgM heavy chain gene expression increased with increase in age of fish. However, a full length transcript sequence could shed further light on the nature of other domains. These results could be useful to further characterize the IgM heavy chain gene and its function in rohu and other related species.

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