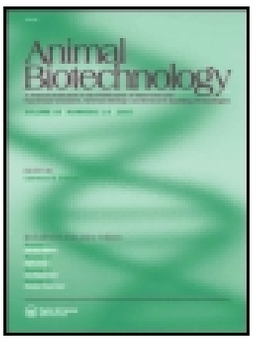

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Molecular characterization and expression analysis of secretory immunoglobulin M (IgM) heavy chain gene in rohu, *Labeo rohita*

K. Saravanan[a,b], K. V. Rajendran[a], P. Gireesh-Babu[a], C. S. Purushothaman[a,c], and M. Makesh[a,d]

[a]ICAR-Central Institute of Fisheries Education, Mumbai, Maharashtra, India; [b]ICAR-Central Island Agricultural Research Institute, Port Blair, Andaman and Nicobar Islands, India; [c]ICAR-Central Marine Fisheries Research Institute, Kochi, Kerala, India; [d]ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India

ABSTRACT

Immunoglobulin M (IgM) is the major isotype among teleost immunoglobulins. The present study was aimed to explore IgM heavy chain gene and its expression profile in rohu. Full-length IgM heavy chain cDNA of rohu consisted of 1994 bp encoding a polypeptide of 576 amino acid residues including a leader peptide, variable (VH) and constant (CH1–CH2–CH3–CH4) domains confirming the secretory form of IgM. The sequence carries conserved residues such as cysteine, tryptophan and amino acid motifs like 'YYCAR' and 'FDYWGKGT–VTV–S'. The predicted 3 D model confirmed various domains of rohu IgM heavy chain. Phylogenetic tree analysis revealed that IgM heavy chain gene of rohu shared the same cluster with that of other cyprinid fishes. Tissue distribution analysis showed the predominant level of IgM heavy chain gene expression in kidney, spleen and intestine. IgM heavy chain gene expression in rohu kidney was found to be up-regulated and reached a maximum at 7 days post-challenge with *Aeromonas hydrophila*. These findings demonstrate the first report of full-length secretory IgM heavy chain gene in rohu. Besides, IgM heavy chain gene was highly expressed in major lymphoid tissues and bacterial challenge influenced its expression which further confirmed its role in the adaptive humoral immune response.

KEYWORDS

IgM heavy chain; adaptive immunity; gene expression; *Labeo rohita*; Aeromonas hydrophila

Introduction

The adaptive immune system of teleost is a well specialized and regulated process. The major humoral component of the adaptive immune system belongs to immunoglobulins (Igs). Ig comprised of two heavy and light chains each and encoded by variable and constant domains which are stabilized by disulfide bridges. Different isotypes of Ig are determined by the constant domains of the heavy chain.[1] Three major isotypes of immunoglobulins have been reported in teleosts, namely IgM,[2,3] IgD[4–6] and IgZ or IgT,[7,8] among which the major isotype belongs to IgM. Further, IgM is involved in systemic and mucosal immunity of host against disease-causing organisms,[9] but IgT and IgD are involved in mucosal protection.[10,11]

Teleost IgM exists as a tetramer with eight antigen binding sites.[12] IgM heavy chain molecules present in two different forms, namely, secretory IgM (sIgM) and membrane IgM (mIgM).[13,14] The heavy chain of sIgM consists of one variable region and four constant domains, whereas mIgM comprises of one variable region, three constant domains and two additional transmembrane domains such as TM1 and TM2.[13,15] IgM heavy chain genes were characterized from various fish species such as *Danio rerio*,[16] *Siniperca chuatsi*,[17] *Paralichthys olivaceus*,[18] *Anguilla anguilla* [19] and *Megalobrama amblycephala*.[3]

Considerable information is available on the adaptive immune genes, especially immunoglobulins of various fishes but the information on structural and functional properties of immunoglobulin genes in rohu and other Indian Major Carps (IMCs) is lacking. Knowledge on the adaptive immune system including structural and genetic organization of immunoglobulins is of potential importance to devise effective prophylactic measures to manage diseases in cultured fish. Till date, the research on IgM of rohu has been confined to serum IgM isolation, partial characterization and few aspects on immunological characterization.[20–23] With this background, here we have characterized the

CONTACT M. Makesh mmakesh@ciba.res.in ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India
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Table 1. List of primers used in this study.

Primer	Sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)
Primers for amplifying partial IgM heavy chain cDNA			
RoHCF1	GGACGCTTCACCATCTCCAG	48	600
RoHCR1	ATCATGAAACCACTTAAACG		
RoHCF2	AGCAACCTTCATGTGTGTAGCCC	52	683
RoHCR2	CAAGCCAAGACACAAACACCTC		
IgM specific primers for 5′ RACE			
Oligo (dG)	GGGGGGIGGGIIGGGIIG		
5′RACEIR	GTTCCTTTCCCCCAGTAGTCG	51	
5′RACEIIR	GCGTCCCTGAACAGACTGAGAG	54	319
IgM specific primers for 3′ RACE			
Adapter oligo (dT)	GGCCACGCGTCGACTAGTAC(dT)17		
Adapter	GGCCACGCGTCGACTAGTAC		
3′RACEIF	GAAGAATGGAGCAACGGCACTG	53	
3′RACEIIF	AGAGGTGTTTGTGTCTTGGCTTG	54	508
IgM specific primers for amplifying ORF			
ORFF	GTCTGTGACTTTGATGTATCAGC	54	1790
ORFR	GACACCACATACACAACGCT		
Primers for real-time PCR			
IgMqRTF	CAGGGACGCTTCACCATCTC	51	149
IgMqRTR	GTTCCTTTCCCCCAGTAGTCG		
β-actinqRTF	GACTTCGAGCAGGAGATGG	55.3	138
β-actinqRTR	CAAGAAGGATGGCTGGAACA		

full-length IgM heavy chain gene of rohu. Further, tissue distribution analysis of IgM heavy chain gene expression in various tissues of healthy rohu and fishes challenged with bacteria was also analyzed.

Materials and methods

Animals

Healthy rohu fingerlings weighing 30–40 g were procured from a commercial fish farm near Mumbai, India and acclimatized in plastic tanks (500 liter capacity) for one month under disease-free condition. The fish were fed once a day with commercial fish feed pellets. After acclimatization, the fishes were euthanised using an overdose of MS-222 (Tricaine methanesulfonate, Sigma-Aldrich, Saint Louis, MO, USA) and the tissues were collected aseptically for total RNA isolation.

Isolation of total RNA and reverse transcription

Total RNA was isolated from rohu kidney for the characterization of full-length IgM heavy chain cDNA and from various tissues for tissue distribution analysis using TRIzol®) as per the manufacturer's protocol. The total RNA obtained was quantified using Nanodrop 2000 (Thermo Scientific, Carlsbad, CA, USA). To remove genomic DNA contamination, the quantified RNA was treated with DNase I (Thermo Scientific, USA). Reverse transcription of DNase treated RNA was performed using the First-strand cDNA synthesis kit (Thermo Scientific, USA) following the manufacturer's instructions.

Amplification of full-length IgM heavy chain cDNA

Primers were designed on the basis of conserved IgM heavy chain gene sequences from the closely related species using DNASTAR® software (DNASTAR Inc., Madison, WI, USA). Real-time PCR primers were designed based on the sequence obtained from our earlier study.[23] *β*-Actin primers previously reported for rohu[24] was used to amplify the reference gene (Table 1).

PCR reaction was performed with gene-specific primers to obtain a partial sequence of IgM heavy chain gene using thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture consisted of 12.5 µl of 2× master mix (Taq polymerase 0.05 U/µl, 4 mM $MgCl_2$ and 0.4 mM each dNTPs), 10.5 µl of nuclease-free water, forward and reverse primers of 0.5 µl each (25 p mole) and 1 µl of cDNA. The PCR thermal profile was 95 °C for 4 mins, 35 cycles of 95 °C for 30 s, appropriate annealing temperatures for 30 s and 72 °C for 2 mins and finally 72 °C for 10 mins. The obtained PCR products were resolved by agarose gel electrophoresis and then cloned into pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Scientific, USA) and sequenced at Bioserve Biotechnologies Pvt. Ltd., India using ABI 3730xl DNA analyzer. The partial IgM heavy chain gene sequence was used to design primers for 5′ and 3′ Rapid Amplification of cDNA Ends (RACE) PCR.[25]

For 5′ RACE, 2 µg of total RNA was reverse transcribed using oligo dT primer as mentioned earlier. MinElute PCR purification kit (Qiagen, USA) was used to purify the synthesized cDNA. Homopolymeric C-tailing was added at the 3′ end of purified first strand cDNA using recombinant terminal transferase

enzyme and dCTP (Thermo Scientific, USA). The dC-tailed cDNA was used for the first step PCR with oligo dG and gene-specific (5′RACEIR) primers. Further, 2 µl of the first step PCR product was used as a template for nested PCR along with forward primer (Oligo (dG)) and reverse primer (5′RACEIIR) as mentioned earlier. Likewise, in 3′ RACE, 2 µg of total RNA was used for synthesizing first strand cDNA with oligo dT-adapter primer as mentioned earlier. First step PCR was performed using adapter oligo (dT) primed first strand cDNA as a template along with forward primer (3′RACEIF) and reverse (adapter) primer. As mentioned earlier, nested PCR was also performed using forward nested primer (3′RACEIIF) and reverse (adapter) primer. PCR was also performed to amplify the ORF region of IgM heavy chain gene of rohu using a forward primer (ORFF) and reverse primer (ORFR) as mentioned earlier. After confirmation on agarose gel electrophoresis, the amplified products were cloned using InsTAclone PCR cloning kit (Thermo Scientific, USA) and then sequenced.

Sequence analysis

Basic Local Alignment Search Tool (NCBI; http://www.ncbi.nlm.nih.gov/blast/) was used to find similarity of the obtained IgM heavy chain gene sequence of rohu with other sequences. The amino acid sequence was predicted using a translator program at open reading frame finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). UniProt (http://www.uniprot.org/) and SMART (http://smart.embl-heidelberg.de) databases were used to identify the protein domains. Functional domains of IgM heavy chain was identified on the basis of sequence comparisons with zebrafish IgM heavy chain gene in the IMGT reference directory using IMGT/DomainGapAlign tool (http://www.imgt.org/3Dstructure-DB/cgi/DomainGapAlign.cgi). NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) was used to predict N-linked glycosylation sites. Multiple sequence alignment was performed using Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Neighbor-joining tree was prepared using MEGA6 software, (University Park, PA)[26] employing pairwise deletion and Poisson correction method.[27] SWISS-MODEL (http://swissmodel.expasy.org/) was employed to predict the 3 D model of rohu IgM heavy chain gene. The predicted 3 D model was validated using the Ramachandran plot in the RAMPAGE server (http://www.mordred.bioc.cam.ac.uk/*rapper/rampage.php).

Quantitative real-time PCR

Various tissues such as kidney, intestine, liver, gill, spleen, and skin were collected from apparently healthy rohu (30–40 g body weight) to study the expression of IgM heavy chain gene. All the tissue samples were collected in triplicate and each replicate contained the tissue pooled from three healthy fishes. For tissue harvesting, the fishes were euthanised using an overdose of MS-222 (Tricaine methanesulfonate) (Sigma, USA) and the tissues were collected aseptically for total RNA isolation. Uniform quantity of RNA was reverse transcribed using oligo d(T)18 primer as mentioned earlier. Quantitative real-time PCR was performed using the ABI 7500 Real-time PCR System (Applied Biosystems, USA). The PCR reaction mixture consisted of 12.5 µl of Maxima^{TM} SYBR Green qPCR Master Mix (Thermo Scientific, USA), 10.5 µl of nuclease-free water, forward and reverse primers of 0.5 µl each (25 p mole) and 1 µl of cDNA (equivalent to 20 ng). The thermal cycling profile for PCR amplification consists of 95 °C for 10 mins, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The threshold cycle (Ct) value was determined using the automatic setting on the ABI 7500 real-time PCR system. Relative expression of IgM heavy chain gene in relation to ß-actin was derived using $2^{-\Delta Ct}$ method.[28]

Rohu fishes were injected with *Aeromonas hydrophila* intraperitoneally to study the IgM heavy chain gene expression in response to bacterial infection. The bacteria were cultured using brain heart infusion broth (Himedia, Mumbai, India), washed and resuspended in phosphate-buffered saline (pH 7.4). A total of 54 numbers of healthy fish (30–40 g body weight) were challenged intraperitoneally with 10^7 CFU of *A. hydrophila* fish^{-1} in 100 µl volume. Kidney tissue was collected aseptically from nine fishes each at 1, 3, 7, 10 and 14 days post-challenge (dpc). Samples from nine uninfected fishes injected with 100 µl of PBS fish^{-1} were used as control. Total RNA was isolated and synthesized into cDNA and then quantitative real-time PCR was carried out. The transcriptional level of IgM heavy chain gene was normalized to β-actin and calibrated with the control sample to obtain the fold change in expression according to $2^{-\Delta\Delta CT}$ method.[29]

Statistical analysis

All the real-time PCR assays were carried out in triplicate and the data were expressed as the mean ± standard error. Statistical analysis was executed using SPSS 16.0 software (SPSS Inc., Armonk, NY, USA).

One-way ANOVA and Duncan's multiple range test was employed to establish a significant difference among the mean and p values <0.05 regarded to be significant.

Results

Characterization of full-length IgM heavy chain cDNA of rohu

The present study revealed 1994 bp full-length IgM heavy chain cDNA of rohu comprising 5′-untranslated region (5′-UTR, 55 bp), 3′-untranslated region (3′-UTR, 208 bp) and an open reading frame (ORF) of 1731 bp which encodes a polypeptide of 576 amino acid residues which is further divided into leader peptide, variable domain including framework regions (FRs) and complementarity-determining regions (CDRs) and CH domains (Figure 1). Nucleotide sequence of IgM heavy chain cDNA of rohu revealed the sequence identity of 83 and 80% with *Ctenopharyngodon idella* and *M. amblycephala,* respectively. Likewise, the amino acid sequence of IgM heavy chain gene of rohu showed 70 and 60% similarity with *C. idella* and *M. amblycephala,* respectively. Full-length IgM heavy chain cDNA sequence of rohu was submitted to NCBI GenBank under the accession no. KX268324.

The deduced amino acid sequence of IgM heavy chain gene of rohu comprised a leader peptide, variable region (V) and four constant regions (CH1–CH4). Variable region also contains FRs and CDRs (Figures 1 and 2). Amino acid sequence analysis of IgM heavy chain of rohu revealed the presence of conserved cysteine (Cys) residues and motifs. IgM heavy chain of rohu contained three conserved cysteine residues each in variable (Cys^{23}, Cys^{44}, Cys^{117}), CH1 (Cys^{153}, Cys^{165}, Cys^{224}), CH3 (Cys^{349}, Cys^{375}, Cys^{430}) and CH4 (Cys^{476}, Cys^{536}, Cys^{574}) domains, except CH2 domain which contained two cysteine residues (Cys^{269}, Cys^{330}). A total of seven numbers of N-linked glycosylation sites i.e. one in VH, two each in CH2, CH3 and CH4 were located in the coding region of IgM heavy chain of rohu. The highly conserved motifs; 'YYCAR' and 'FDYWGKGT–VTV–S' were found in rohu IgM heavy chain. Besides, putative polyadenylation signal, 'AATAAA' was found 13 bp upstream of polyA tail (Figure 1).

Sequence analysis of rohu IgM heavy chain cDNA

Deduced amino acid sequence alignment of rohu IgM heavy chain with related species showed that cysteine residues (shadowed) are completely conserved and involved in disulfide bond formation (Figure 3). The tryptophan residues (boxed) are also conserved and responsible for the formation and stabilization of the tertiary structure of the protein. The structural features of rohu VH gene were essentially same as it found in other vertebrates (Figure 3), having a leader peptide followed by four FRs and three CDRs between FRs; the 'YYCAR' block and 'FDYWGKGT–VTV–S'. Further, 28 amino acid residues in the variable region and 59 residues in the constant region were found to be conserved (Figure 3).

Phylogenetic tree analysis revealed that IgM heavy chain gene of *L. rohita* is closely related to those of *C. idella, M. amblycephala* and *D. rerio*, which were different from the cluster associated with *Lutjanus sanguineus* (Figure 4). The 3 D model of rohu IgM heavy chain is comprised of a variable domain (VH) and four constant domains such as CH1, CH2, CH3 and CH4 (Figure 5). The predicted 3 D model was further validated using Ramachandran plot (Figure 6).

IgM heavy chain gene expression profile in rohu

IgM heavy chain gene expression was observed in all the tissues taken for study with higher level of expression in kidney, followed by spleen, intestine, gill, skin and liver (Figure 7). The relative expression of IgM heavy chain gene in rohu kidney against *A. hydrophila* infection showed up-regulation, which gradually increased from initial time period and attained maximum (22.35 fold) at 7 days post-challenge (dpc) (Figure 8).

Discussion

The present study identified 1994 bp of full-length IgM heavy chain cDNA in rohu comprising 55 bp of 5′-untranslated region (5′-UTR, 55 bp), 208 bp of 3′-untranslated region (3′-UTR, 208 bp) and 1731 bp of an open reading frame (ORF, 1731 bp) which encodes a polypeptide of 576 amino acid residues. Xia et al.[3] reported 1961 bp of full-length IgM heavy chain cDNA in blunt snout bream which comprises 49 bp of 5′-UTR, 202 bp of 3′-UTR and an ORF of 1710 bp encodes for a polypeptide of 569 amino acid residues. Likewise, full-length IgM heavy chain cDNA of grass carp comprised of 1940 bp which encodes for 576 amino acids.[30] This corresponds with the earlier study that IgM heavy chain of teleost exhibits variation in gene number, sequence diversity and genetic organization of putative elements.[14,31]

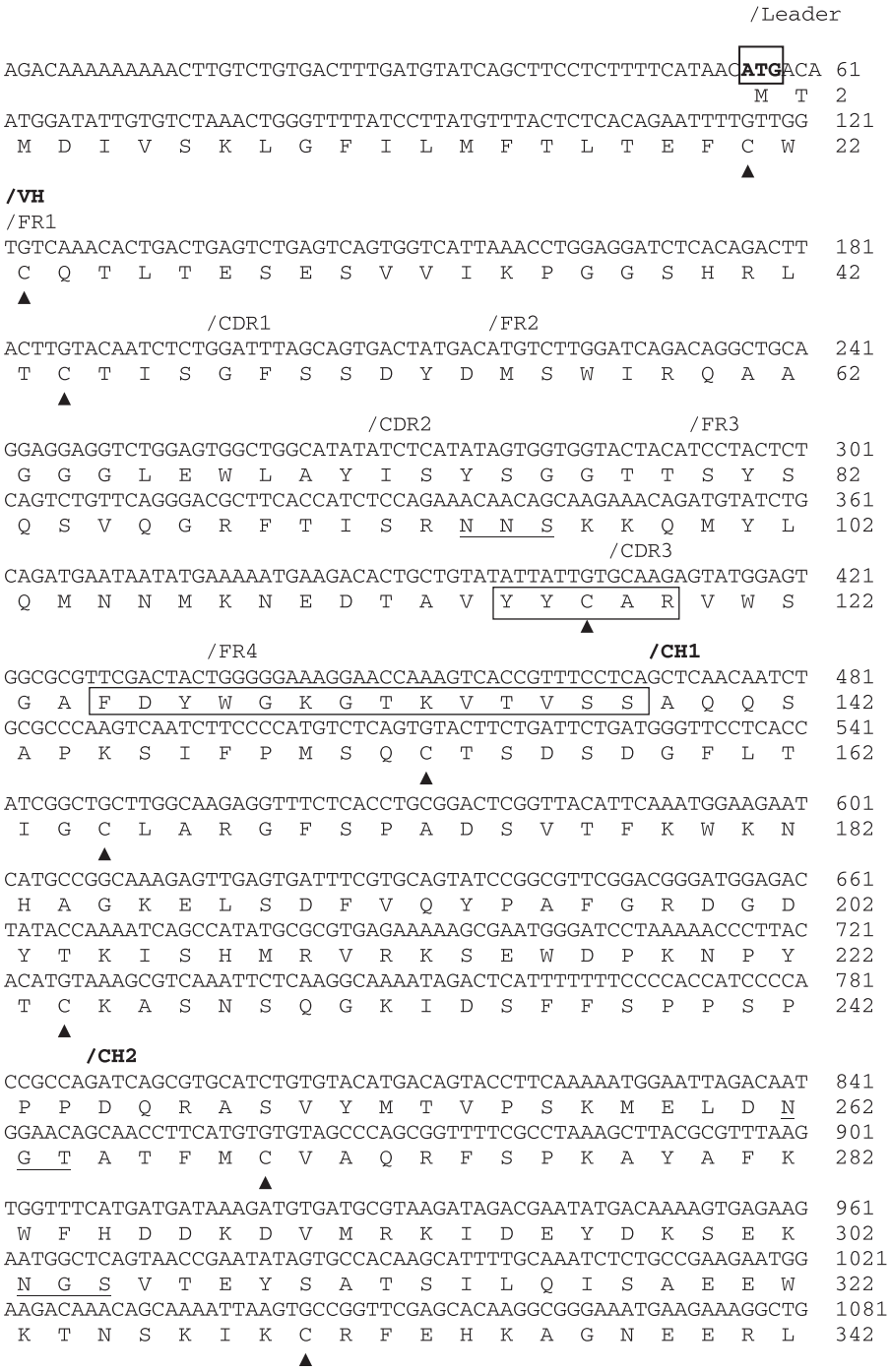

Figure 1. Complete nucleotide (upper row) and deduced amino acid (lower row) sequences of the IgM heavy chain of *Labeo rohita* (GenBank accession number KX268324). The deduced amino acid sequence is reported in one-letter code and the open reading frame of 1731 bp encodes a protein of 576 amino acid residues which is divided into leader peptide, variable domain (VH) including, CDRs and FRs and constant domains (CH1 to CH4). The cysteines (C) are denoted by triangle. The putative N-glycosylation sites are designated by underline. The initiation codon (ATG), stop codon (TAG) and the highly conserved motif 'YYCAR' and 'FDYWGKGT–VTV–S' were designated in box.

Deduced amino acid sequence of rohu IgM heavy chain consists of typical structural features of IgM heavy chain family members of the teleosts. The structural organization of gene elements in the full-length IgM heavy chain of rohu includes a leader peptide, VH domain (FR1 to FR4 and CDR1 to CDR3) and CH domain (CH1 to CH4) which is similar to that of other teleosts like *Epinephelus coioides*, *M. amblycephala* and *Oreochromis niloticus*.[3,15,31] As far as teleost immunoglobulin is considered, CH4 domain along with CH1–CH3 domains is assumed to be secretory form[13]. Since CH4 is present only in

```
                    /CH3
GTTGAAAATACAGATGACTGCAATCCTGAAATAGATCCCGATATAGTGCCTCCCTCTCCT  1141
 V  E  N  T  D  D  C  N  P  E  I  D  P  D  I  V  P  P  S  P   362
                   ▲
GAAGACATGCTGAAAAATAGAGTAGGATTGCTGAAGTGCAAAGCTTCAGCAGAAAATGCA  1201
 E  D  M  L  K  N  R  V  G  L  L  K  C  K  A  S  A  E  N  A   382
                                     ▲
GGATTTGTGAAAATAACAATAAAAGCAAATAATAACATCATCGCTAACAAATCAGGCGAC  1261
 G  F  V  K  I  T  I  K  A  N  N  N  I  I  A  N  K  S  G  D   402
GAATATTTCCAGAACAGAAAATCTGTGGAACTTGATGCACCTATAGGCTATGAAGAATGG  1321
 E  Y  F  Q  N  R  K  S  V  E  L  D  A  P  I  G  Y  E  E  W   422
AGCAACGGCACTGAATTTACATGCAGCATTGAACACCGCGAGCTAGCAGAGCCCAAGGAG  1381
 S  N  G  T  E  F  T  C  S  I  E  H  R  E  L  A  E  P  K  E   442
                   ▲
                   /CH4
AAAACGTTCAGCAGAGAAAATGGTAAAGAACCCAAACAACCCACTGTTTTCATAATAGCA  1441
 K  T  F  S  R  E  N  G  K  E  P  K  Q  P  T  V  F  I  I  A   462
CCCCCAGAGCACAAACCGGGAGAACCTGTGACCCTGACATGTTATGTGAAGGACTTCTAC  1501
 P  P  E  H  K  P  G  E  P  V  T  L  T  C  Y  V  K  D  F  Y   482
                                        ▲
CCCAAAGAGGTGTTTGTGTCTTGGCTTGTTGATGATGGACCTCTGCCTGCTGAGTACAGT  1561
 P  K  E  V  F  V  S  W  L  V  D  D  G  P  L  P  A  E  Y  S   502
TACAGCACTAGCCAGCCAATTAAGAATGGTCAAAACTTCTCAGCCTACAGTCAGCTAACA  1621
 Y  S  T  S  Q  P  I  K  N  G  Q  N  F  S  A  Y  S  Q  L  T   522
GTTGGTTATTCTGAGTGGAAGAGCGGTGCAGTGTTCAGTTGTGTTGTGTACCACGAAGGC  1681
 V  G  Y  S  E  W  K  S  G  A  V  F  S  C  V  V  Y  H  E  G   542
                                        ▲
ATTGATGACCATATGCGCGTACTGGCTAGATCAATTGATGATAATGTGGAGAAGGCAGGT  1741
 I  D  D  H  M  R  V  L  A  R  S  I  D  D  N  V  E  K  A  G   562
GTAATTAATCTAAGTATGAATACCCCTGCATCTTGCAAGGACTAGAGCGTTGTGTATGTG  1801
 V  I  N  L  S  M  N  T  P  A  S  C  K  D  *                 576
                                  ▲
GTGTCTTCCTGTTATGCCTGTTAAGTGTTCATCTTGTTTTCTTTTTCTTTTGGTTTTTGA  1861
ATTGCATGTCCATATTTGTAATTGTGTTGGTCTTTAATGTTTGTGGCTTCTTGCTTTTTT  1921
GAGATATTTTCATGCACGTTACATTAGGAAAAGATTAATAAAAAAGAAACAAAGCAAAAA  1981
AAAAAAAAAAAAA                                                 1994
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Figure 1. Continued.

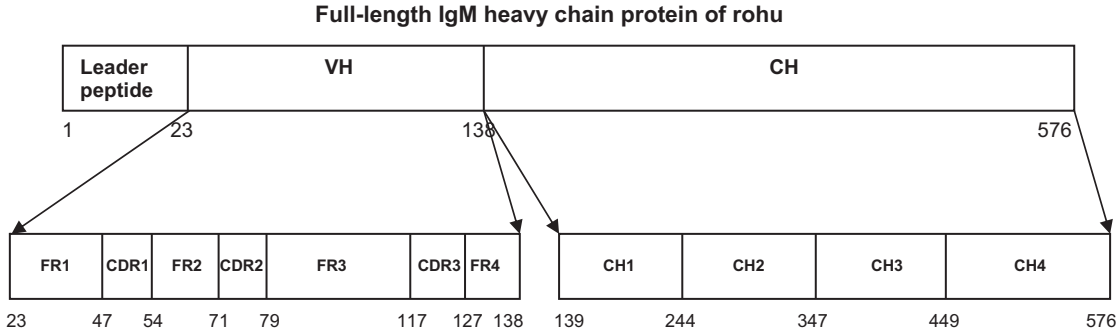

Figure 2. Schematic representation of full-length IgM heavy chain protein of rohu. The IgM heavy chain protein with a leader peptide, variable domain (VH) and constant domain (CH) were represented schematically as bars with their corresponding amino acid numbers. Different segments of the V-domains and various constant domains were also represented separately.

secretory IgM,[13–15] the rohu IgM heavy chain obtained in this present study is considered as a secretory form of IgM.

Cysteine (Cys) residues are responsible to form intra-domain disulfide bridges and L-chain binding.[32] IgM heavy chain of rohu contained three conserved cysteine residues each in variable, CH1, CH3 and CH4 domains, whereas, CH2 domain contained two residues. Among these residues, a cysteine residue (Cys^{153}) present in CH1 region is responsible for connecting heavy chain with light chain, Cys^{349} present in CH3 region is responsible for establishing disulphide bond with heavy (H–H) chains and Cys^{574} present in CH4 region is responsible for forming

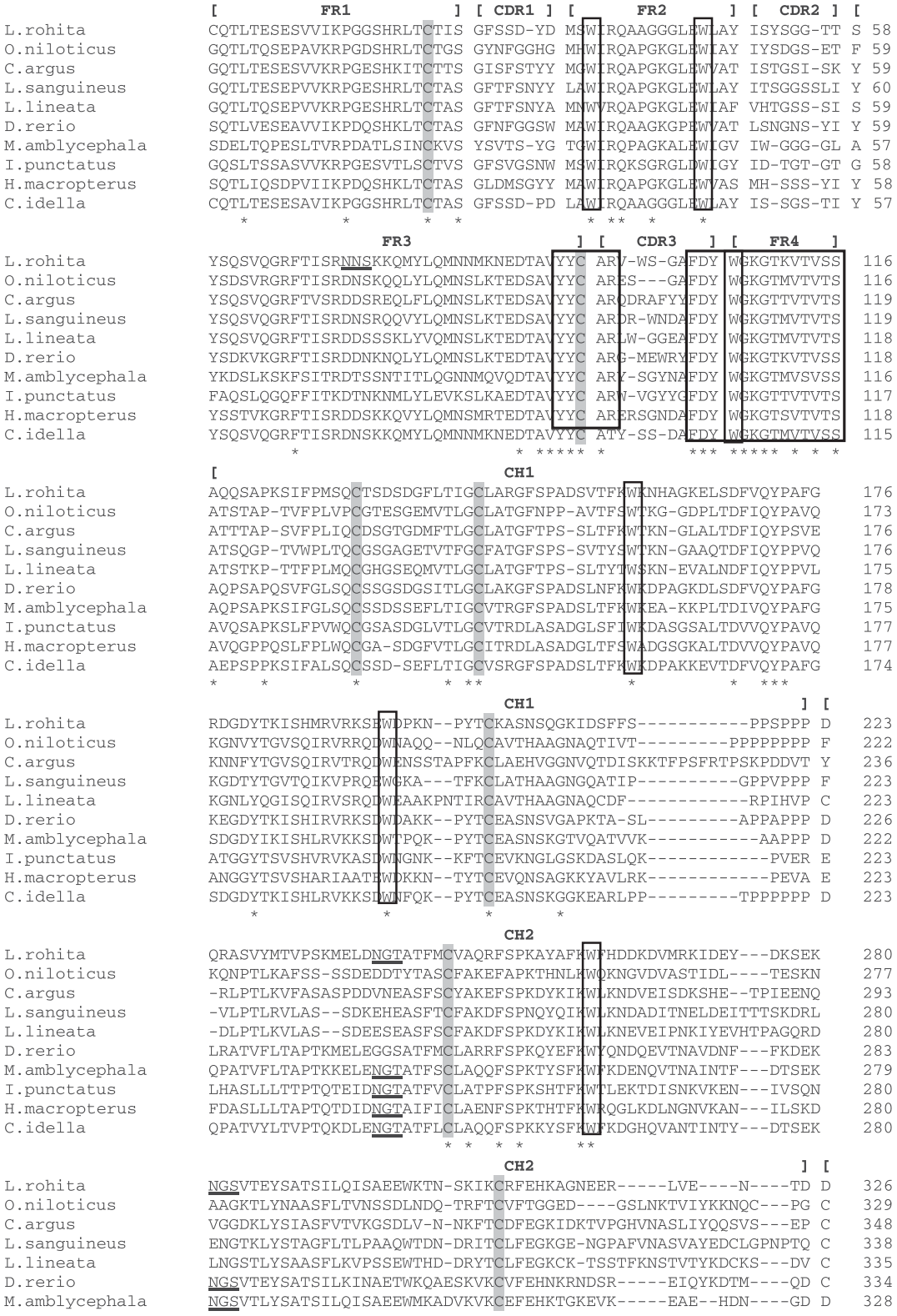

Figure 3. Multiple sequence alignment of deduced amino acid sequence of rohu IgM heavy chain with related species such as *Oreochromis niloticus* (AHY86391), *Channa argus* (ACF49353), *Lutjanus sanguineus* (ADX01345), *Latris lineata* (ADC45387), *Danio rerio* (AAH67633), *Megalobrama amblycephala* (AGR34023), *Ictalurus punctatus* (A45804), *Hemibagrus macropterus* (AEH84415) and *Ctenopharyngodon idella* (ABD76396). The gap positions were indicated by hyphens. Identical amino acid residues found in all of the compared sequences are indicated by asterisk. Conserved cysteine residues are shadowed and the conserved tryptophan residues are boxed. The putative N-glycosylation sites are underlined. Highly conserved motifs namely 'YYCAR' and 'FDYWGKGT–VTV–S' were designated in the boxes. Boundaries between each domain are shown beyond the sequences estimated by analogy to the other sequences.

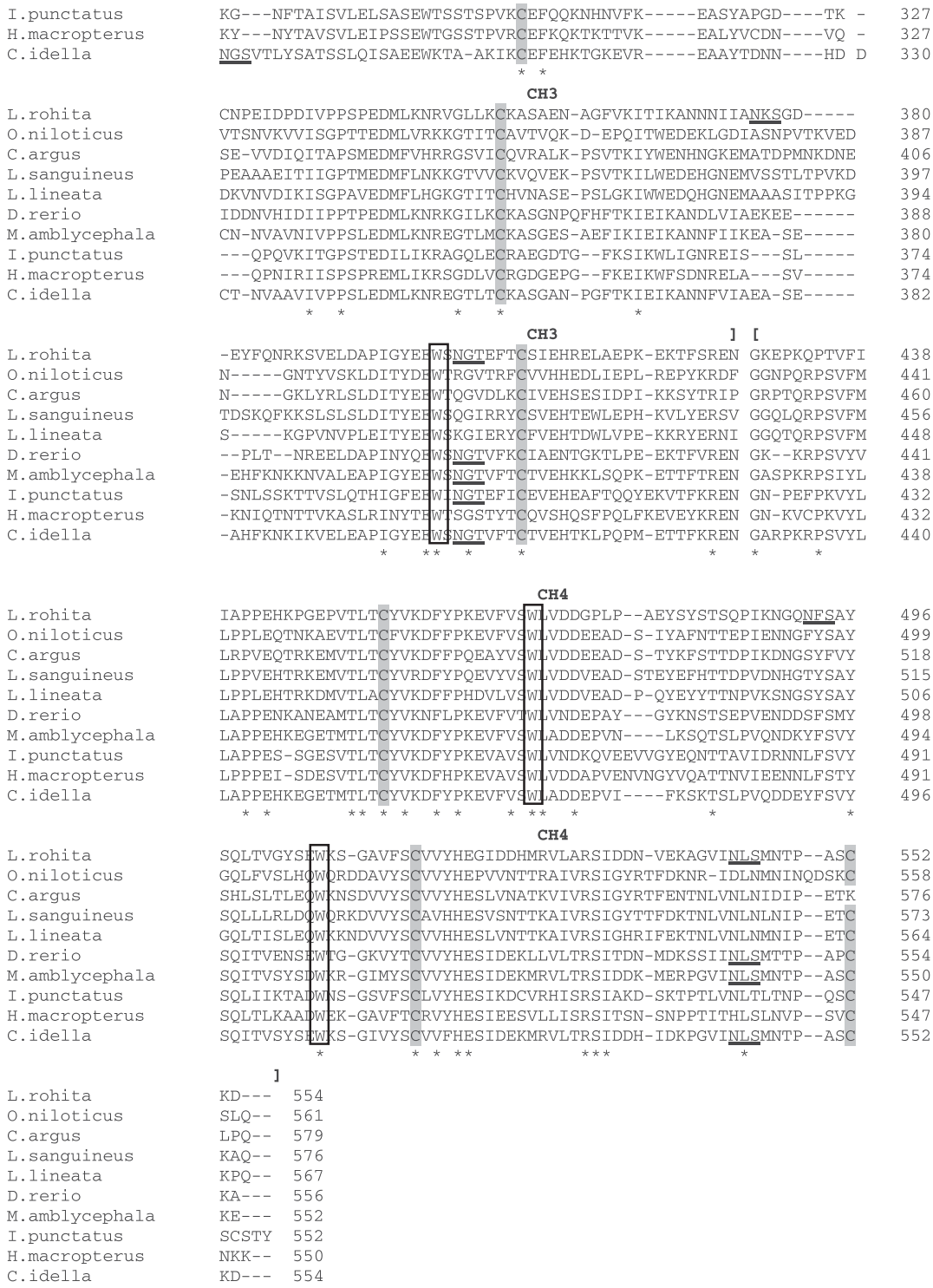

Figure 3. Continued.

multimers as reported earlier for medaka and blunt snout bream.[3,33] Cysteine residues present in IgM heavy chain of rohu are essential for its polymer structure.[3,34] Particularly, cysteine residues located within CH4 domain of rohu IgM heavy chain are found to be responsible for the formation of tetrameric structure or dimerization of two heavy chains[35–37] and provides further evidence to the earlier suggestions that rohu IgM molecule is a tetramer.[20,21] Besides, constant region of rohu IgM heavy chain consisted of seven conserved tryptophan (W) residues, which are responsible for the formation and

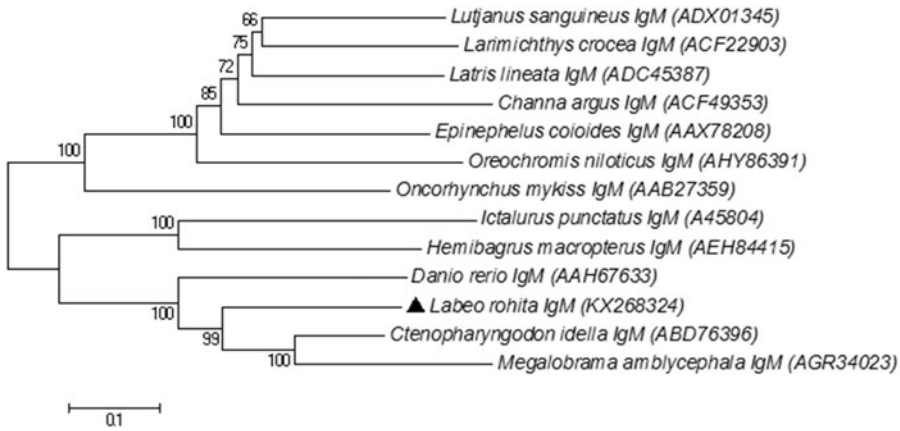

Figure 4. Phylogenetic tree constructed using the deduced amino acid sequence of the IgM heavy chain gene of *Labeo rohita* with other species retrieved from the NCBI GenBank. The tree was constructed using neighbor-joining method and bootstrapped 1000 times using MEGA6 software. Values within parentheses represent GenBank accession numbers. Numbers next to the branches indicate bootstrap values. Scale-bar represents evolutionary distance.

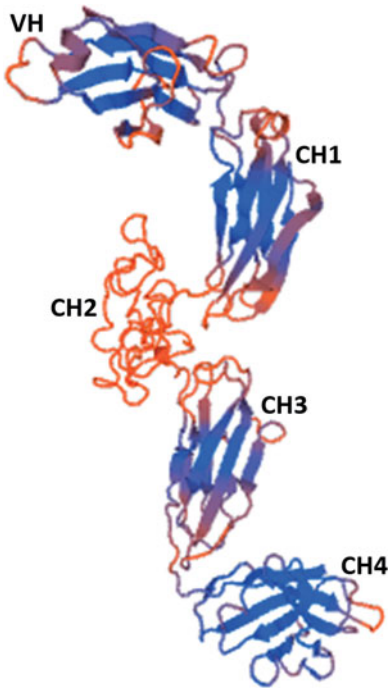

Figure 5. Putative 3 D model of IgM heavy chain of rohu showing VH, CH1, CH2, CH3 and CH4 domains.

stabilization of tertiary structure of proteins.[31,35] The function of N-linked glycosylation sites is to provide structural stability and biological function and the constant regions of immunoglobulin heavy chain should contain at least one or more N-linked carbohydrates.[31] Two N-linked glycosylation sites each present in CH2, CH3 and CH4 domains of rohu IgM heavy chain may be responsible for maintaining the effector functions.[38] Another feature of putative polyadenylation signal (AATAAA) was identified 13 bp upstream of the polyA tail in rohu IgM heavy chain as conserved in other teleost species.[3,13,19,39,40]

The maximum IgM heavy chain gene sequence homology was exhibited among *L. rohita, C. idella* and *M. amblycephala* as all the three species belong to the same family in the taxonomical hierarchy (Cyprinidae). The IgM gene sequences are highly conserved within the family Cyprinidae as in *C. idella*[30] and *M. amblycephala*.[3] Moreover, several conserved amino acids were also found in various domains of rohu IgM heavy chain as reported in other teleosts.[13,31,41] The conserved motif (YYCAR) and other canonical residues (C, S, W, Q, D, L) responsible to form antigen binding sites by proper folding of VH domain[13,14] were also conserved in rohu.[3,42] Likewise, another motif 'FDYWGKGT–VTV–S' existing in VH domain is also well conserved in rohu as in other teleosts with unknown function.[14,31]

The phylogenetic tree analysis showed that IgM heavy chain gene of rohu formed the same cluster along with other cyprinid fishes like *D. rerio, C. idella* and *M. amblycephala* and different cluster with *L. sanguineus*. This result is well supported by the earlier findings where a very high level of amino acid identity was found among the members within a family and very less identity existed between the families.[31,40,43] The structural organization of 3 D model of IgM heavy chain in rohu was found to be similar with *Epinephelus akaara*.[44] Ramachandran plot revealed the good quality 3 D model of rohu IgM protein in which more residues fall in favored (86.9%) and allowed (10.5%) regions than very less residues in the outlier region (2.6%).

IgM heavy chain gene expression was observed in all the tested tissues of rohu with a higher level of expression in the kidney followed by spleen, intestine, gill, skin, and liver. This result suggests that IgM

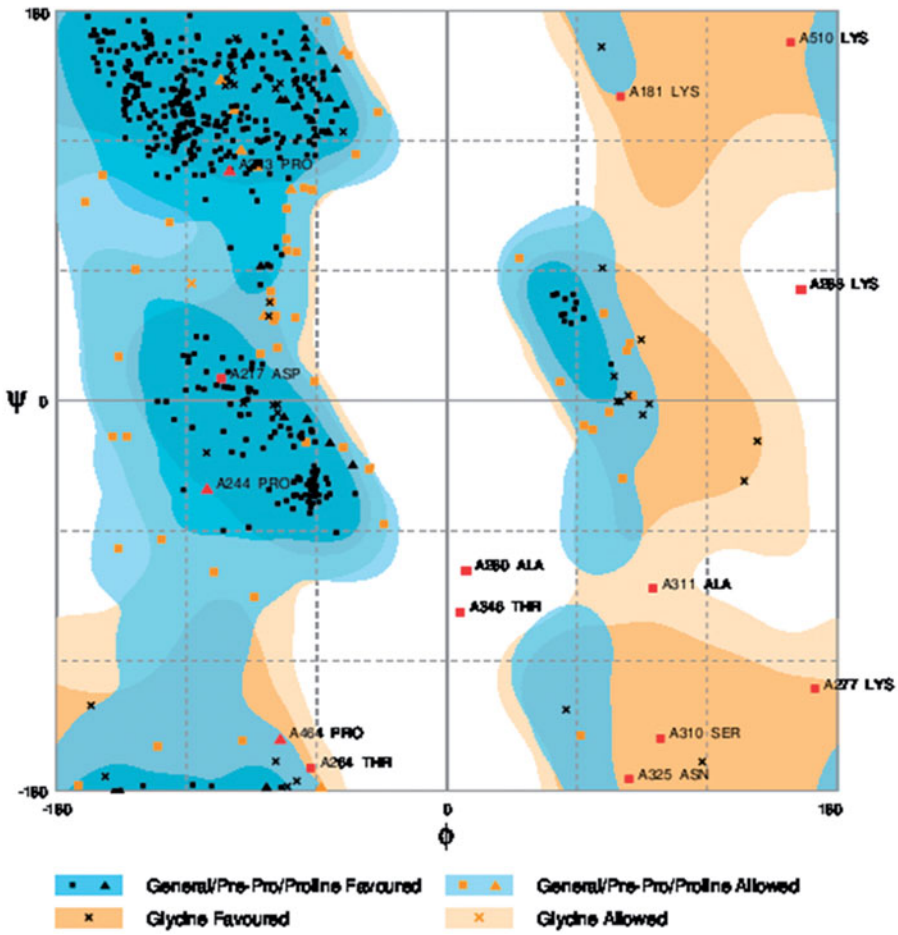

Figure 6. Ramachandran plot for the predicted 3 D model of rohu IgM heavy chain.

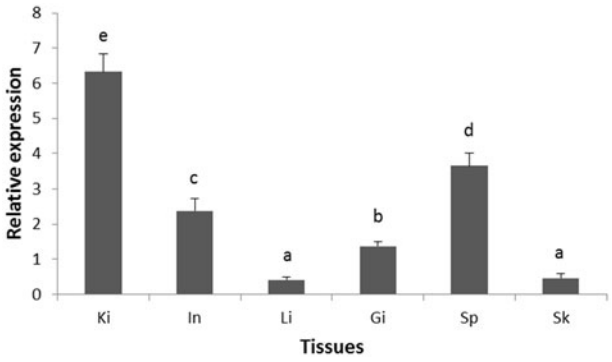

Figure 7. Relative expression of IgM heavy chain gene in different tissues of healthy rohu. Ki: Kidney; In: Intestine; Li: Liver; Gi: Gill; Sp: Spleen, and Sk: Skin. Bars represent mean values (±S.E) of nine samples per tissue. The significant differences ($p < 0.05$) in gene expression are indicated by different letters (a, b, c, d, e) over the bars.

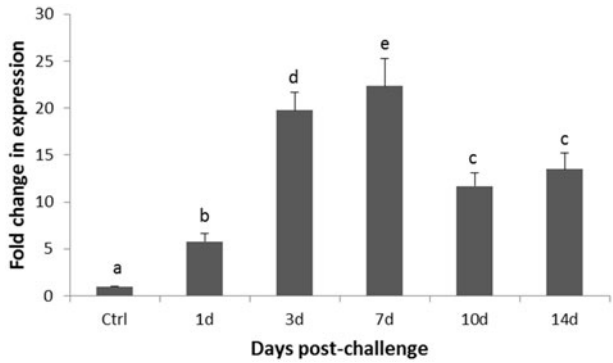

Figure 8. Relative expression of IgM heavy chain gene in rohu kidney in response to *Aeromonas hydrophila* infection. Bars represent mean values (±S.E) of nine samples per time point. The significant differences ($p < 0.05$) in gene expression are indicated by different letters (a, b, c, d, e) over the bars.

heavy chain gene is highly expressed in major lymphoid organs such as kidney, spleen and intestine in which antibodies are synthesized by B cells.[14,19,30,45]

Recently, more focus is given by the researchers on expression analysis of immune-related genes of fish after vaccination or challenge with pathogen[46] and also found that the host immune response varied with different factors like fish species, pathogen, etc.[47] *Aeromonas hydrophila* is responsible for many disease conditions such as haemorrhagic septicaemia and

abdominal dropsy of carps in freshwater aquaculture, causing mortality and economic loss and hence it is necessary to understand the immunological response and defense mechanism of carps against *A. hydrophila*. Kidney tissue was selected for this study based on the fact that as a major lymphoid organ, it involves in the process of clearance of bacteria with the support of macrophages.[48] The IgM heavy chain gene expression in rohu kidney showed a gradual increase over time and reached maximum level (22.35 fold) at 7 days post-challenge (dpc). This result corroborates with the earlier reports that the peak level of IgM expression was observed at 7 days post-immunization in rainbow trout[11] and also in blunt snout bream.[3] It is reported that the IgM secreting cells are mostly found in teleost kidney and further the plasma cells present in lymphohaematopoietic tissues like kidney are involved in the process of antigen trapping and lymphocyte stimulation during the host immune response.[49] Thus, IgM heavy chain expression in rohu showed up-regulation during the process of *A. hydrophila* infection.

In conclusion, we have characterized the full-length IgM heavy chain cDNA of rohu for the first time. Analysis of constant domains showed that the characterized IgM heavy chain gene of rohu belongs to the secretory form of IgM. The IgM heavy chain gene expression was mainly found in lymphoid organs and also in the mucosal tissues, suggesting its role in adaptive humoral immunity. Besides, the IgM heavy chain gene expression was influenced by challenge with bacteria which indicated that IgM could possibly have a key role to play in the immune response during bacterial infections in rohu.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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