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Ontogenic expression of adaptive immune genes in Rohu, *Labeo rohita* (Hamilton-Buchanan, 1822)

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The ontogenic expression of selected adaptive immune genes in *Labeo rohita* was studied using semi-quantitative RT-PCR and quantitative real-time PCR. Adaptive immune genes, viz., Ikaros, MHC-II and Rag-1 were cloned and sequenced using self-designed primers. The partial nucleotide sequences of *L. rohita* Ikaros gene (510 bp), MHC-II gene (310 bp) and Rag-1 gene (142 bp) showed 94%, 93% and 99% homology with the sequences of *Danio rerio, Barbus intermedius* and *Catla catla*, respectively. Total RNA extracted from fertilized eggs and larvae at 1, 3, 7, 10, 14, 17, 21, 24, 28, 35 and 42 days post-hatch (dph) was subjected to real-time PCR using self-designed real-time primers to amplify the adaptive immune genes, viz., IgM heavy chain gene, Ikaros, Rag-1 and MHC-II. The expressions of IgM heavy chain gene, Rag-1 and MHC-II were first detected at 1 dph and thereafter increased gradually, and the onset of statistically significant ($P < 0.05$) increase in expression was observed from 24 dph (18.6 fold), 17 dph (16.7 fold) and 28 dph (10.7 fold), respectively, whereas, Ikaros gene expression was observed even at the fertilized egg stage and significant ($P < 0.05$) increase in expression was observed from 28 dph (7.1 fold) onwards. The findings of the present study suggest that the initial development of immunocompetence may occur by 3-4 weeks post-hatch in *L. rohita*.

Keywords: *Labeo rohita*, adaptive immunity, ontogeny, gene expression, immunocompetence

Introduction

Vertebrate immune system consists of two major components of innate and adaptive immune responses. The adaptive immune system is unique to jawed vertebrates[1] and refers to the antigen-specific defense mechanisms that confer the ability to recognize and remember specific pathogens, and to mount stronger attacks each time the pathogen is encountered[2]. Adaptive immune system is a highly specialized and regulated process involving the interaction of both immune-relevant molecules and lymphocytes. Ikaros gene is a key component of adaptive immune system and encodes a transcription factor which is used as an early lymphoid marker[3]. It plays a vital role in vertebrate hematopoietic stem cell differentiation, and the generation of lymphocytes and natural killer cells[4,5]. In addition to Ikaros, the key molecules in the adaptive immune system include the recombination activating genes (Rag), immunoglobulin (Ig), B cell receptor (BCR), T cell receptor (TCR) and major histocompatibility complex (MHC)[6]. Rag-1 gene encodes a protein involved in genomic rearrangement {termed V(D)J recombination} of the TCR and immunoglobulin (Ig) loci, and is a suitable marker for maturing lymphocytes[7]. Rag-1 is critical to the differentiation of pre-B and pre-T cells; its expression within an associated primary lymphoid organ can serve as a developmental marker[8].

Immunoglobulins are the primary humoral component of the adaptive immune system[9]. So far, six isotypes namely, IgM, IgD, IgZ, IgT, IgM-IgD chimera and IgM-IgZ chimeras, of immunoglobulins have been reported in teleost fish[10-15]. IgM is the major isotype of fish immunoglobulins. In bony fishes, immunoglobulin occurs as a tetramer[16,17]. MHC Class II molecule plays an important role in immune response by presenting the foreign protein antigens to the T-cell receptors[18]. MHC Class II molecules present exogenously derived peptides to $CD4^+$ T cells to activate $CD4^+$ helper T cell-mediated humoral immunity[19,20].

Ontogenesis is a sequence of molecular and cellular events regulated by time and space, leading to the

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development of a fully functional organism[21]. The ontogeny of the adaptive immune response is important from the fish health and welfare point of view as it can help to identify the most appropriate developmental stage for vaccination[22]. Ideally, fish should be vaccinated as early in the culture process as possible in order to increase survival and decrease the use of antibacterial drugs[23]. However, if fish are vaccinated prior to becoming immunologically competent, a phenomenon known as immunological tolerance can occur, leading to a decrease in the subsequent secondary response and efficacy of vaccination[9,24,25]. Therefore, it is important to identify the ideal stage for vaccinating fish which has a functional response developed[22].

Labeo rohita (Rohu) is the most preferred, widely cultured species of Indian major carps (IMCs) in Southeast Asia and serves as the economic back bone of Indian aquaculture. In rohu, the study on ontogeny of immune system is mainly limited to the innate immune system[21,26]. Many reports are available on the ontogeny of the adaptive immune system in other cyprinids such as common carp[27,28] and zebrafish[29] and other teleost species like haddock[30], seabass[31] and killifish[32]. However, there is no report available on the ontogeny of adaptive immune genes of rohu as well as other IMCs. Hence, the present study focused on studying the ontogenic expression of adaptive immune genes, viz., IgM, Ikaros, Rag-1 and MHC-II in rohu to get a detailed picture on the development of adaptive immune system in *L. rohita*.

Materials and Methods

Animals

Rohu (*L. rohita*) fingerlings weighing 30-40 g were procured from a fish farm in Mumbai and maintained in the wet lab of the Central Institute of Fisheries Education, Mumbai, India. The fish were maintained in a 500-l fibreglass tank with continuous aeration and 20% water exchange daily. The water temperature varied between 25°C and 30°C throughout the experiment. The fish were fed once a day with commercial fish feed pellets.

The early developmental stages of rohu were sampled from a fish seed hatchery in Mumbai at the following stages of development: Fertilized eggs and larvae at 1, 3, 7, 10, 14, 17, 21, 24, 28, 35 and 42 days post-hatch (dph). Samples of each age group were collected in triplicate and rapidly submerged in RNAlater (Ambion, USA) and then stored at -20°C until used for RNA extraction. RNA was isolated from the triplicate lots of samples and each lot consisted of pooled larvae ranging from 30-40 for 1 and 3 dph to 2-3 larvae for 42 dph at a concentration of not more than 100 mg tissue per millilitre of Trizol reagent (Invitrogen, USA).

Isolation of Total RNA and Synthesis of cDNA

Total RNA was isolated from the fertilized eggs, post-hatch larval samples and the kidney of rohu fingerlings with 1 ml Trizol reagent following the manufacturer’s instructions. The total RNA obtained was quantified using Nanodrop 2000 (Thermo Scientific, USA) and treated with DNase1 (Thermo Scientific, USA) prior to reverse transcription to remove residual genomic DNA contamination. DNase treatment reaction was carried out in 20 µl volume containing 5 µg of total RNA, 2 µl DNase1 (1 u/µl), 2 µl 10X reaction buffer with $MgCl_2$ and DEPC water up to 18 µl. The reaction mixture was mixed thoroughly and incubated at 37°C for 30 minutes and then 2 µl EDTA (50 mM) was added and incubated at 65°C for 10 minutes. cDNA was synthesized using the First-strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer’s instructions. The reverse transcription reaction was carried out in 20 µl volume containing 1 µg of DNase treated RNA, 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 hour. The reaction was terminated by heating at 70°C for 5 minutes. The resulting cDNA was stored at -20°C.

Primer Design

Primers were designed to amplify and sequence the adaptive immune genes (Ikaros, MHC-II and Rag-1) based on the conserved gene sequences from the related species using DNASTAR software. To analyse the expression of the adaptive immune genes, real-time PCR primers (IkarosqRT, MHC-IIqRT and Rag-1qRT) were designed based on the sequences obtained in this study. Primers were designed to analyse IgM heavy chain gene (IgMqRT) expression based on our earlier study[33] and β-actin primers β-actin was choosen as the reference gene as it is constitutively expressed in

Table 1 — Primers used to amplify adaptive immune genes.

Target gene	Sequence (5'- 3')	Annealing temperature (°C)	Amplicon size (bp)	Reference/ Accession no.
Ikaros	F-CATCTGCGCACTCATTCGGTTGG R-GTGCTCTGCGGCGCTGTCTTTAG	55	510	Self-designed
MHC-II	F-GTGACTGGTTTCTATCCTGCT R-CTTTGATGAGGAAGAAGGTTC	51	310	Self-designed
Rag-1qRT	F-AGGCACCGGCTATGATGAAAAGA R-GTGGCTGCGTGTGATGGAATGTA	50	142	Self-designed
IgMqRT	F-CAGGGACGCTTCACCATCTC R-GTTCCTTTCCCCCAGTAGTCG	51	149	HM581639
IkarosqRT	F-CTGCGCACTCATTCGGTTGG R-TGCTCATTCTGGCTGTTCTCTTCC	51	170	KC958576
MHC-IIqRT	F-AGAGGGAGACATTTACAGCCG R-AAGAAGGTTCCAGCAGCGAC	50	162	KC958577
β-actinqRT	F-GACTTCGAGCAGGAGATGG R- CAAGAAGGATGGCTGGAACA	55.3	138	26 (Mishra *et al*, 2009)

all the developmental stages of rohu and is an ideal reference gene for developmental studies[21].

Cloning and Sequencing of Adaptive Immune Genes

Adaptive immune genes, viz., Ikaros, MHC-II and Rag-1 were PCR-amplified, cloned and sequenced. A 25 µl PCR mix consisting of 12.5 µl of 2X master mix (*Taq* polymerase 0.05 u/µl, 4 mM $MgCl_2$ and dNTPs 0.4 mM each), 10.5 µl of nuclease-free water, 25 pM of forward and reverse primers and 1 µl template (kidney cDNA) was prepared. All amplification reactions consisted of an initial denaturation at 95°C for 4 minutes prior to 35 cycles of 95°C denaturation for 30 seconds, different annealing temperature for 30 seconds (Table 1) and 72°C extension for 2 minutes, followed by a final 72°C extension for 10 minutes in a thermal cycler (Applied Biosystems, USA). The generated PCR products were then analyzed through electrophoresis on 2% agarose gel.

The amplicons in the agarose gel were eluted using GeneJET gel extraction kit (Thermo Scientific, USA) following the manufacturer's protocol. The purified PCR fragments were cloned into pTZ57R/T vector (InsTAclone PCR cloning kit, Thermo Scientific, USA) and transformed into *Escherichia coli* DH5α cells, following the manufacturer's protocol. The plasmid DNA was isolated using GeneJET plasmid miniprep kit (Thermo Scientific, USA) and sequenced using M13F and M13R primers in ABI 3730xl DNA analyzer (Bioserve Biotechnologies Pvt. Ltd., Hyderabad).

Sequence Analysis

The generated sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) program in the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast/) GenBank nucleotide database for finding homology with other sequences. The multiple sequence alignment of partial adaptive immune gene sequences of rohu with other species was constructed using Clustal omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/), and subjected to consensus analysis in BoxShade sever (http://www.ch.embnet.org/software/BOX_form.html). A neighbor-joining phylogenetic tree was constructed based on the nucleotide sequence of the adaptive immune genes of *L. rohita* with other sequences retrieved from the GenBank using MEGA6 software[34], employing pairwise deletion and Kimura - 2 method[35].

Expression of Adaptive Immune Genes During Early Development of Rohu

Real-time PCR primers (IgMqRT, IkarosqRT, Rag-1qRT and MHC-IIqRT) designed for adaptive immune genes were used in this study. The semi-quantitative estimation of adaptive immune gene expression was carried out for various developmental stages of rohu starting from fertilized egg to 42 dph. The reaction mixture consisted of 12.5 µl of 2X master mix (*Taq* polymerase 0.05 u/µl, 4 mM $MgCl_2$ and dNTPs 0.4 mM each), 10.5 µl of nuclease-free water, forward and reverse primers 0.5 µl each (25 pM) and 1 µl of cDNA (equivalent to 50 ng). All amplification reactions consisted of an initial denaturation at 95°C for 4 minutes prior to 35 cycles of 95°C denaturation for 30 seconds with different annealing temperature for 30 seconds and 72°C extension for 2 minutes, followed by a final 72°C extension for 10 minutes in a thermal cycler (Applied Biosystems, USA). The generated PCR products were then analyzed by electrophoresis on 2% agarose gel.

Similarly, quantitative real-time PCR was carried out by using the cDNA prepared from all the samples. 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 0.5 µl each (25 pM) and 1 µl of cDNA (equivalent to 50 ng). Real-time PCR was carried out on an ABI 7500 Real-time PCR System (Applied Biosystems, USA). The thermal cycling profile used for PCR amplification was 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. The threshold cycle (Ct) value was determined using the automatic setting on the ABI 7500 Real-time PCR System. The transcriptional level of target genes was normalised to β-actin and calibrated to the sample with the lowest level of transcription to obtain the fold change in expression according to $2^{\wedge-\Delta\Delta CT}$ method[36].

Statistical Analysis

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

Results

PCR Amplification and Sequencing of Adaptive Immune Genes of Rohu

The amplification of adaptive immune genes, viz., Ikaros, MHC-II and Rag-1 from rohu kidney using self-designed primers yielded the expected size products (Fig. 1). Partial sequence of Ikaros gene (510 bp) upon BLAST revealed maximum homology (94%) with *Danio rerio* Ikaros gene sequence; MHC-II gene (310 bp) revealed 93% homology with *Barbus intermedius* and Rag-1 gene (142 bp) revealed 99% homology with *Catla catla*. The multiple sequence alignment of adaptive immune genes with other species was performed using Clustal Omega program (Fig. 2). The adaptive immune gene sequences were submitted to GenBank under the accession numbers KC958576 (Ikaros), KC958577 (MHC-II) and KC958575 (Rag-1). Phylogenetic tree was constructed using the adaptive immune genes of rohu with the sequences of other fish species retrieved from the GenBank (Fig. 3).

Expression of adaptive immune genes during early development of Rohu

In the semi-quantitative RT-PCR method, the target amplicons first appeared as very faint bands up to a certain early age group indicating very low expression levels. Subsequently, the intensity of the ethidium bromide stained bands gradually increased as the expression level increased with age for all the adaptive immune genes studied (Fig. 4). Real-time PCR assay was performed to confirm the level of adaptive immune gene expression observed in semi-quantitative PCR. IgM heavy chain gene expression was first detected at 1 dph and thereafter, increased gradually, and the onset of statistically significant ($P < 0.05$) increase in expression was observed from 24 dph (18.6 fold) onwards. On the other hand, Ikaros gene expression was first observed moderately at the fertilized egg stage and significantly higher expression was observed from 28 dph (7.1 fold) onwards. Rag-1 expression started from 1 dph onwards and reached significant expression level on 17 dph (16.7 fold). Likewise, MHC-II gene reached the increased expression level (10.7 fold) on 28 dph (Fig. 5).

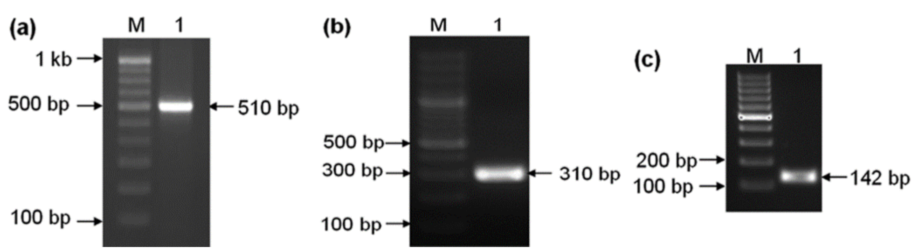

Fig. 1(a-c) — RT-PCR products of rohu partial adaptive immune genes. (a). Ikaros (510 bp); (b). MHC-II (310 bp) and (c). Rag-1 (142 bp). M: 100 bp molecular weight marker; Lane 1: RT-PCR products.

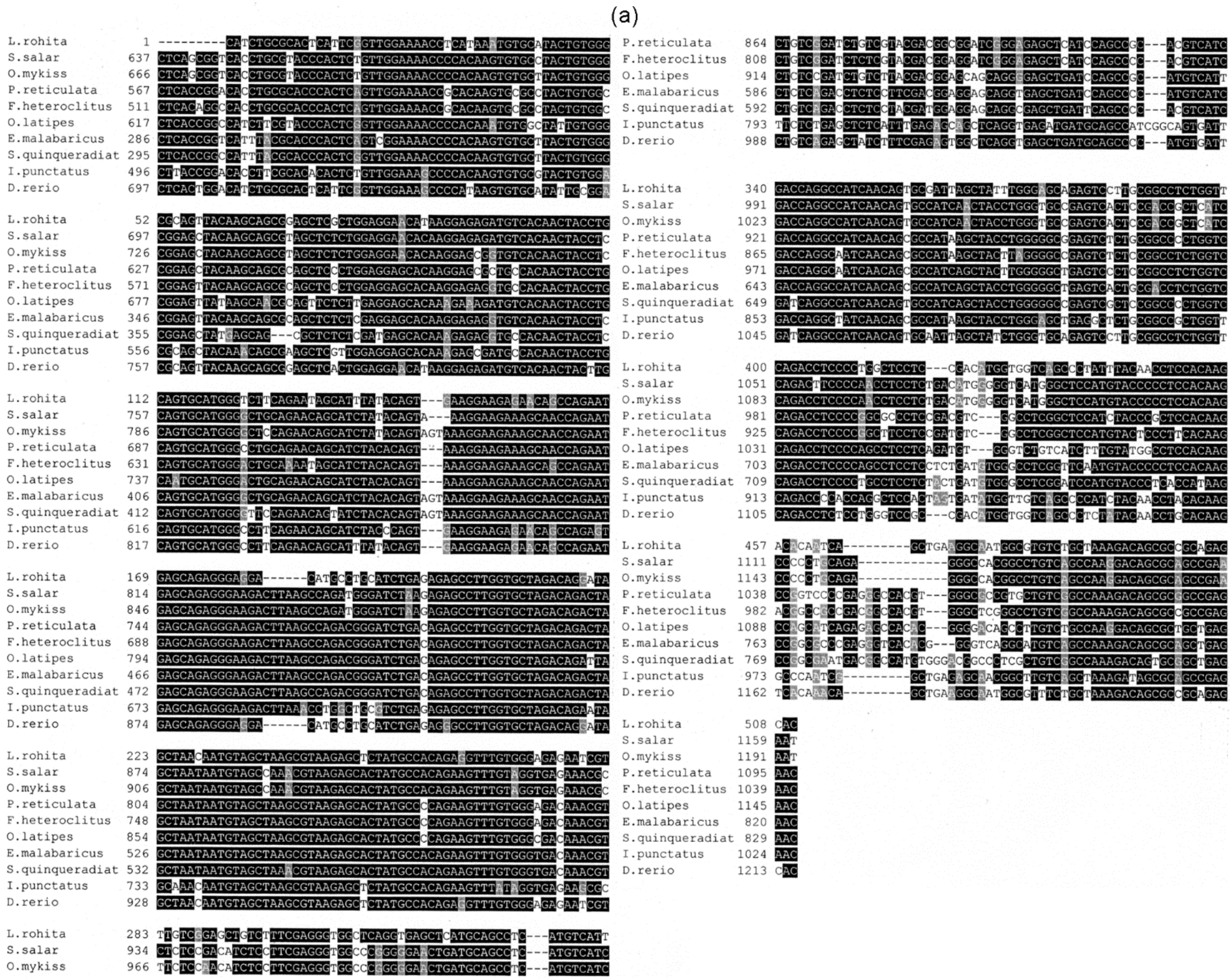

Fig. 2 (a) — Multiple sequence alignment of rohu partial adaptive immune genes with related species. (a). Alignment of rohu partial Ikaros gene (KC958576) with *Salmo salar* (NM_001173899), *Oncorhynchus mykiss* (NM_001124712), *Poecilia reticulata* (XM_017309018), *Fundulus heteroclitus* (XM_012856773), *Oryzia latipes* (XM_011493140), *Epinephelus malabaricus* (DQ054836), *Seriola quinqueradiata* (AB060640), *Ictalurus punctatus* (XM_017463676) and *Danio rerio* (BC162270)

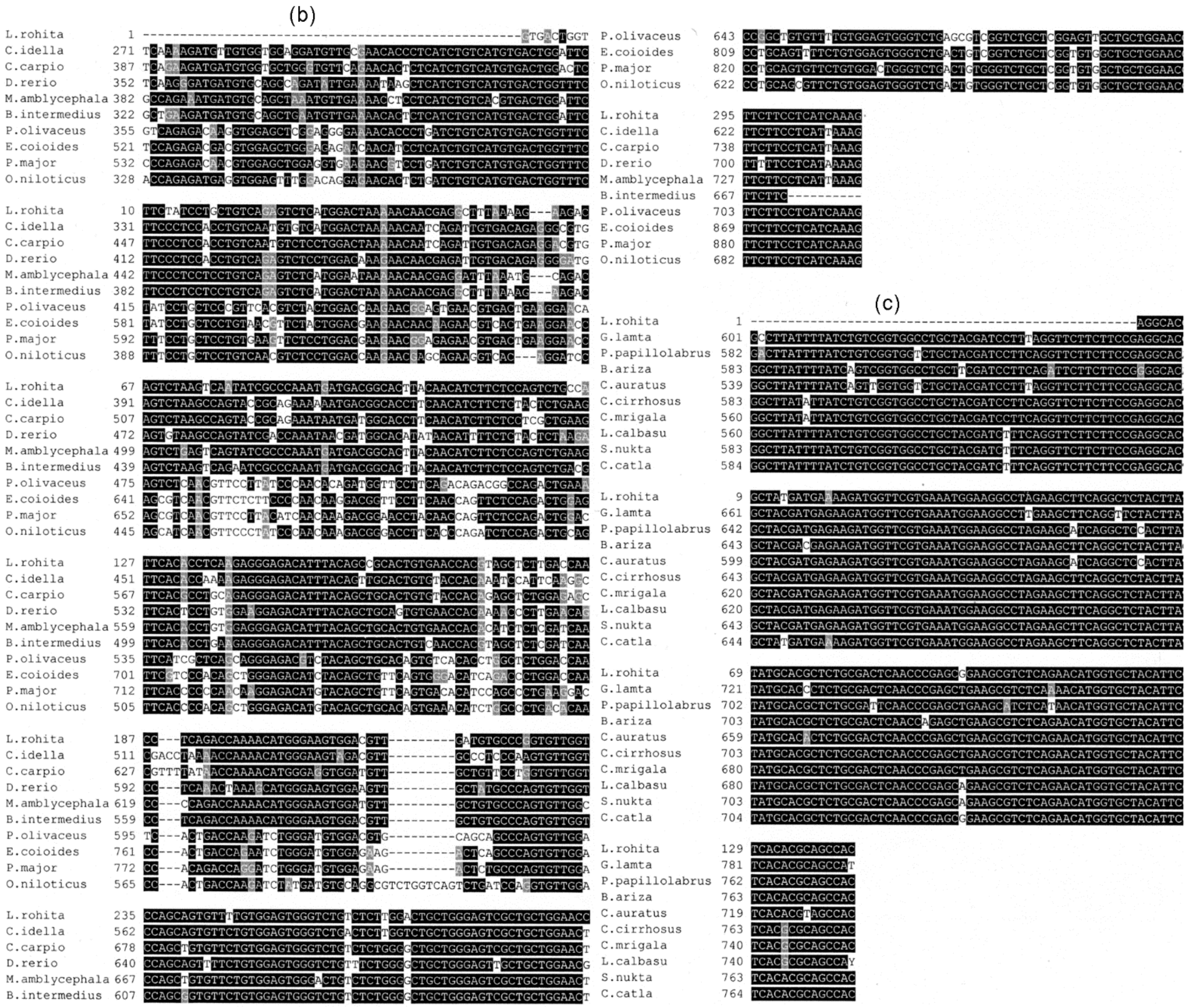

Fig. 2 (b-c) — Multiple sequence alignment of rohu partial adaptive immune genes with related species. (b). Alignment of rohu partial MHC-II gene (KC958577) with *Ctenopharyngodon idella* (EU186147), *Cyprinus carpio* (HQ380379), *Danio rerio* (NM_131490), *Megalobrama amblycephala* (KF193864), *Barbus intermedius* (AJ507006), *Paralichthys olivaceus* (AY997530), *Epinephelus coioides* (GU988722), *Pagrus major* (AY698064) and *Oreochromis niloticus* (JN983475). (c). Alignment of rohu partial Rag-1 gene (KC958575) with *Garra lamta* (JX074458), *Pseudocrossocheilus papillolabrus* (GU086513), *Bangana ariza* (JX074481), *Carassius auratus* (DQ196520), *Cirrhinus cirrhosus* (JX074468), *Cirrhinus mrigala* (GQ913452), *Labeo calbasu* (GQ913472), *Schismatorhynchus nukta* (JX074480) and *Catla catla* (JX074465). The consensus sequences were shown in black shadow and the similar sequences were highlighted in grey shadow and dashes (-) indicate gaps.

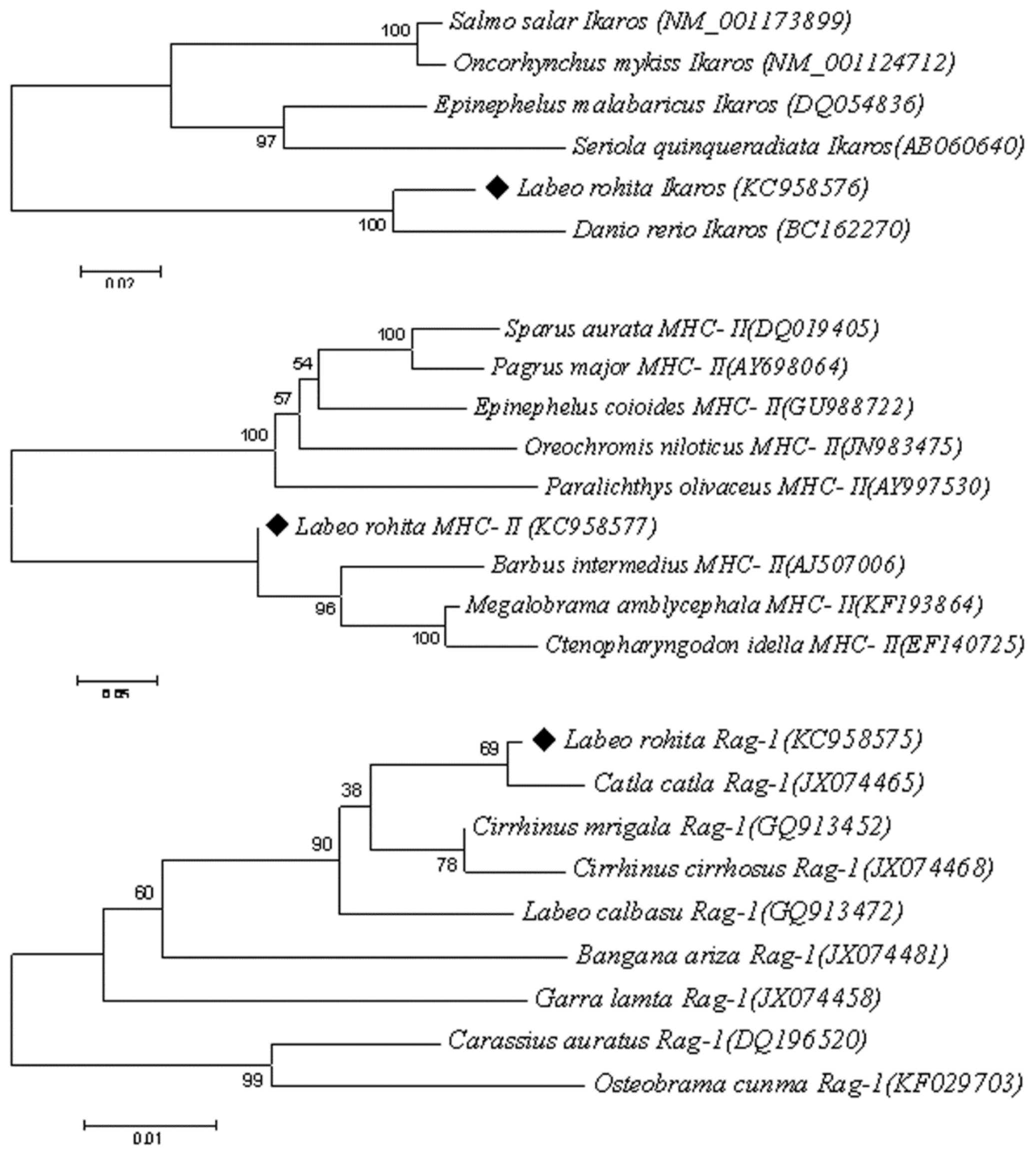

Fig. 3 (a-c) — Phylogenetic tree analysis of the nucleotide sequences of rohu partial adaptive immune genes, viz., Ikaros (a), MHC-II (b) and Rag-1 (c) with other reported sequences from GenBank. The rooted tree was constructed by the Neighbor-joining method and was bootstrapped 1000 times using MEGA6 software. Numbers next to the branches indicate bootstrap values. Scale-bar represents evolutionary distance. Values within parentheses represent GenBank accession numbers.

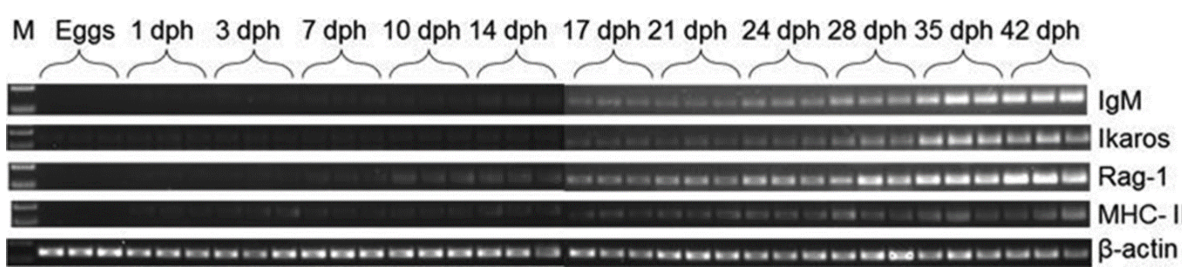

Fig. 4 — Ontogenic appearance of mRNA transcripts of adaptive immune genes of rohu. M: 100 bp molecular weight marker, dph: days post-hatch. The samples were run in triplicate.

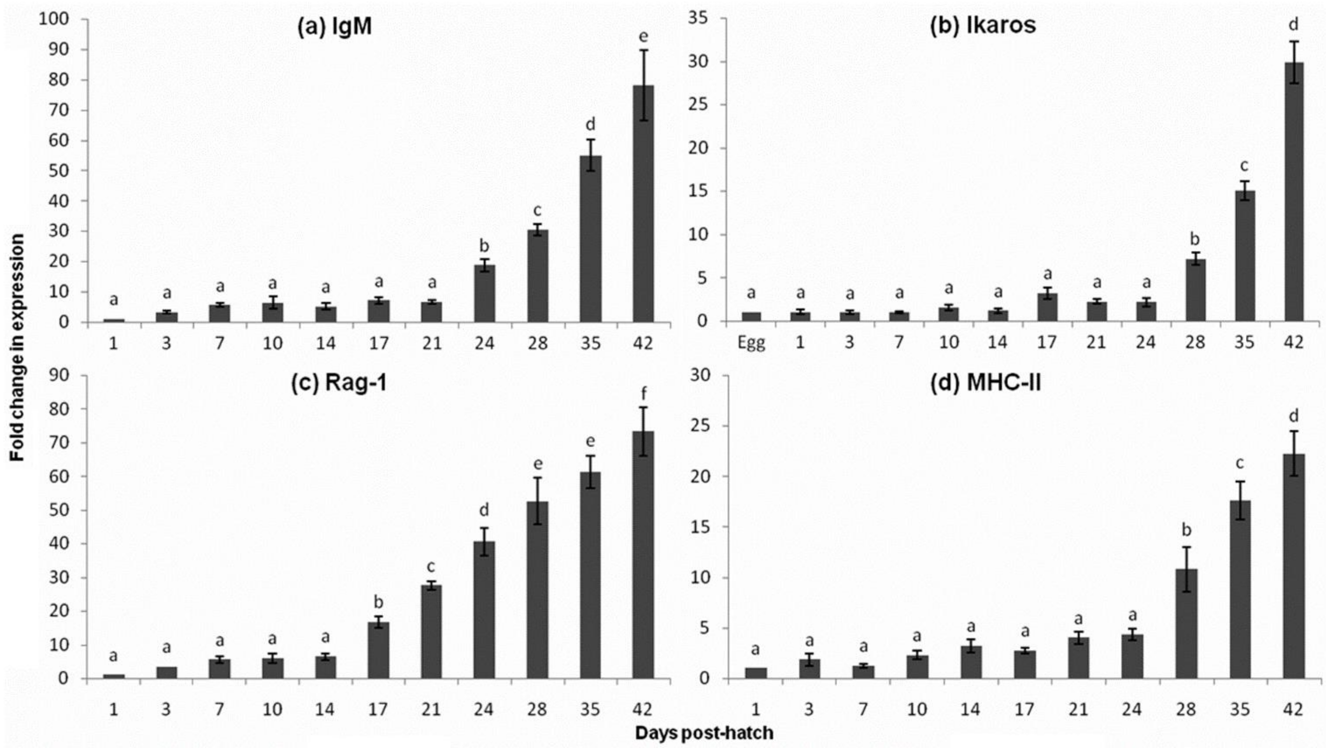

Fig. 5 (a-d) — Relative expression of adaptive immune genes, viz., IgM (a), Ikaros (b), Rag-1 (c) and MHC-II (d) in different larval stages of rohu. The transcriptional level of the adaptive immune gene was normalised to β-actin and calibrated to the sample with the lowest level of transcription (i.e., 1 dph for IgM, Rag-1 and MHC-II and fertilized eggs for Ikaros). Data are presented as mean ± S.E. (n=3). The significant differences ($P < 0.05$) in gene expressions are indicated by different letters (a, b, c, d, e, f) over the bars.

Discussion

The expression of adaptive immune genes is of significance for indicating the state of lymphopoiesis and maturation of the adaptive immune system during the development of an organism. In this study, the cDNA sequences encoding adaptive immune genes, viz., Ikaros, MHC-II and Rag-1 were amplified from the total RNA isolated from the kidney tissue of rohu. Kidney tissue was selected for the amplification of adaptive immune genes as it is reported to be the major site of expression of adaptive immune genes in fishes such as rohu[33], common carp[15], striped trumpeter[22], tongue sole[37] and sea bass[38]. Ikaros and MHC-II genes of rohu have been partially sequenced for the first time in this study. The characterized adaptive immune gene sequences of rohu in this study showed high homology with the sequences from fishes belonging to the family Cyprinidae. This corresponds with the earlier report where high degree of immune gene sequence conservation was observed among the members of this family[39].

Humoral adaptive immunity in fish is mediated by immunoglobulins and IgM class is the primary immunoglobulin in most teleosts[40]. The expression of rohu IgM heavy chain gene transcripts were found at very low level until 21 dph and significantly increased from 24 to 42 dph. Similarly, the expression of Ig light chain isotypes in zebrafish by RT-PCR was weak at 3 days post-fertilization (dpf) and thereafter, increased steadily while strong signals were observed only between 21 and 28 dpf[29]. Significantly elevated antibody titre was recorded in the spawn of rohu and catla of 3 weeks post-hatch (wph) exposed to a bacterin by bath immunisation and it was suggested that the development of immunological competence in spawn starts after 3 wph[41]. Hence, the significant increase in IgM heavy chain gene expression beyond 24 dph in the present study might indicate the initial development of immunocompetence in rohu.

Rag-1 is critical to the differentiation of pre-B and pre-T cells, and its expression within an associated primary lymphoid organ can serve as a developmental marker[8]. In the present study, Rag-1 expression was detected from 1 dph onwards and increased significantly during 17 to 42 dph. This result is in accordance with the report of Huttenhuis *et al*[42] where Rag-1 expression was first detected with RQ–PCR in the head of common carp embryos at 3 dpf and also with the report of Hunt and Rice[32] where an elevation in Rag-1 expression of killifish was detected at 2 wph, followed by an increase to the maximum intensity at 3–4 wph. Finally, it is concluded that the increased expression of Rag-1 in rohu at 17 dph onwards may be contributed by the expansion of the differentiating thymocyte population and/or the expansion of maturing (Rag-1 expressing) lymphocyte populations in the head kidney as evident from the earlier reports[29,32].

The generation of T, B and NK lymphocyte lineages from pluripotent hematopoietic stem cells is dependent upon the early expression of the Ikaros locus which by means of alternative splicing produces a variety of zinc finger DNA binding transcription factors[5]. In the present study, Ikaros gene transcripts were first observed in fertilized eggs and no significant increase in expression was found until 24 dph. A significant ($P < 0.05$) increase in expression was observed from 28 to 42 dph. The result is in line with the earlier report that Ikaros expression has been found at 14-somite stage during development in wild-type zebrafish embryos by whole-mount *in situ* hybridization[3] and in trout, beginning roughly at 3–4 days in the yolk-sac stage[5]. Moreover, it was reported in Japanese medaka[43] and zebrafish[29] that Ikaros expression is moderate and only a relatively small change occurs during the early life stages. It was suggested that as Ikaros encodes a transcription factor, its expression is well-regulated and transient in a limited pool of lymphoid progenitors which is rapidly down-regulated when Ikaros-expressing cells switch over to Rag-1 expression, thus limiting any further increase of Ikaros expression[29]. This may be the cause for the relatively small change observed in Ikaros gene expression during the early life stages of rohu in the present study.

MHC is functional in the primary immune defence system and plays an important role in the immune response to antigenic peptides in vertebrates[18]. MHC-II transcripts were first detected at 1 dph and the expression reached the maximum between 28 and 42 dph in the present study. This result corroborates the earlier findings that MHC class-II transcripts are detectable in common carp embryo at 1 dpf, and the transcript levels increase up to 2 weeks in whole fish larvae and lymphoid tissues of 28 dpf carp larvae by semi-quantitative PCR[27]. The early expression of MHC class-II in carp larvae suggests a fast developing immune system[44].

With the work carried out in the present study, it can now be confirmed that the adaptive immune genes express in the early stages of life cycle, i.e., from fertilized eggs to 42 dph and the onset of significant increase in expression is observed between 3 and 4 wph, viz., IgM (24 dph), Ikaros (28 dph), Rag-1 (17 dph) and MHC-II (28 dph). These results suggest that the initial development of immunocompetence occurs during 3-4 wph in rohu. However, at this point, the immune system of the larvae is still likely to be immature as larvae will only become immunocompetent after the lymphoid organs become mature[45]."

Adaptive immune system is not functional in fish during the time of hatching and it depends on maternal immunity to combat the microbes found in the aquatic environment. Response to vaccination depends on adaptive immune system of fish which starts maturing during early development stages. Hence the study on ontogeny of adaptive immune system in fish helps to identify the most appropriate developmental stage for vaccination. Vaccination at the correct stage prevents disease outbreaks and has a considerable impact on aquaculture industry and environment by preventing loss due to diseases and indiscriminate use of antibiotics. The generated information may contribute to the better understanding of the adaptive immune system in embryonic and larval stages of rohu. However, further investigations at protein level need to be carried out to get detailed information on the ontogeny of adaptive immune system.

Further investigation on the ontogeny of adaptive immune system of rohu would be helpful to decide the earliest age at which rohu can be immunized. It would be of interest to investigate on vaccinating the larvae after immunocompetence could result in the production of quality, disease-resistant larvae and improved survival during early life stages of rohu.

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