Sequence information, ontogeny and tissue-specific expression of complement component C3 in Indian major carp, *Labeo rohita* (Hamilton)

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The complement system is one of the first line of immune defence mechanisms as well as a modifier of acquired immunity. C3 is the central complement component primarily synthesized in liver. The local synthesis of C3 in tissues other than liver may play an important role in local inflammatory processes. The present study aims at looking into ontogeny of C3 in *Labeo rohita* and its tissue-specific expression that is yet to be explored for Indian carps. Unfertilised eggs, and eggs after 0, 1, 3, 6, 12 h post-fertilization and hatchlings at 24 h, and 3 and 7 days post-fertilization were collected from three brood fish of *L. rohita* (rohu). Total RNA was extracted from ~50 mg of tissue and subjected to RT-PCR using heterologous carp primers to amplify C3 fragment. A product of 155 bp size of rohu C3 was amplified, the deduced amino acid sequence of which had 91.1% similarity to that of *Cyprinus carpio* C3. C3 mRNA was not detected in unfertilized and 6 h post-fertilised eggs. C3 transcripts were detected 12 h post-fertilisation. Similarly, tissues from liver, spleen, kidney, muscle, brain, gonads, intestine, blood, heart and gills collected from juveniles of rohu were subjected to detection of C3 transcripts by RT-PCR and C3 mRNA was detected in all the tissues. Thus, it is concluded that there is extra-hepatic synthesis of complement (C3) in *L. rohita* and the synthesis of this component occurs only 6 h post-fertilisation.

**Keywords:** Complement factor C3, *Labeo rohita*, Ontogeny, Tissue-specific expression
pattern, around the nuclei, while production was located all over the yolk sac in older embryos of common carp. The activity of complement has only been studied and detected in the serum of Indian major carps that forms mainstay of Indian aquaculture.

However, no such attempt has been undertaken to see the existence of any of the complement components during development in Indian carp species. In this backdrop the present study aims at ontogeny and tissue-specific expression of C3 in rohu, *Labeo rohita* (Hamilton).

**Materials and Methods**

**Fish** — *L. rohita* were collected from the farm of Central Institute of Freshwater Aquaculture, Bhubaneswar. For ontogenic study, three pairs of sexually mature female and male rohu were taken. The three pairs of fish were bred following standard induce breeding technique using "Ovaprim" (Biomeda MTC Animal Health, Canada).

**Tissue sample collection** — Eggs were collected from each female by stripping after 6 h of the hormone injection. The males were stripped individually to collect sperm and the same was used to fertilize eggs collected from individual females. Fertilized eggs and hatchlings were collected 0, 1, 3, 6, 12, 24 h, and 3 and 7-days post-fertilization. For tissue-specific expression study, three healthy advanced fingerlinge of rohu (∼200 g) were taken. Tissue from nine different organs such as liver, kidney, heart, gill, muscle, intestine, brain, spleen and blood were collected from each of the three fish separately after anaesthetizing them with MS-222 (tricane methane sulphonate) (Sigma). All the tissue samples (eggs, hatchlings and tissue) were collected from the farm of *L. rohita* (Ambion) aseptically and preserved at –20°C until extraction of RNA.

**RNA extraction** — Total RNA was extracted from 50-100 mg each of above tissue samples. RNA was extracted from three samples of each stage/tissue by using TRI reagent (Sigma), as per manufacturer's instruction. RNA was dissolved in DEPC-treated water and stored at –20°C until further use. The concentration of the nucleic acid in the sample was quantified by measuring absorbance at 260 nm. Purity of the sample was checked by measuring the ratio of OD260/OD280 nm.

**First strand cDNA synthesis** — Total RNA (1 μg) was used for first strand cDNA synthesis using thermocycler (Eppendorf Mastercycler gradient) by incubating with 1 μl of random hexamer (50 μM) at 72°C for 5 min. The reaction was cooled at 25°C for 10 min to allow primers to anneal to the RNA after which the following components were added to the reaction in order: 2 μL of 10× MMLV-RT buffer, 0.25 μL of RNase inhibitor (40 UμL⁻¹), 2 μL 100 mM dNTPs, 4.75 μL of DEPC water and 1.0 μL of MMLV-RT (200 UμL⁻¹, Promega). The reagents were gently mixed and incubated for 1 h at 42°C. The reaction was terminated by heating at 95°C for 5 min.

**Polymerase chain reaction and agarose gel electrophoresis** — The PCR was performed to amplify C3 and β-actin (a house-keeping gene) to check the integrity of RNA) using heterologous primer pairs obtained from Genie, India. The sequences of the primers used were *C3CCF5*′*C3CCR5*′ for C3 and *CCBA1F*′*CCBA1R* for β-actin. All amplification reaction consisted of an initial denaturation at 95°C for 3 min prior to 28 cycles of 95°C denaturation for 45 sec, 60°C annealing for 1 min 30 sec (55.3°C for β-actin), and 72°C extension for 45 sec, followed by a final 72°C extension for 10 min using 1.5 units of Taq DNA polymerase (Genei, India). The generated PCR products (8 μl) were analyzed by electrophoresis on 1.0% agarose gel.

**Cloning** — The C3 gene PCR product was purified by PCR product purification kit (Genei, India) and sub-cloned into pGEMT vector (Promega) as per manufacture’s instructions. The plasmid DNA from the above was further purified by standard phenol-chloroform extraction method before sequencing. Three positive clones for each species PCR product carrying the desired gene inserts were used for sequencing.

**Sequencing** — Sequencing was done using the cycle sequencing kit (Bigdye Terminator V.3.1, ABI, USA) with T7 universal primer (New England Biolab) in 310 Genetic Analyzer, ABI, U.S.A. Nucleotide sequences of C3 cDNAs were determined from three separate clones of each species.

**Sequence analysis** — The nucleotide sequences of C3 cDNA of rohu and deduced amino acid sequences were analyzed using the BioEdit Sequence Alignment Editor (version 7.0.5.3)11. Multiple alignments of the derived amino acid sequence of rohu C3 along with other published C3 sequences were carried out by the
above program using CLUSTAL W. A sequence identity matrix was created also using the above software to find the percent homology between the C3 amino acid as well as nucleotide sequences of different species. A phylogenetic tree was constructed by the bootstrap neighbor-joining method using CLUSTAL X (version 1.83) in PHYLIP format and visualized by TreeView.13

Results

Amplification of C3 mRNA of rohu and sequence analysis — Initially total RNA was extracted from liver samples of three rohu individuals and subjected to RT-PCR using heterologous C3 primer pairs. A band size of 155 bp was obtained in each case. The PCR products (three) cloned into pGEMT vector carrying the desired inserts of expected size (as determined by plasmid isolation and restriction analysis) were sequenced. The three sequences were aligned and the final rohu C3 mRNA sequence information was obtained (Fig. 1). The same sequence was confirmed to be as C3 using BLAST with other species C3 gene based on the similarity index.

Alignment of rohu C3 deduced amino acid sequence with C3 sequences of other species — Using BioEdit Sequence Alignment Editor, the rohu C3 amino acid sequence was aligned with published C3 sequences of Hippoglossus hippoglossus, Anarchichas minor, Paralichthys olivaceus, Bos taurus, Gallus gallus, Rattus norvegicus, Oryctolagus cuniculus, Mus musculus, Homo sapiens, Oncorhynchus mykiss, Oryzias latipes, Cyprinus carpio and Ctenopharyngodon idella. Highly conserved amino acid blocks were found in all species examined (Fig. 2.). The deduced amino acid sequence

Fig. 1 — Partial nucleotide sequence of L. rohita complement component C3 gene.

![Fig. 1](image.png)

Fig. 2 — Multiple alignment of L. rohita complement component C3 deduced amino acid sequence with that of other carps. Identical (*) and similar residues (: or .) identified using CLUSTAL W are indicated. Accession numbers are: Hippoglossus hippoglossus, AAW72004; Anarchichas minor, CAC29154; Paralichthys olivaceus, BAA88901; Bos taurus, CAJ32149; Gallus gallus, NP_990736; Rattus norvegicus, NP_058690; Oryctolagus cuniculus, P12247; Mus musculus, AAH43338; Homo sapiens, AAR89906; Oncorhynchus mykiss, AAB05029; Oryzias latipes, BAA92285; Cyprinus carpio, BAA36619; Ctenopharyngodon idella, AAQ74974 and Labeo rohita, AM773825.

![Fig. 2](image.png)
of rohu C3 after alignment showed identity of 52.9% with Hippoglossus hippoglossus, 58.8% with Anarhichas minor, 56.8% with Paralichthys olivaceus, 47.0% with Bos taurus, 39.2% with Gallus gallus, 49.0% with Rattus norvegicus, 47.0% with Oryctolagus cuniculus, 49.0% with Mus musculus, 49.0% with Homo sapiens, 56.8% with Oncorhynchus mykiss, 58.8% with Oryzias latipes, 91.2% with Cyprinus carpio, and 86.2% with Ctenopharyngodon idella (Table 1).

Phylogenetic tree analysis — The phylogenetic tree based on the C3 coding sequence and taking into consideration of length of nucleotide sequence of rohu showed a close relationship of Labeo rohita (rohu) with Cyprinus carpio and Ctenopharyngodon idella (Fig. 3). The C3 gene of the five marine fishes and five endothermic species forms two different clusters. The C3 gene of avian species Gallus gallus forms a separate sub-cluster close to endothermic species.

Ontogenic appearance of C3 mRNA — C3 transcripts of rohu were not detected in unfertilized eggs and up to 6 h post-fertilisation. C3 mRNA transcripts were detected by RT-PCR from 12 h post-fertilization to 7 day post-hatch (Fig.4).

Tissue-specific expression of C3 mRNA in rohu — RT-PCR analysis revealed presence of C3 mRNA transcripts in intestine, heart, gill, muscle, liver, spleen, brain and blood of fish. However, strong amplification of C3 product was observed in liver, blood and muscle (Fig. 5).

Discussion
Being a central component of all activation pathways, C3 is crucial for a complete innate immune response to occur. For lysis of pathogens to occur, all components of the membrane attack complex (MAC) are essential. C3 leads to inflammatory reaction such as chemotaxis, opsonization and lysis of pathogens. Liver is the richest source of complement, while other tissues also contribute to its synthesis and this varies from species to species4,5.

The C3 primer pairs used here were based on common carp C3 sequence. A similarity to the tune of 92.1% at deduced amino acid level was

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Table 1— Similarity indices for partial complement component C3 amino acid sequences of different species with that of L. rohita.

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<th>Hippoglossus hippoglossus</th>
<th>Anarhichas minor</th>
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<th>Bos taurus</th>
<th>Gallus gallus</th>
<th>Rattus norvegicus</th>
<th>Oryctolagus cuniculus</th>
<th>Mus musculus</th>
<th>Homo sapiens</th>
<th>Oncorhynchus mykiss</th>
<th>Oryzias latipes</th>
<th>Cyprinus carpio</th>
<th>Ctenopharyngodon idella</th>
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obtained with sequence of C. carpio. The product was further confirmed of C3 gene by drawing sequence information of the obtained product and further aligning the same with commonly related species.

The presence of C3 component is present before or after hatching or in unfertilized eggs in the developing rohu embryo was not known. In the present study, the developing rohu embryo showed C3 mRNA transcripts at 12 h post-fertilization. There are differential findings regarding presence of C3 mRNA transcripts before or after fertilization among different fish species. C3 mRNA was not detected in unfertilized eggs of rohu as marked in Atlantic salmon\(^4\). In another study, rainbow trout eggs, embryos and hatchlings were assayed for the onset and duration of C3-1, C3-3, C3-4, C4, C5, C7, factor B and factor D transcription using real time reverse transcription-polymerase chain reaction (RT-PCR). The complement transcript levels increased steadily in rainbow trout from day 18-post fertilization to hatch followed by a decrease during yolk sac resorption\(^5\). In fertilized cod eggs (G. morhua) C3 β-chain was detected from day 7 post-fertilization\(^1^4\) while in spotted wolfish C3 RT-PCR products were detected at 49 days post fertilization\(^1^5\). Similarly, immuno-blotting technique revealed presence of C3 protein in the unfertilized eggs of Atlantic salmon (Salmo salar).
but C3 mRNA was not detected after fertilization by real-time RT-PCR\(^4\). Lange et al.\(^6\) also demonstrated presence of C3 in yolksac membrane from day 1 post hatching and in several other organs from day 2 post hatching in Atlantic cod by immunohistochemistry. In carp (\textit{Cyprinus carpio}) the C3 protein (only the \(\beta\)-chain) was found in unfertilized eggs. At two days post fertilization, another small band (~40 kDa), in addition to \(\beta\)-chain was detectable using Western blot. However, C3 mRNA was expressed from about 12 h post-fertilization as in the present study, which is relatively long before hatching, indicating its significant role in immunity of the embryos. C3 expressions peaked at 1-2 days post-fertilization in \textit{Cyprinus carpio} ensuring sufficient levels around hatching time\(^10\). Three sub-types of C3 transcripts were found at increasing levels pre-hatching and at decreasing levels in the period during yolk sac resorption in embryos of rainbow trout eggs\(^14\). Similar to rohu C3 development, C3 mRNA appeared before hatching in the fertilized eggs of the halibut. The levels of C3 mRNA appeared relatively consistent in the period after hatching\(^9\).

It is well known that liver is the major site of C3 synthesis, however, the information was not available with rohu. Tissue-specific expression of C3 mRNA in the juveniles of this species by RT-PCR analysis revealed presence of C3 mRNA transcripts in intestine, heart, gill, muscle, liver, spleen, brain and blood. However, strong amplification occurred in liver, blood and muscle. This result showed the expression of C3 mRNA transcripts in extra-hepatic tissues. Similar studies on rainbow trout showed that the highest extra hepatic levels of synthesis occurred in the gills, skin, skeletal muscle and heart\(^4\). Lange\(^6,7\) showed the expression of C3 in the cord, eye, intestine, oesophagus and in kidney of 30, 50 and 99 day old halibut larvae by in \textit{situ} hybridization. Whereas, RT-PCR analysis revealed the presence of C3 mRNA expression in the liver and a small amount was found within spleen of adult halibut\(^9\). In common carp, C3 is produced in the yolk syncytial layer of embryos. Huttenhuis\(^10\) et al. found that C3 production started in a circular pattern, around the nuclei of the yolk syncytial layer, while production was located all over the yolk sac in older embryos. The yolk syncytial layer is a structure unique to fish and is not found in other vertebrate embryos. After the absorption of the yolk material C3 is produced in the liver. The presence C3 in brain tissue of rohu may be correlated to the presence of C3 protein in the cerebrospinal canal in common carp embryos. The brain and spinal cord are effectively shielded from plasma components by the blood brain barrier, which implicates that these components are synthesized in the brain itself as was shown in mammals\(^8\).

Although the study is very preliminary in nature it adds a lot to generate basic information on C3 gene that can be subsequently exploited for any immunomodulation, disease resistance or molecular pathogenesis studies related to this fish species. In addition, this immune related gene expression profile could be used as rohu health reference marker.

**Acknowledgement**

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**References**


