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Studies of marker for the development of adaptive immune system during the larval ontogeny of angelfish

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Abstract
The early larval stages of fish are susceptible to microbial infections until the maturation of lymphoid organs. Fishes are capable of responding the pathogens specifically by activating the adaptive immune system. During the maturation of lymphoid organs, phenomenon of V(D)J recombination is mediated by recombination activating genes (RAGs), which act as the marker of physiological maturity of the adaptive immune system. In the present study, the RAG-1 nucleotide sequence was partially cloned (272bp) in order to assess its pattern of mRNA expression in different tissues and during the larval developmental stages of *Pterophyllum scalare*. The RAG-1 mRNA expression was detected in early larvae right from the first day after hatching. Later, an up-regulation in the expression was observed upto 30 dph followed by a declined trend till the end of experiment. Moreover, the tissue distribution of RAG-1 revealed a significantly high expression level in kidney than other tissues which demonstrate it to be a major lymphoid organ. Hence, the current study concludes that the complete maturation and development of lymphoid organs based on the mRNA expression of RAG-1 occurs at 27 to 30 DPH in *P. scalare*.

Keywords: *Pterophyllum scalare*; RAG-1; kidney; immune system

1. Introduction
The adaptive immune response of vertebrates involves a variety of antigenic receptors encoding immunoglobulin (Ig) and T-cell receptor (TCR) genes during the early development of B- and T-cells [1]. The repertoire of Ig and TCR genes is arranged by the phenomenon of V[D]J recombination which is triggered by the recombination activating gene 1 (RAG-1) [2, 3, 4].
The amino acid sequence of recombination activating gene-1 (RAG-1), is highly conserved among species from elasmobranchs to mammals [5, 6, 7] and has been reported as a significant marker of physiological maturity of the immune system [8]. The RAG-1 protein is crucial for the differentiation of immature B- and T-cells and is expressed in different lymphoid organs [9]. Therefore, this expression makes the RAG-1 gene important for studying the developmental pattern of adaptive response of immunity in several fishes [10].
Among the aquarium fishes, *P. scalare* (angelfish) belonging to Cichlidae family is considered as the most attractive and graceful group of fish. Due to mixtures of strains and the ease of maintenance, freshwater angelfish are also the most popular tropical aquarium fish. Early developmental stages of fish are prone to several infectious diseases and significant loss due to larval mortalities in various fishes has been reported throughout the world [11]. Thus, this study aimed to evaluate the mRNA expression of RAG-1 gene in different developmental stages and tissues of angelfish. The study could be helpful to provide the crucial information regarding the ontogeny of the immune system, with implications for vaccination regimes.

2. Materials and Methods
2.1 Sample collection
To study the expression of RAG-1 in different normal healthy tissues, adult angelfish (6-7 cm) were sacrificed after anaesthetizing with clove oil and tissues like liver, kidney, spleen, intestine, gill, skin and brain were collected. The tissues were pooled from six animals into three separate samples. Larval developmental stages were collected from Ornamental Fish Breeding Unit, Division of Aquaculture, ICAR-Central Institute of Fisheries education,

Mumbai. Larval developmental stages including unfertilized egg (UF) and 0 day post-hatch (dph) and fifteen subsequent stages (3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42 and 45 dph) were obtained for the study. The first day after hatching was considered as 0 dph. The larval developmental stages were pooled in three separate samples and were used for RNA extraction. All the samples were collected aseptically in RNA stabilizer (Himedia) and preserved at -80 °C until RNA isolation.

2.2 RNA extraction and cDNA synthesis
Total RNA was extracted from all the collected samples using TRIzol™ Reagent (Invitrogen, USA). The isolated RNA was treated with DNase I (Thermo Scientific, USA) in order to remove the residual genomic DNA as per the manufacturer's protocol. The purity and concentration of the treated RNA were then determined at OD of 260/280 by using NanoDrop-2000 spectrophotometer (Thermo Scientific, USA). One microgram of DNase-treated RNA was reverse transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) primed with Oligo dT primer.

2.3 Primer designing, cloning and sequencing
Primers for partial amplification of RAG-1 were designed (Table 1) from the reported conserved sequences of these genes from closely related species, using the Gene Runner software (v. 4.0.9.62). Partial amplification reaction was carried out by PCR, using a total volume of 20 µL containing 2.5 µL of 10X Taq buffer, 2.5 µL of 25 mM $MgCl_2$, 0.5 µL of 10 mM dNTP mix, 1 µL each of 10 pmol forward and reverse primers, 1 µL of cDNA, 0.25 µL of 5 U/µL Taq polymerase and 11.25 µL of nuclease-free water. The reaction follows the conditions: initial denaturation at 95 °C for 4 min, denaturation of 35 cycles of 95 °C for 30 sec, followed by annealing at 56 °C for 30 sec and extension at 72 °C for 40 sec and a final elongation of 10 min at 72 °C by 96-well ABI 7500 RT-PCR system (Applied Biosystems, USA).
PCR products were cloned into the pTZ57R/T vector, using Ins. TAclone™ PCR Cloning Kit (Thermo Fisher scientific, USA) as per the manufacturer's protocol, sequenced and confirmed by BLAST analysis.

2.4 Homology and Phylogenetic analysis of RAG-1 gene
The homology analysis of the RAG-1 putative protein was done by BLASTp algorithm of the National Centre for Biotechnological Information (NCBI). Phylogenetic analyses of the RAG-1 putative protein with that of already reported sequences of the same protein of other fish species and mammals obtained from NCBI was deduced using MEGA 7.0 software.

2.5 qRT-PCR primer designing and PCR efficiency
Primers for qRT-PCR were designed from the partially amplified sequence of *P. scalare* RAG-1 (Table 1), using Gene Runner software (v. 4.0.9.62) and synthesized by Eurofins Genomics India Pvt. Ltd. The predicted amplification size of the designed qRT-PCR primers was 100 to 200 bp in length. Before performing any qRT-PCR analysis, the efficiency (E) and correlation coefficient (R^2) of the primers were determined by preparing 10-fold serial dilutions (10, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) of kidney cDNA and were used to generate the standard curves. The efficiency was calculated using the formulae: $E\ (\%) = (10^{1/slope} - 1) \times 100$. Desired E value ranged between 90% and 110%.

Table 1: Primers used for the amplification and qRT-PCR analysis of RAG1 gene

Primer name	Primer sequence (5'–3')	Tm (^{0}C)	Purpose
RAG1 F	ATGTTGGCATTATTAATGGGCTCTC	56	Partial amplification
RAG1 R	CAGTTCTGAATTTGGCTTTGGCTC		
β-actin qRT F	GTACGTTGCCATTCAGGCTGTGC	57	Quantitative PCR
β-actin qRT R	GGGCAGGGCATAACCCTCATAGA		
qRT-RAG-1 F	GCCCCTCCACGATGTCCTCCTCC	63	Quantitative PCR
qRT-RAG-1 R	TTCCTCGGTGGATGACGCCCCA		

2.6 qRT-PCR analysis
The mRNA expression levels of RAG-1 during various above mentioned developmental stages and tissues of *P. scalare* was carried out in AriaMx Real-time PCR system (Agilent, United States). The 25 µL reaction mixture was prepared containing 12.5 µL of 5X SYBR® Premix Ex Taq™ II-Clontech (Takara, India), 1 µL each primer (10 pmol), 1µL of cDNA and 9.5 µL nuclease-free water. Then, each sample was divided into two wells with a final volume of 10 µL. The conditions used for PCR amplification were 95 °C/10 min, followed by 40 cycles of 95 °C/20 sec, annealing at 63 °C (RAG-1) for 30 sec and extension at 72 °C/30 sec respectively. The relative level of mRNA expression was analysed using $2^{-\Delta Ct}$ method [12].

2.7 Ethics Statement
The care and sampling of animals used in this study were carried following the guidelines of the CPCSEA (Committee for Control and Supervision of Experiments on Animals), Ministry of Environment and Forests (Animal Welfare Division), Govt of India on care and use of animals in scientific research.

2.8 Data analysis
Data were analysed by one-way ANOVA using SPSS 22.0 software (SPSS Inc., USA) and the significance level determined at $P < 0.05$. Shapiro-wilk's test was performed to carry out the normality and, equality of variances was assessed by Levene's test before performing ANOVA.

3. Results and Discussion
The immune system of the early developmental stages of fish is not fully developed [13], and the study on the mRNA expression of RAG-1 is of significance for specifying the state of maturation of the immune system in *P. scalare*. RAG-1 is an essential gene for the adaptive immune response, hence, it's up-regulation can help in mitigating the problem of high mortality in early developmental stages [13, 14].

3.1 Sequence analysis and PCR efficiency of RAG-1 gene
The partial sequence of RAG-1 was cloned and sequenced in the present study with a nucleotide base pair length of 272bp (Fig.1). The sequence was submitted to the NCBI, GenBank database under the accession number *KY413808*. In the BLASTp algorithm of NCBI, the RAG-1 putative protein

sequence displayed 52-54% and 72-97% sequence similarity with mammals and other finfish respectively. The selected genes of *P. scalare* were found to be conserved across the vertebrates, especially cichlid fishes which can be seen from the phylogenetic neighbour joining tree analysis (Fig. 2).

The RAG-1 gene was amplified by qRT-PCR from different tissues and developmental stages of *P. scalare.* The PCR efficiency values of RAG-1 gene ranged from 98% to 102% and the R^2 values ranged between 0.982 and 0.995.

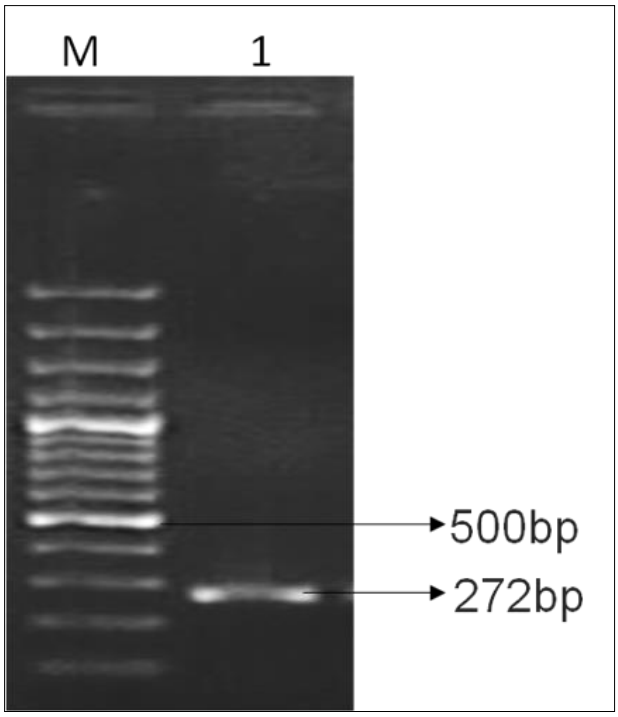

Fig 1: PCR amplified product, Lane M: 100 bp plus ladder (Fermentas); Lane 1: RAG-1 (272 bp)

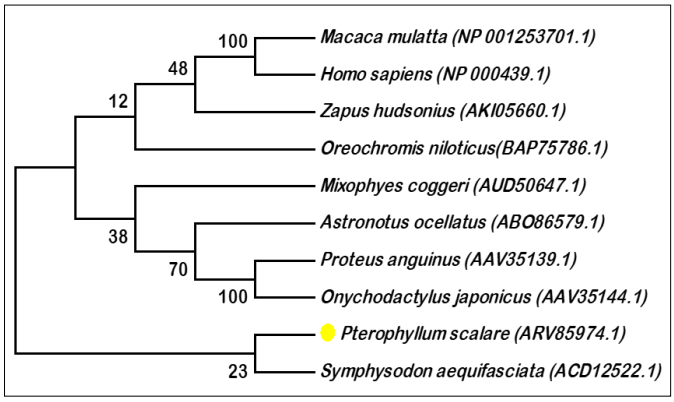

Fig 2: Phylogenetic analysis of *P. scalare* RAG-1 protein was done using MEGA 7.0 software. Phylogenetic relationship with other piscine and selected mammalian RAG-1 proteins. The evolutionary tree was formed using the neighbor-joining method and a bootstrap analysis with 1000 replicates was used to assess the strength of modes in the tree. Accession numbers are given in the brackets

3.2 mRNA expression of RAG-1 in larval stages

Till now, the present study is the first report on the mRNA expression of RAG-1 in *P. scalare* which was investigated from 0 dph to 45 dph developmental stages along with unfertilized egg. The mRNA expression pattern of RAG-1 was evaluated at different developmental stages of *P. scalare* and a variable level of expression was observed between the selected time points. The RAG-1 expression was detectable during the early stages from the first day after hatching including the unfertilized egg stage although at insignificant level (Fig.3). The mRNA expression level started a significant increase from 12 dph onwards and reaches its peak at 27 dph where a significantly high transcript level of RAG-1 was observed. Later, the mRNA expression gradually started down regulating from 33 dph till the end of experiment at 45 dph.

The detection of mRNA expression in unfertilized egg could be due to the maternal transfer as previously mentioned in the report of Lee *et al.* [14]. The appearance of the mRNA expression level during the different developmental stages of olive flounder and Loach were previously observed [13, 14]. A similar pattern of RAG-1 expression has been reported with slight variations as that of present study [10]. Our results were in agreement with the study where zebrafish showed an increase in RAG-1 expression levels from 3 to 17 days post fertilization (dpf) with a peak at 21 to 28 dpf and then a gradual decrease in expression over time during its ontogenetic development [15]. The study was supported by the previous report in which mRNA expression of RAG-1 appeared at 0 dph then increased till 21 dph in haddock fish [16].

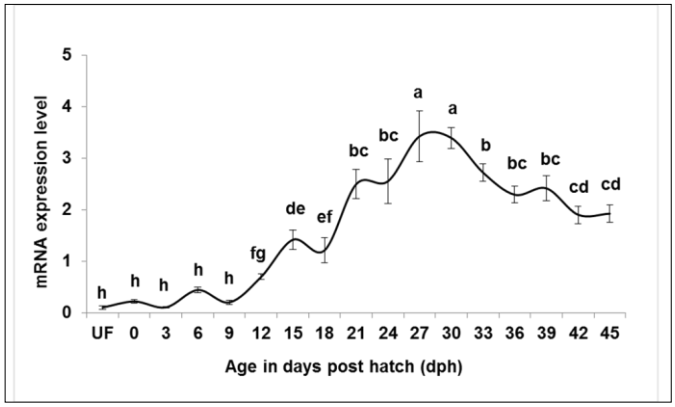

Fig 3: qRT-PCR analysis of RAG-1 mRNA expression during *P. scalare* larval development (mean ± SEM, n=3). Different superscript letters indicate significant differences ($P < 0.05$). UF-unfertilized eggs

3.3 mRNA expression of RAG-1 in different tissues

The expression of RAG-1 mRNA transcript level was investigated in different selected tissues of adult *P. scalare* using qRT-PCR. The RAG-1 expression was found ubiquitously in all the tissues including kidney, spleen, intestine, skin, liver, brain and gill of *P. scalare*, where a varied level of mRNA expression was observed (Fig.4). Among the selected tissues, kidney showed significantly high expression followed by spleen, brain, liver and intestine. The mRNA transcript level of RAG-1 was not detected in skin tissue.

The results demonstrated the ubiquitous expression of RAG-1 in all the tissues examined and significantly expressed in the kidney, supporting the idea that kidney is the major lymphoid organ in teleosts [7]. Several studies have also reported the expression of RAG-1 in different immune related organs [7, 17, 18, 19]. However, the RAG-1 mRNA has been previously reported to be expressed in non-lymphoid organs comprising gill, intestine and muscle of fish [13, 14]. Hence, the study concludes the development and maturation of lymphoid organs at around 27 dph in angelfish with kidney as the major lymphoid organ based on the mRNA expression pattern of RAG-1.

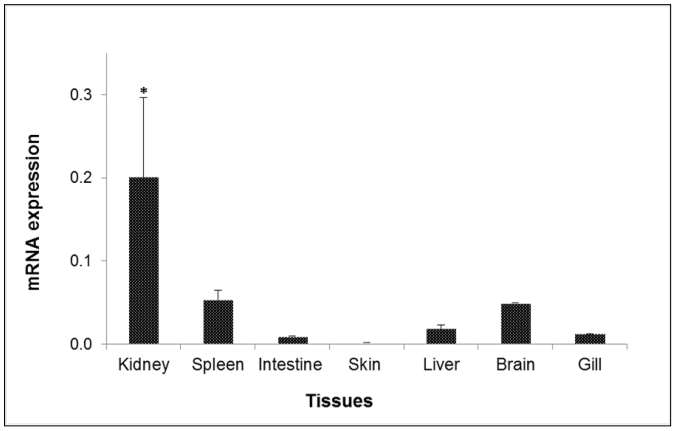

Fig 4: qRT-PCR analysis of RAG-1 mRNA expression in different tissues of *P. scalare* (mean ± SEM, n=3). Asterisk (*) superscript indicate significant differences ($P < 0.05$)

4. Conclusion

As per the records, this study was the first to conclude the time of development of lymphoid organs in angelfish at 27 dph based on the mRNA expression profile of RAG-1 for promoting the advanced response of immunity. Further, the integrative studies like the histological organisation of lymphoid organs and their histochemical localisation studies are required to understand the development of organs related to adaptive immune system completely.

Conflict of interest

The authors report no conflict of interest.

5. Acknowledgements

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