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Short communication

Transcriptional changes in three immunoglobulin isotypes of rohu, *Labeo rohita* in response to *Argulus siamensis* infection

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ABSTRACT

Immunoglobulin heavy chains of three isotypes viz., IgM, IgD and IgT/IgZ are described in teleosts. In this study, a challenge experiment with an ectoparasite *Argulus siamensis* was conducted to evaluate the changes in adaptive immune response by quantitation of expression of Ig heavy chains in skin, head kidney and mucus of infected rohu, *Labeo rohita*. Rohu were challenged with 100 metanauplii of *A. siamensis*/fish. Head kidney, skin and mucus samples were collected at 0 h, 12 h, 24 h, 3 d, 7 d, 15 d and 30 d by sacrificing four fish each from infected and control groups at each time point. The expression of IgM, IgD and IgZ in these tissues were measured by reverse transcription real time quantitative PCR. IgM level was found to reach its peak significantly 30 d post-infection in head kidney tissue, while IgM transcripts were below detectable range in skin and mucus at all time points. IgZ and IgD levels were significantly up-regulated post-infection in all the three tissue samples. Early up-regulation of IgD was observed in skin and mucus, compared to head kidney. This study showed that parasitic invasion can trigger varied expressions of immunoglobulin types to provide systemic as well as local protection in the host. In particular, the appearance of high level of expression of IgZ and IgD in skin and mucus will pave the way for vaccine development against *A. siamensis* which feeds on those tissues.

1. Introduction

Phylogenetically, fish are the most primitive group of vertebrates to possess an adaptive immune system capable of generating an effective antibody response to pathogenic challenges [1]. The immunoglobulin (Ig) genes encode these defense proteins, antibodies which are the major effective molecules of humoral immunity in vertebrates. The Ig molecule is composed of a basic four chain unit in which two heavy (IgH) chains are held together and two light (IgL) chains are associated with the heavy chains. These associations are generally stabilized by disulfide bridges, although some exceptions do exist [2,3]. In mammals, immunoglobulin heavy chains are of five isotypes, namely IgM, IgG, IgA, IgD and IgE [4]. In teleosts, four types of Igs have so far been reported, namely, IgM [5,6], IgD [7–9], IgZ or IgT [10,11], and IgM–IgZ chimera [4]. IgM was the first immunoglobulin to be studied in many species of teleosts such as salmon (*Salmo salar*) [12], zebrafish (*Danio rerio*) [13] and Atlantic cod (*Gadus morhua* L.) [14]. The existence of IgD in teleosts was first reported in catfish (*Ictalurus punctatus*) [7] and Atlantic salmon [8]. Recently, new Ig isotypes IgZ, IgT and a chimera of IgM–IgZ have been identified from zebrafish [10], rainbow trout (*Oncorhynchus mykiss*) [11], fugu (*Takifugu rubripes*) [15] and common carp (*Cyprinus carpio* L.) [4], respectively. The production of humoral antibodies in response to piscine parasites has received some attention in the past [16] though the information of their gene expression in response to parasitoses is still scarce.

Argulus siamensis, the fish louse feeds on host mucus, skin tissue, tissue fluid and blood. The parasites usually cause only abrasive wounds on the host skin but nevertheless lead to systemic stress responses and modulations of the immune system and physiology [17–20]. *A. siamensis* is controlled mainly by pesticides and at present only a few types are in wide use like deltamethrin and avermectins [21]. However, future of this strategy is questionable due to development of pesticide resistance, occurrence of treatment failures, and undesirable environmental impacts. Hence, there is a need to develop new control methods. Since there exists a possibility of immunization against parasites of the genus *Argulus*

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[22,23]; a better understanding of acquired immune responses becomes imperative for vaccine development. It is essential to identify protective antibody responses following infection and challenge. Until recently, studies have addressed relatively few immune parameters and immune related genes with respect to *Argulus*-host system [17,18,23,24]. Knowledge of immune responses of the host to the parasite, *Argulus* and their role in protection against the parasite is still limited and this host-parasite system is yet to evolve as a research domain.

In this study, we report the modulation in the transcriptional changes in genes encoding three immunoglobulins, namely IgM, IgZ and IgD in response to *A. siamensis* infection over a period of 30 days post-infection. Gene expression profiling was carried out in skin, mucus and head kidney tissues using real-time RT-qPCR analyses.

2. Material and methods

2.1. Animals

Rohu (*Labeo rohita*) juveniles (55.89 ± 0.99 g) showing no signs of disease (under gross and microscopic examination of skin, gill, intestine and kidney tissues of representative samples) and no previous history of parasitic infections were obtained from the farm of the Central Institute of Freshwater Aquaculture, Bhubaneswar, India. Fishes were acclimatized in plastic tanks of 1000 L capacity with tap water, for 15 days before conducting the experiment. They were fed with commercial pellet diet at 2.5% of body weight. About 10% of water was removed daily along with the left-over feed and fecal matter. The basic physico-chemical water parameters were measured systematically at seven-day intervals to maintain at optimal level throughout the experiment. The water temperature in the tanks varied from 25 to 28 °C during the experiment.

2.2. Parasite

A population of *A. siamensis* was maintained on stock rohu (approximately 500 g) in tanks of wet laboratory. The eggs deposited on the sides of the tank were collected in beakers containing tap water and incubated at 28 °C with daily refreshment of water for hatching in a previously standardized way [25]. Upon hatching, the metanauplii were counted and maintained in similar conditions as the eggs till used in the challenge test. Care was taken to use the metanauplii within 6–8 h of hatching.

2.3. Experimental design and sampling procedure

For expression analysis of various immunoglobulin genes, twenty-eight fishes were divided into seven groups (seven time points 0 h, 12 h, 24 h, 3 d, 7 d, 15 d and 30 d) of four fish in each and maintained in separate tanks for the experiment. Another lot of twenty eight fish were also maintained in similar conditions to serve as control for each time point. The rohu juveniles were challenged with 100 numbers of metanauplii each as per earlier standardized protocol [20]. For sample collection, at each time point four fish were collected from the tanks. The mucus (approximately 500 μl) was collected aseptically from the integument of the fish using a glass slide carefully after anaesthetizing the fish with MS222 (Argent Chemical, Redmond, USA) and transferred to TRI reagent for storing at −70 °C until RNA extraction. Skin from the site of parasite attachment (generally around pelvic fin base or ventral surface) and head kidney tissue samples were subsequently collected at 0, 12, 24 h, 3 d, 7 d, 15 d and 30 d post-infection (p.i.) aseptically after sacrificing the fish with overdose of anaesthesia and stored in RNA*later* (Sigma, USA) at −70 °C until extraction of RNA.

2.4. RNA isolation and quantification

One hundred milligrams of skin and head kidney tissues of each fish stored in RNA*later* were utilized for extraction of total RNA using TRI reagent (Sigma, USA) following the manufacturer's instructions. RNA was also extracted from the mucous samples stored directly in TRI reagent. The resulting RNA was treated with RNase-free DNase I (Fermentas, USA) followed by inactivation of DNase I according to the manufacturer's instructions. The concentration of the total RNA in the sample was quantified by measuring absorbance at 260 nm. The purity of the samples was also checked by measuring the ratio at OD260 nm and OD280 nm using NanoDrop ND1000 (Thermo Scientific, USA) with expected values between 1.9 and 2.0. The integrity of RNA was checked by electrophoresis on 1% agarose gel containing 0.5 μg/ml ethidium bromide at 100 V and also screening through RT-PCR using β-actin expression. Out of the four RNA samples collected for each time point, three best samples were used for further expression study.

2.5. cDNA synthesis

One microgram of total RNA was used for generation of first-strand complementary DNA by reverse transcription using Thermo Scientific RevertAid First Strand cDNA synthesis kit (Thermo Fischer Scientific Inc., USA) following the manufacturer's instructions. Briefly, template RNA (1 μg) was added to random hexamer primer (1 μl) and nuclease free water to make the volume to 12 μl. Subsequently, 5× reaction buffer (4 μl), Ribolock RNase inhibitor (1 μl), 10 mM dNTP mix (2 μl) and RevertAid M-MuLV RT (200 U/μl) (1 μl) were added to make the final volume of the reaction mixture to 20 μl. The mixture was incubated at 25 °C for 5 min followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min. The reverse transcription reaction product was stored at −20 °C for further use in the expression studies.

2.6. Real time quantitative PCR

The primer pairs for IgM (F-5′ACGCTTCACCATCTCCA3′ and R-5′AGCCACCGTAGCCTCTT3′), IgZ (F-5′CAGCCCTAAACTCGG3′ and R-5′GGTTGTGCCCTCTATGT3′) and IgD (F-5′GGGACTCAAAGCAAAGAA3′ and R-5′TAACCTCACAGGCAAAGAC3′) were self-designed using Primer Premier 5 (version 5.0, Premier Biosoft International, Palo Alto, CA) from transcriptome data of rohu [26] and homologous sequences of related species available in NCBI database (http://www.ncbi.nlm.nih.gov). β-actin was used as the reference gene based on our earlier observations (20). Real time quantitative PCR was carried out using cDNA samples of mucus, skin and head kidney of rohu for IgM, IgZ and IgD expression analysis in Light Cycler 96 SW 1.1 (Roche, Germany) using FastStart Essential DNA GreenMaster (Roche, Germany) according to the manufacturer's instructions after standardization. Briefly, 1 μl of cDNA synthesized was used as a template in a total reaction mixture of 10 μl containing 5 μl of 2× Light cycler SYBR green I mix, 0.5 μl each of primer pairs (5 pmole) and 3 μl of H_2O provided in the kit. The qPCR program consisted of pre-denaturation at 95 °C for 10 min and 45 cycles of amplification at 95 °C for 10 s, annealing temperature Ta (58 °C for IgZ and 59 °C for IgM and IgD) for 10 s, and 72 °C for 20 s. All reactions were performed simultaneously for each gene with β-actin, in the same plate in triplicate. qPCR specificity was verified by melt curve analysis at a temperature of 95 °C for 10 s, 65 °C for 1 min and 95 °C for 1 min. No-template controls were also included in each run. Tm analysis was done to check primer specificity.