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DIFFERENTIAL EXPRESSION OF TOLL-LIKE RECEPTORS (TLRS) IN GOLD FISH, *CARASSIUS AURATUS* INFESTED WITH FRESH WATER LICE OF *ARGULUS* SP.

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ABSTRACT

Toll like-receptors (TLRs) are pattern recognition receptors (PRRs) that are involved in the recognition of the pathogens based on their conserved motifs, called pathogen associated molecular patterns (PAMPs). As the information on the TLR/s involved in the recognition of the parasitic ligand is limited, the objective of this study was to assess the expression profiles of six different TLRs (2, 3, 4, 7, 9 and 22) in various organs *viz.,* skin, intestine, liver and kidney of gold fish, *Carassius auratus* in response to parasitic infestation with freshwater lice of *Argulus* sp., by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Observations of the study showed tissue-specific and differential expression of TLRs in tissues of *C.auratus*. TLRs 2, 4 and 7 expressions were upregulated in all the tissues studied. TLR 9 expression was down regulated in all the tissues studied. TLR22 was upreglated significantly in liver and in skin the organ immediately affected by the parasite. Expression of TLRs 2, 3, 4, 7, 9 and 22 in gold fish are modulated during infestation by *Argulus* sp., in *C.auratus*

KEY WORDS: **Gold fish, TLR expression, *Argulus*, parasitic ligand, semi-quantitative RT-PCR.**

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INTRODUCTION

Immunity in a host is of two types namely, innate and acquired immunity. Innate immunity is non-specific and is always present in the host. Adaptive immunity is the specific immunity produced in response to infections caused by the pathogens[1]. Fishes largely rely on the non-specific immune system which forms the first line of defense to protecting them against the invading pathogens. Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) involved in the recognition of the pathogens based on pathogen associated molecular patterns (PAMPs) and alerts the host about the pathogens to induce the immunity in a host. About 17 different TLRs have been reported in fish[2], each with its own specificity in recognizing the pathogens. Increasing economic importance of fish parasitoses for aquaculture and fisheries has aroused the interest in the research related to the defense mechanisms of fish against parasite infestations[3]. Although the involvement of the TLRs in recognizing the human protozoa like *Trypanosoma* sp., *Toxoplasma gondi*, *Leishmania major* and *Plasmodium falciparum* have been reported [1,3,4], information on the involvement of TLRs in the recognition of parasites in fishes is scanty. Hence, this study was carried out to improve our understanding on the immune response during parasite infestation which would help to develop strategies to control the losses in aquaculture due to parasite infestation.

MATERIALS AND METHODS

(i) *Experimental design*

Healthy gold fishes of approximately 10g size showing no clinical signs of diseases or previous history of parasitic infestations were obtained from a local fish farm in Chennai, TamilNadu, India. Gold fish infested with *Argulus* sp., were collected from various ornamental fish rearing units in and around Chennai. The fishes were acclimatized and maintained in lab conditions with adequate feeding and aeration. The healthy fishes were divided into two groups *viz.,* control and treatment group for conducting experiment. Each group was maintained in triplicates in experimental troughs with 10 numbers of fishes each. In each experimental trough of the treatment group, about twenty numbers of matured *Argulus* parasite were collected from the parasite infested fishes were introduced. The fish were grossly observed daily for the parasitic infestation. After 7 days, the fishes of control and treatment groups were dissected and the tissue samples of skin, liver, intestine, and kidney were collected aseptically and were either used immediately for total RNA extraction or stored at -80°C until use.

(ii) *Total RNA extraction and cDNA synthesis*

Total RNA was extracted from tissue samples using a commercial total RNA extraction reagent (Invitrogen U.S.A.) following the manufacturer’s protocol. Total RNA from the tissues were quantified and the purity was ascertained by spectrophotometry (optical density, 260/280 ratio). About 2μg of total RNA from each of the tissue samples was reverse transcribed using high capacity cDNA synthesis kit (Applied Biosystems, U.S.A.) following the manufacturer’s instructions.

(iii) *RT-PCR analysis*

RT-PCR amplification of TLRs was carried out using TLR-specific PCR primers designed based on the sequence information of TLRs in the GenBank database (www.ncbi.nlm.gov.in). The details of the primer codes of the PCR primers used for the amplification of specific TLRs presented in Table 1. PCR was performed with initial denaturation at 94°C for 2min, 30 cycles of denaturation at 94°C for 45 sec; annealing for 45 sec at varying temperatures for TLRs

(55°C for TLR3, 4, 9 and β-actin; 56°C for TLR2 and TLR22; 57°C for TLR7) and extension at 72°C for 45 sec followed by a final extension at 72°C for 5min at the end of 30 cycles. PCR amplified TLR products were confirmed by agarose gel electrophoresis, nucleotide sequencing and analysis.

Table1
The details of the primers used for the TLR expression studies

Target genes	Primer code
TLR2	SDDL/TLR2/350F and SDDL/TLR2/350R
TLR3	SDDL/TLR3/803F and SDDL/TLR3/803R
TLR4	SDDL/TLR4/152F and SDDL/TLR4/152R
TLR7	SDDL/TLR7/550F and SDDL/TLR7/550R
TLR9	SDDL/TLR9/709F and SDDL/TLR9/709R
TLR22	SDDL/TLR22/180F and SDDL/TLR22/180R
β-actin	SDDL/BA150F and SDDL/BA/ 150R

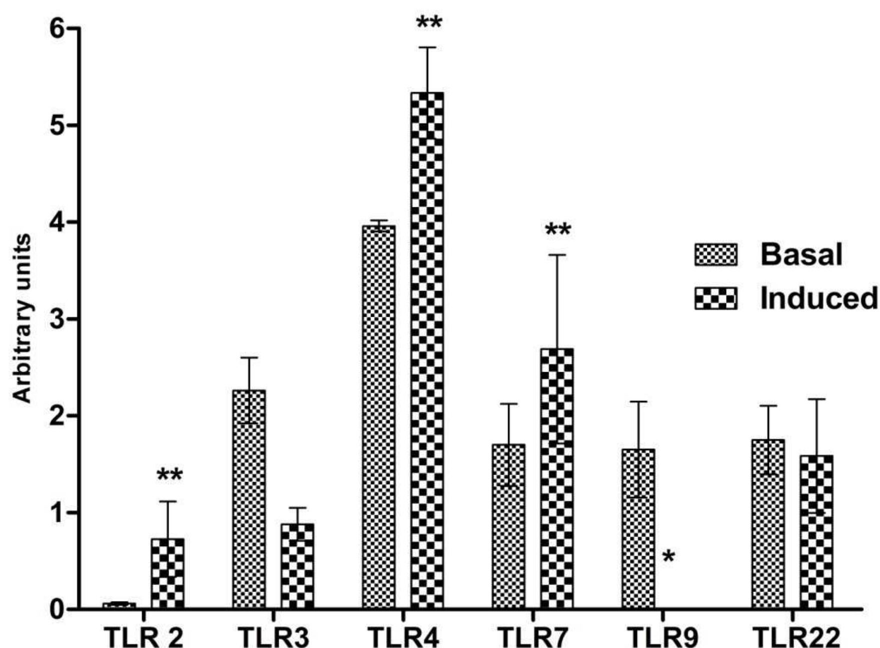

Figure 1
Expression of TLRs in kidney tissue of Carassius auratus infested with Argulus sp.

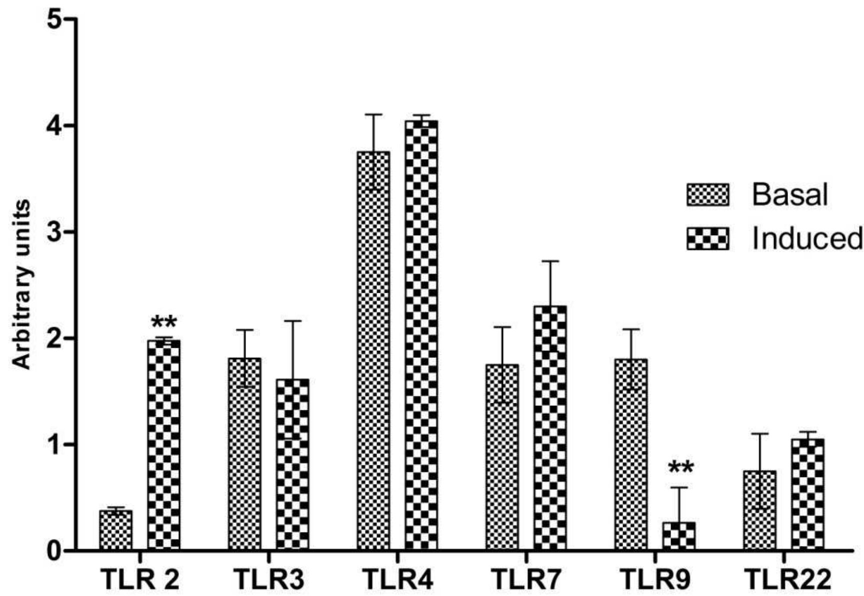

Figure 2
Expression of TLRs in liver tissue of Carassius auratus infested with Argulus sp.

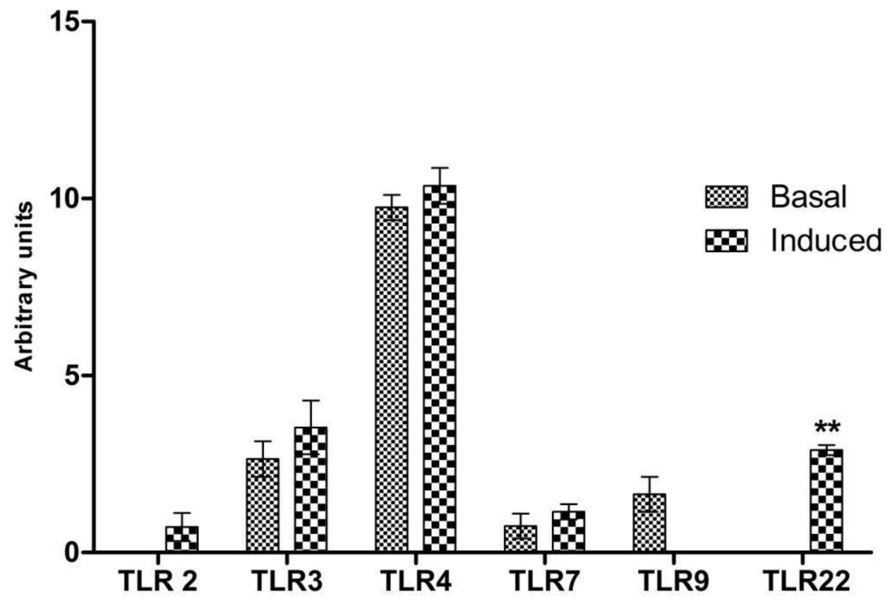

Figure 3
Expression of TLRs in skin tissue of Carassius auratus infested with Argulus sp.

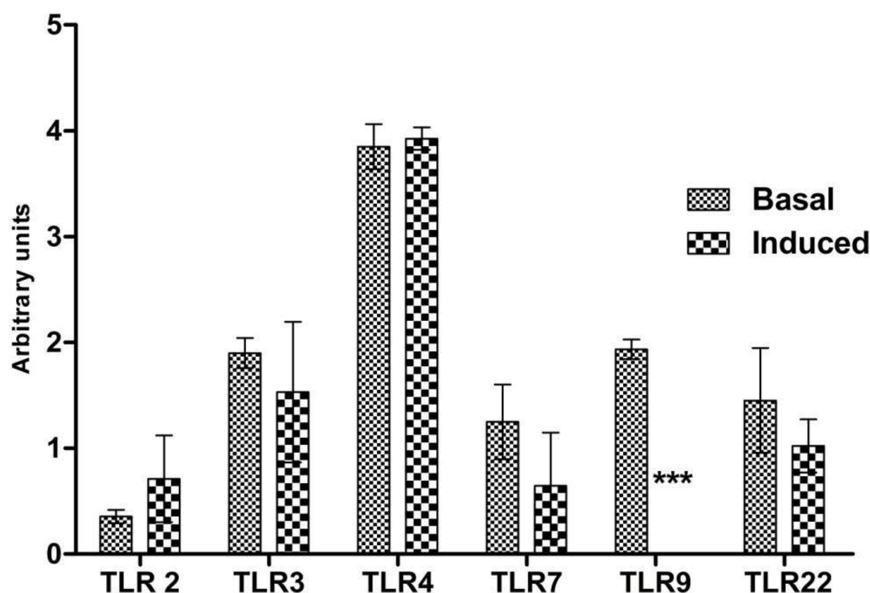

Fig. 4
Expression of TLRs in intestine tissue of Carassius auratus infested with Argulus sp. Data presented in figures are the mean+ SD. ***denotes (P<0.001) and ** denotes (P<0.01).

(iv) ***Expression analysis by semi-quantitative RT-PCR***
PCR amplified products were resolved in a 2% agarose gel and stained with ethidium bromide and the relative level of expression of each TLR with respect to β-actin (internal control) expression was analyzed by densitometry using quantity one ^{TM} image acquisition software (BioRad INC., U.S.A). The ratios of TLRs/β-actin product were subsequently calculated in terms of arbitrary units.

(v) ***Statistical analysis***
Mean value (±S.D.) of the level of expression of TLRs relative to the β-actin expression from the tissue of the healthy control and parasite infested fishes were calculated and analyzed using two way ANOVA test.

RESULTS AND DISCUSSION

Basal and tissue specific expression of TLRs (TLR2, TLR3, TLR4, TLR7 TLR9 and TLR22) was observed in the kidney, liver, skin, and intestine of healthy control fishes (Fig. 1-4). Tissue- specific basal expression of TLRs has been reported in various species of fishes *viz.,* Fugu [5] Japanese flounder [6], Trout [7] Seabream [8], Common carp [9], gold fish [10], rohu [11] and channel catfish [12]. Significant variation in expression levels of TLRs was observed in the tissue of parasite infested fishes in treatment groups. Kidney tissue showed significant (P<0.01) upregulation in the expression of TLRs (TLR2, TLR4 and TLR7). Increased expression of TLRs has been documented in kidney and spleen of gold fish stimulated with LPS[10] and in various organs of fishes experimentally induced with LPS[10], *E.ictaluri* [12] , Poly I:C, PGN, [13], *A. hydrophila* [8] and *Argulus* [14]. Liver and kidney tissue showed significant upregulation (P<0.01) of TLR2 alone, although other TLRs showed increased expression at various levels. Modulation in TLR expression in the internal organs like kidney or liver are affected by the action of released toxins by the parasite [14,15]. Skin tissue which is the major site of damage by the ectoparasite showed significantly

(P<0.01) higher expression of TLR22 (Fig.3). Similar observation has been recorded in *Argulus* infested rohu [14] as skin acts as the first barrier against infections [16]. Hence, this increased expression of TLR22 could be attributed due to parasite induced reaction as observed in the study with *Argulus* infested rohu [14]. TLR9 expression was significantly (P<0.05) down regulated in all the tissues studied. Down regulation of TLRs during infections and parasite infestations have been recorded and explained due to lowered leukocyte activity and TLR expression due to the action of toxins released by parasite[14]. Down regulated expression of TLR9 in zebra fish experimentally infected with *A. hydrophila* had been documented [17]. Some of the expressed TLRs in *C.auratus* observed in this study were also confirmed by nucleotide sequencing and their identity were confirmed and submitted to GenBank JN126251 (TLR2), JN126252 (TLR3) and JF808130 (TLR7). In conclusion, the observations of this study showed that the immune system of gold fish, *C.auratus* is modulated by the *Argulus* parasite infestation which is evident from the differential expression profiles of TLRs observed in various tissues.

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