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DIFFERENTIAL EXPRESSION OF TOLL-LIKE RECEPTOR18 (TLR18) MRNA IN *PANGASIUS PANGASIUS* INDUCED WITH *EDWARDSIELLA TARDA*

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ABSTRACT

Toll-like receptors (TLRs) are a family of germline-encoded pattern-recognition receptors involved in recognizing diverse molecular patterns of the invading pathogens called pathogen associated molecular patterns (PAMPs). TLRs play an important role as regulators of innate and adaptive immune responses in fishes. TLRs specifically recognize the ligands and activate various cascades of pathways to destroy the pathogens. About 17 different TLR types (TLR 1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23) have been identified in teleost fishes with specificity in identifying the ligands. Studies on the expression profiles of TLR18 in teleost fishes are scanty. Hence, this study was carried out with an objective to assess the expression profile of TLR18 in *Pangasius pangasius* (a freshwater cat fish species) by inducing with *Edwardsiella tarda*, a gram-negative bacterial pathogen infecting fish. Real-time quantitative PCR analysis was performed to study the differential expression of TLR18 in skin, gill, , liver, intestine, kidney and spleen tissue of *P.pangasius*. The results of real-time quantitative PCR analysis showed differential and induced expression of TLR18 in various tissues of *P.pangasius* at various time intervals, 2h, 6h, 24h and 48h post exposure with *E.tarda*.

KEY WORDS: Toll-like receptors, pathogen recognition receptors, mRNA expression, Real time quantitative PCR

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INTRODUCTION

Fish does not have well developed immune system thereby the biological system of fish are deficient in adaptive immune responses, lack immunological memory, and mostly depend on innate or nonspecific immune responses [1]. Initiation of immune defence mechanisms is the recognition of danger signals and the subsequent activation of signalling cascades [2]. Key activators are the pattern recognition receptors (PRRs) that recognize conserved microbial components called ''pathogen-associated molecular patterns'' (PAMPs) [3]. The PRRs also detect endogenous structures released after tissue damage called ''damage-associated molecular patterns'' (DAMPs) [4]. TLRs are family of type I trans-membrane receptors that either act alone or in concert with other molecules, generate innate immune responses to counter invade the pathogens [5]. TLRs of fish are unique and diverse in nature which is likely to be derived from their distinct environment [6]. TLRs recognise various PAMPs from different pathogens like lipopolysaccharide, lipoprotein, peptidoglycan, flagellin, dsRNA and ssRNA [2&7]. Six non-mammalian TLRs were identified in fishes include TLR14 (similar to TLR1 and TLR2), TLR19, TLR20, TLR21, TLR22, TLR23 [6]. *Edwardsiella tarda* is a gram-negative bacterial pathogen causing Edwardsiellosis in fishes like carp, tilapia, eel, catfish, mullet, salmon, trout and flounder [8]. Studies on the expression of TLR18 and its tissue specific expression profile in teleost fishes are scanty. Hence, an attempt was made in this study to assess the expression profiles of TLR18 in *P.pangasius* induced with *E.tarda*.

MATERIALS AND METHODS

(i) Collection and maintenance of fishes

P.pangasius fish samples of 20±10g weight with no previous history of infections were collected from various fish farms in and around Chennai, Tamilnadu, India. Fish samples collected were transported in aerated polythene bags and brought to the wet lab facilities of shrimp disease diagnosis laboratory of Tamilnadu Veterinary and Animal Sciences University, Chennai. The fishes were acclimatized to lab conditions with good aeration and *ad libitum* feeding. Treatment and control fish groups (15 fishes each) were maintained in 15 litre capacity troughs in triplicates.

(ii) Bacterial culture and experimental exposure of P.pangasius with E.tarda

An overnight culture of *E.tarda* was centrifuged at 5,000 rpm for 10 min. Bacterial cells were washed twice with physiological saline and resuspended in the same solution to obtain a bacterial suspension with a concentration of 2×10^7 CFU/ml. 100µl of this culture was injected intraperitonealy to each fish of the treatment group. Fishes of control group were maintained as *placebo* and a same volume of physiological saline was used instead of bacterial suspension [9].

(iii) Tissue sampling

Tissue samples were collected aseptically by dissecting the fishes from treatment and control groups at 2h, 6h, 24h, and 48h post-exposure. The tissue samples collected include skin, gill, liver, intestine, kidney and spleen.

(iv) RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue samples collected using one-step RNA reagent (Bio Basic Inc, Canada) following the manufacturer's protocol. The extracted RNA was then quantified using a Biophotometer (Eppendorf, Germany) and the purity was confirmed (1.8-2.0 at OD 260/280). About 1 µl (6µg) of RNA from each tissue sample was used for cDNA synthesis using a cDNA Reverse Transcription Kit (Applied Biosystems, USA).

(v) Real Time PCR analysis

The expression of TLR18 was studied by real-time quantitative PCR using self-designed TLR18 -specific PCR primers (TLR18C/156F and TLR18C/156R) designed

based on the sequence information of *Ictalurus punctatus* (Acc No : HQ677721) so as to yield a PCR product of 156 bp size. The primer sequences used to study the TLR18 expression is shown in Table 1.

Table 1
PCR primer codes and sequences used in real-time PCR

Primer set no	Primer code	Primer sequence
1.	TLR18C/156F TLR18C/156R	GTG CTT TTC CAC TGG ATG GT GCT TGG TTG CAT GGT ATG TG
2.	βACT/150F βACT/150R	GAT TTG GCT GGT CGT GAT CT GGC CAT CTG CTG GAA GT

The RT-qPCR was performed in a ABI 7500 Standard Real Time PCR equipment (Applied Biosystem, USA). The reaction was carried out in a total volume of 10 µl containing 5 µl of SYBR green master mix, 1µl of forward primer (10pmol), 1 µl of reverse primer (10pmol), 0.2 µl of 1X Rox dye, 1.3 µl of nuclease free water, 1.5 µl of cDNA (6µg/µl) template. cDNA was amplified using a PCR amplification profile with an initial denaturation at 94°C for 4 min; followed by 40 cycles of denaturation at 94°C for 45 sec; annealing at 54°C; and extension at 72°C for 45 sec followed by final extension at 72°C for 4 min.

(vi) Statistical analysis

Relative quantification of gene expression was assessed using Ct values. For each sample, the Ct value of the target gene (TLR18) was subtracted with the values of internal control gene β-actin to arrive DCt values. Higher values indicated higher TLR18 expression levels. Analysis of 40-DCt mean values was carried out using two way ANOVA table with mean ± SD.

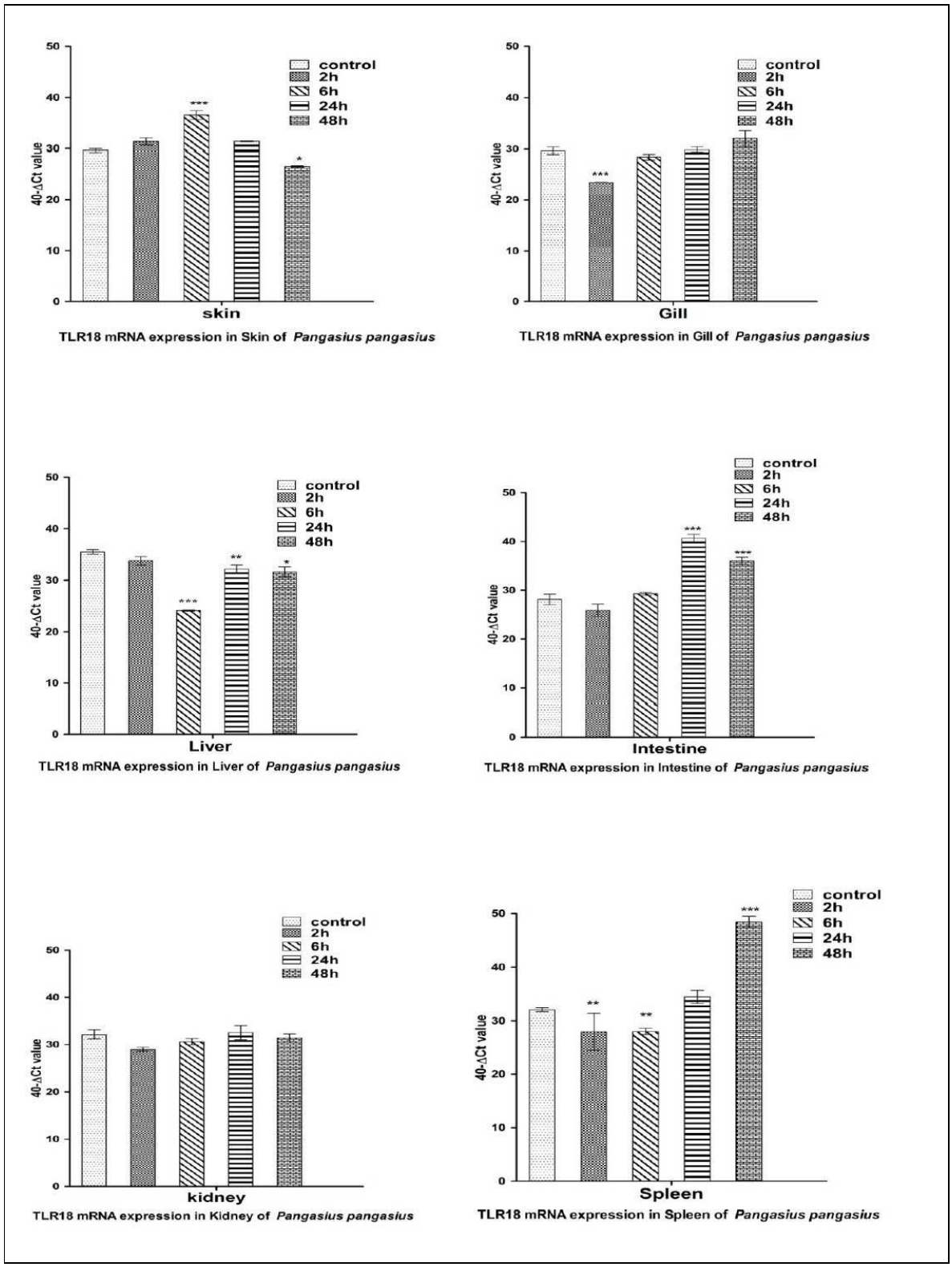
RESULTS

Differential expression of TLR18 was observed in various organs of *P.pangasius* when induced with *E.tarda* as ligand at various time intervals. Significant up-regulation of TLR18 was found in the tissues namely skin (2h), liver (24h), intestine (24h) and spleen (48h) whereas significant down-regulation of TLR18 was found in the tissues namely skin (48h), gill (2h), liver (6h and 48h), intestine (48h) and spleen (2h and 6h) (Fig.1)

DISCUSSION

Studies on the tissue specific expression of TLR18 in fishes have not been reported so far. Sequence information of TLR18 is available only for *Danio rerio* (Acc nos: BC162732.1, BC163840.1, NM_001089350.1, AY389455.1, AL954306.10) and *Ictalurus punctatus* (Acc no: HQ677721.1). Although induced expression of TLR18 has been reported in zebrafish, induced with *Mycobacterium marinum* [10&11], tissue specific expression was not studied.

Figure 1
Quantitative expression (by Real Time PCR) of TLR18 mRNA in** P. pangasius **induced with Edwardsiella tarda



Differential expression of TLR18 was observed in various organs of *P.pangasius* studied. The highest level of basal expression was observed in liver and the least expression was observed in intestine. Liver plays a vital role in controlling systemic innate immunity through biosynthesis of various soluble pathogen-recognition receptors [12]. Moreover lymphocytes in liver are enriched in natural killer and natural killer T cells which play a critical role in first line of immune defence [13]. Up-regulation in the expression of various TLRs has been reported in the liver tissues of Mrigal (TLR5); Rohu (TLR2); and Japanese flounder (TLR5S) exposed with *E.tarda* [7, 14 & 15]. Although the basal expression levels were lesser in intestinal tissue, induced expression was significantly higher at various time intervals of post-exposure. Similar observations have been reported in Mrigal (TLR5) and Rohu (TLR2) [14&15]. The immunological role of intestinal tissue is reported to be attributed to the expression of class I and class II MHC antigens, presentation of antigens to lymphocytes, expression of adhesive molecules mediating interaction with intraepithelial lymphocytes and components of extracellular matrix, production of cytokines and probable participation in extrathymic T cell development of intraepithelial lymphocytes [16].

TLR18 expression in skin of *P.Pangasius* exposed with *E.tarda* was up-regulated in our study which is similar to that of the TLR2 expression in skin of Rohu exposed with *E.tarda* [15]. Skin is a protective interface between the outer environment and the internal organ. Dermis layer of skin are traversed by a network of lymphatic and blood vessels containing lymphocytes, leukocytes, mast cells and tissue macrophages [17]. The scales, skin and surface of mucous membrane are also reported to have antimicrobial substances that are active against infection in early stages [18]. In this study, no significant induced expression was observed in gill tissues of *P.pangasius* when induced with *E.tarda*. Down-regulation of TLR5 and expression levels similar to basal expression was observed in the gills of induced Mrigal (TLR5) and Rohu (TLR2) induced with *E.tarda* [14 &15]. Similar to the gill tissue, insignificant induced expression profiles could be observed in kidney tissues of *P.pangasius* induced with *E.tarda* as reported in TLR2 of Rohu [15]. Kidney is an important central organ for immune-endocrine interactions and even neuroimmuno endocrine connections [19]. The spleen is the largest secondary immune organ responsible for initiating immune reactions to blood-borne antigens and for filtering the blood of foreign material, old or damaged red blood cells [20]. Next to liver, highest level of basal expression of TLR18 was observed in the spleen of *P.pangasius* in our study as reported earlier [15], in Rohu exposed with *E.tarda*. Significant up-regulation in the expression of TLR18 could be recorded in spleen in our study after 24h post-exposure. The induced up-regulation in the expression of TLR18 in various organs viz., Skin, gill, liver, intestine, kidney and spleen of *P.pangasius* clearly demonstrates the involvement of these organs in activating the immune response against *E.tarda*.

CONCLUSION

Tissue-specific expression profile of TLR18 will be helpful to understand the role of immune genes encoded in the germline of organisms. Real-time PCR results showed differential expression of TLR18 in skin, gill, liver, intestine, kidney and spleen of *P.pangasius*. Results showed that *E.tarda*, a gram-negative bacterial pathogen enhances the protective immunity in *P.pangasius*. This improves our knowledge on the immunity in fishes and the methods to manipulate them so as to reduce the production and economic losses in fish farming due to diseases.

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