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Regulation of tissue specific gene expression of toll-like receptor 18 (TLR18) mRNA in freshwater shark *Pangasius pangasius* experimentally induced with Poly I:C

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

Toll-like receptors (TLRs) are important components of innate immune system that are involved in the recognition of pathogens and activation of immune system. In this study, we have reported the expression profile of TLR18 from pangasius catfish, *Pangasius pangasius* (designated as PpTLR18) inducted with poly I:C, a synthetic viral ligand. Quantitative real-time PCR analysis was performed to study the expression of PpTLR18 in skin, gill, liver, intestine, kidney, spleen, brain and muscle tissues of *P. pangasius*. The results showed no significant changes in the expression levels of PpTLR18 gene in skin and intestine. Significant ($P < 0.05$) up-regulation of PpTLR18 was observed in gill, liver and kidney at 24, 48 and 24 h post injection (p.i.) respectively, whereas the expression was significantly ($P < 0.05$) down-regulated in brain (24 h and 48 h p.i.), spleen (8 h p.i.) and muscle (24 h p.i.).This result supplies the basic information for further expansion concerning transduction analysis of pathogen associated indications into the defense related tissues and cells of *P. pangasius*. Further studies have to be conducted to understand the signaling pathways involved in the TLR18-specific recognition of its ligand.

KEYWORDS

Gene expression; *Panagasius pangasius*; Poly I:C; Toll-like receptor

1. INTRODUCTION

Fish lives in an environment, which pre-dispose them to many infectious diseases due to various pathogens. The innate immune system of the fish is the first line defense that acts against the pathogen entry. Fish identify danger signals created by pathogens through the innate immune system with its encoded pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are PRRs present in the extracellular space, membrane-associated variant cell types or in the cytosol (Wei et al., 2011; Rajendran et al., 2012). TLRs identify specific ligands of the pathogens entering the host organisms by directing the activation of MyD88 dependent and independent signaling pathways (Huang et al., 2011; Rebl et al., 2010). Specific pathogen associated molecular patterns (PAMPs) that have been reported to be identified by TLRs include lipololysachharide, triacyl lipoproteins, dsRNA, flagellin, CpG DNA, polyionosinic-polycytidilic acid (Poly I:C) and ssRNA (Zhu et al., 2013). So far, 11 TLRs have been reported from mammals and 17 from fish (1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19,20, 21, 22 and 23) (Su et al., 2009). Palti (2011) reported that among the 17 TLRs from fishes, the ligand specificity have been demonstrated only for a few TLRs in the fishes viz., common carp TLR2 (lipopeptide), fugu TLR3 (dsRNA), rainbow trout TLR5 (flagellin) and TLR22 (dsRNA).

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Pangasius pangasius which is commonly known as freshwater shark belongs to the member of the family *Pangasidae*. It is cultured due to its strong market demand, delicious flesh and fast growth rate. *P. pangasius* is an air-breathing fish that can survive even in oxygen depleted water bodies. Many Asian countries including Thailand, Nepal, Pakistan, India, Bangladesh, Vietnam, Laos, Myanmar, Indonesia, and Cambodia dominate the culture production and the species is the third most important freshwater fish in the aquaculture sector. Since this species is cultured at high stocking densities, disease outbreaks and associated mortality have been reported to occur at various life stages. For example, parasitic infestations due to *Ichthyophthirius* sp and *Trichodina* sp in the fingerling stage (Lazard and Cacot, 1997) and fluke worm (*Clonorchis sinensis*) during the grow-out period (Phu and Hein, 2003) have been reported. In addition to these parasitic infections, a few bacterial infections viz., Bacillary Necrosis of Pangasius (BNP) caused by *Edwardsiella ictaluri* and Motile *Aeromonad Septicaemia* (MAS) caused by *Aeromonas hydrophila*, *A. sobria* and *A. caviae* have also been reported (Roberts and Vidthayanon, 1991). These reports showed that the *P. pangasius* aquaculture industry is under a threat due to the various diseases. Thus research on this commercially important fish species and its immune process is necessary to evolve disease control measures at molecular level. In our earlier study, we have reported (Jawahar raj et al., 2012) that TLR9 recognize the unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides of bacterial DNA motifs and trigger the immune responses during bacterial invasion in *Catla catla*. In another study (Uma et al., 2012), differential gene expression of TLR18 from *P. pangasius* infected with *Edwardsiella tarda* have been recorded. However, the detailed functions and characterization of TLR18 in teleost fishes is poorly understood and there is no report on TLR18 of *P. pangasius*. Hence, to gain insight into the immunological role of TLR18 at molecular level, an attempt was made to study the expression of TLR18 from *P. pangasius* (designated as PpTLR18) during induction with Poly I:C, a synthetic viral ligand.

2. MATERIALS AND METHODS

2.1. Collection and maintenance of fish

Healthy *P. pangasius* (average weight 15 g) were collected from a local fish farm at Chennai, Tamil Nadu, India. They were maintained in flat-bottomed fiber tanks (50 L) with aerated and filtered freshwater at 30± 2 ºC in the laboratory. They were acclimated for 10 days before being injected with poly I:C. A maximum of 15 fish per tank were maintained during the experiment. Five fish were randomly selected at 0, 2, 4, 8, 24 and 48 h post-injection from experimental and control groups.

2.2. Experimental induction of P. pangasius with Poly I:C and tissues collection

Polyinosinic-polycytidylic acid sodium salt (polyI:C), a synthetic analog of double-stranded RNA (dsRNA) has a molecular pattern associated with viral infection [polyI:C is composed of a strand of poly(I) annealed to a strand of poly(C)] thereby induces the mRNA expression. The fish were intra-peritoneally injected with 100 µL of γ-irradiated poly I:C (50 µg/µL) (Sigma-Aldrich, USA) in the experimental group and with 1X PBS in the control group. Skin, muscle, gills, liver, spleen, intestine, kidney and brain tissue samples were collected at 2, 4, 8, 24 and 48 h post-injection (p.i.) from the PBS injected control and poly I:C injected experimental groups for total RNA extraction. Tissues were immediately snap-frozen in liquid nitrogen and stored at -80 ºC until the total RNA was isolated. All samples were obtained and analyzed in triplicates, and the results were determined by the 40-ΔCt (Livak and Schmittgenm, 2001).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from the tissue samples 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 then the purity was confirmed (1.8-2.0 at OD 260/280). About 1 µL (6 µg) of RNA from each tissue sample was transcribed to cDNA using a cDNA Reverse Transcription Kit (Applied Biosystems, USA).

2.4. Real-time PCR analysis

The expression profiles of PpTLR18 was studied by quantitative real-time PCR using TLR18-specific PCR primers(TLR18C/156F:GTGCTTTTCCACTGGATGGT and TLR18C/156R: GCTTGGTTGCATGGTATGTG) designed based on the sequence information of *Ictalurus punctatus* (GenBankAccession No. HQ677721). A self-designed β-actin internal control primer set was

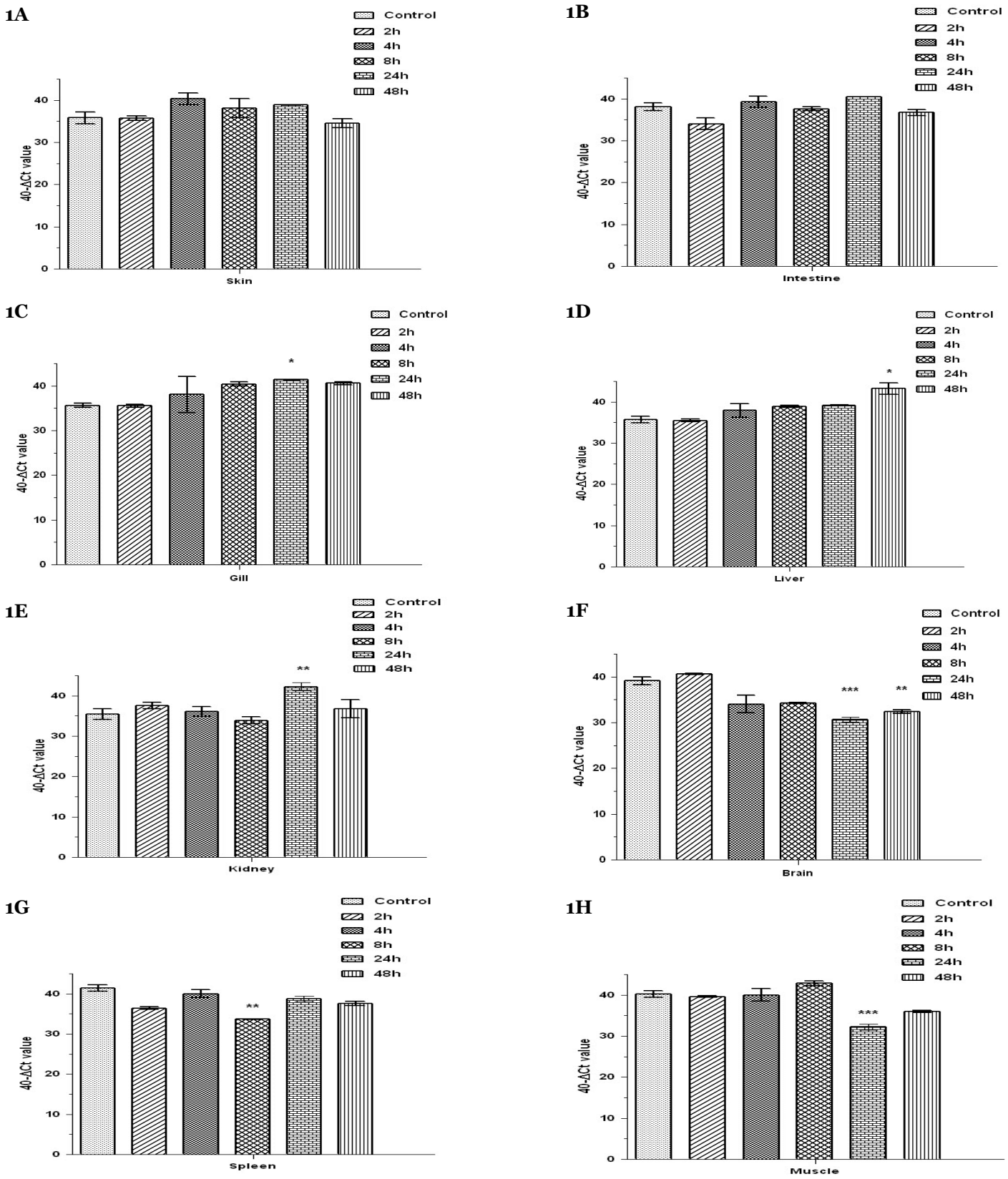

Figure 1. Gene expression patterns of PpTLR18 by qRT-PCR. 1A, 1B, 1C, 1D, 1E, 1F, 1G and 1H: The time course of PpTLR18 mRNA expression in skin, intestine, gill, liver, kidney, brain, spleen and muscle at 2, 4, 8, 24, and 48 h post injection with poly I:C. Data are expressed as mean of three replicates of 40-Δct. The asterisk represents the levels of significance of up-regulation and down-regulation (* - P<0.05; ** - P<0.01 and *** - P<0.001).

used (βACT/150F: GATTTGGCTGGTCGTGATCT and βACT/150R: GGCCATCTGCTGGAAGT). The qRT-PCR was performed in 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, 1 µL of forward primer (10 p mol), 1 µL of reverse primer (10 pmol), 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 following the 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.

2.5. Statistical analysis

Relative quantification of PpTLR18 gene expression was assessed using Ct values. For each sample, the Ct value of the target gene (PpTLR18) was subtracted with the value of internal control (β-actin) to arrive at the ΔCt values. The three replicate value of 40-ΔCt was performed using one-way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using Two way ANOVA with mean + SD.

3. RESULTS AND DISCUSSION

Real-time PCR analysis showed differential expression of PpTLR18 in various tissues including skin, intestine, gill, liver, kidney, brain, spleen and muscle of *P. pangasius* exposed to poly I:C at various time intervals (Fig.1A-1H). No significant change was observed in the expression levels of PpTLR18 in skin and intestine. Significant up-regulation of PpTLR18 was observed in gill, liver ($P < 0.05$) and kidney ($P<0.01$) infected with polyI:C at 24, 48 and 24 h p.i. respectively, whereas the expression was significantly down regulated in brain (24 h and 48 h), spleen (8 h) ($P < 0.01$) and muscle (24 h) ($P<0.001$). Jawahar et al.(2012) recorded significant ($P < 0.05$) up-regulation of TLR18 in skin, liver, intestine and spleen of *P. pangasius* infected with *E. tarda* at 2, 24, 24 and 48 h p.i. respectively, whereas significant($P < 0.05$) down-regulation was observed in skin, gill, liver, intestine and spleen at 4, 6, 48, 24 and 8 h p.i. respectively.

The available literature reported that TLRs are expressed in many tissues of fishes. TLR22 was significantly ($P < 0.05$) up-regulated at 12 h p.i. in kidney and spleen of large yellow croaker *Pseudosciaena crocea* induced with poly I:C (Xiao and Chen, 2011). Huang et al. (2011 & 2012) reported that TLR14 and TLR3 from Japanese flounder *Paralicthys olivaecious* infected with viral hemorrhagic septicaemia virus (VHSV) showed up-regulation in kidney, gills, liver and spleen. Similarly Avunjce et al. (2011) also recorded the highest expression of TLR2 and TLR7 genes in kidney and spleen of *P. olivaecious* infected with VHSV and it is found to be up-regulated at 3 h p.i. and 6 h p.i. respectively. These expressions are possibly indicating their functional relevance to counteract pathogenic infections. Funami et al. (2004) suggested differential expression of the TLRs in various tissues could be attributed due to different fish species, immunological status, developmental stage and genetic background. The authors further reported that the haemopoitic organs like kidney, liver, spleen and gills play a major role in synthesizing TLRs.

The role of TLRs in defense system is complicated and differs from species to species. In earlier findings, TLRs have been shown to react to viral or poly I:C, bacterial and LPS induction (Huang et al., 2011).The significant up-regulation of PpTLR18 noticed in gill, liver and kidney induced with polyI:C showed that PpTLR18 plays a crucial role in protecting fish against double strand RNA viral infection in gill, liver and kidney. Moreover, the down-regulation of PpTLR18in brain (24 h and 48 h), spleen (8 h) and muscle (24 h) after poly I:C induction could be due to the transmission of antigen-presenting cells (APC) among other leukocytes to the intra-peritoneum in the intra-peritoneal injection (Yang and Su, 2010). These findings indicate that the immune response of PpTLR18 in gill, liver, kidney, spleen, muscle and brain to different PAMPs were not simultaneous in *P. pangasius* as stated by Yang and Su (2010). In addition, the results indicated that tissue-specific expression of PpTLR18 gene possibly plays an important role in fish immune system against viral diseases.

4. CONCLUSIONS

Real-time PCR results showed that the expression of PpTLR18 could be induced in various tissues of *P. pangasius* with poly I:C. This result provides the basic information for further expansion concerning transduction analysis of pathogen associated indications into the defense related tissues and cells of *P. pangasius*. Further studies have to be conducted to understand the signaling pathways involved in the specific recognition of its ligand.

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