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DIFFERENTIAL EXPRESSION OF SELECTED TOLL-LIKE RECEPTORS (TLRS) IN TISSUES OF CATFISH, *PANGASIUS PANGASIUS*

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Abstract: Toll-like receptors (TLRs) are one of the important components of the host innate immune system which induce its immunity in response to their recognition of pathogen based on pathogen associated molecular patterns (PAMPs). Studies on the expressions of various TLRs are limited to few species of fishes. Apparently normal expression of TLRs (TLR2, TLR4, TLR9, and TLR22) in tissues from various organs *viz.,* skin, gill, brain, liver, intestine, kidney and spleen of catfish, *Pangasius pangasius* was studied by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Differential expression of TLRs was also studied in the tissues of *P. pangasius.* Among the TLRs studied in various tissues, the highest and the lowest expressions were observed with TLR4 and TLR2 respectively.

Keywords: Toll-like receptors (TLRs); *Pangasius pangasius*; semi-quantitative RT-PCR.

Introduction

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) of type I transmembrane protein [1] which play a crucial role in recognition of microbial pathogens invading the host. Pathogen associated molecular patterns (PAMPs) are the conserved motifs of the pathogens like lipopolysaccharide, peptidoglycan, lipoteichoic acid and CpG DNA which are specifically recognized by TLRs, which in turn induces the immune response in the host by activating the production of cytokines, reactive nitrogen and oxidative radicals and differentiation of cells [2],[3]. 20 TLRs in fish were identified including: TLR1, 2, 3, 4, 5M (membrane bound), 5S (soluble), 7, 8, 9, 13, 14, 18, 19, 20, 21, 22, 23, 24, 25, and 26 [4]. About 17 expressed TLR genes have been identified by expression profiles in fish [5]. Each TLR differ in their localization and in its ability to recognize the ligands. The apparently normal expression pattern and levels of each TLR mRNA in different tissues indicate the natural load of PAMPs in each tissue and its ability to resist pathogen challenge [6]. Although TLRs and their expression had been studied extensively in *Danio rerio,* (zebra fish) [5,7] and

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Takifugu rubripes, (puffer fish) [8], information on TLRs and their expression profiles in tropical fishes is scanty. Hence, this study was carried out with an objective to identify the apparently normal expression profiles of TLRs *viz.,* TLR2, TLR4, TLR9, and TLR22 in *P. pangasius,* a commercially important species of catfish in tropical countries.

2 Materials and methods

2.1 Fish sample collection and total RNA extraction

Samples of apparently healthy *P. pangasius* with no previous history of infection and with an average weight of 50 $\pm$ 10g were collected from a commercial fish farm in Chennai, Tamil Nadu, India. The fishes were dissected aseptically and the tissues from various organs *viz.,* skin, gill, brain, liver, intestine, kidney and spleen were collected and were either used immediately for total RNA extraction or stored at -80°C until use. Total RNA was extracted from tissue samples using a commercial total RNA extraction reagent (Invitrogen U.S.A.) following the manufacturer's protocol. The quantity and purity of total RNA from each tissue were determined by spectrophotometry (optical density, 260/280 ratio). The extracted RNA was stored at -80°c until it was used as the template for RT-PCR.

2.2 RT-PCR analysis

About 2µg of total RNA from tissue samples were reverse transcribed using oligodT primers of cDNA synthesis kit (Applied Biosystems, U.S.A.) following the manufacturer's protocol. RT-PCR amplification of TLRs was carried out using TLR-specific PCR primers designed in this study based on the sequence information of TLRs retrieved from the GenBank database (www.ncbi.nlm.gov.in). TLR-specific primers used in the study are detailed in Table 1. PCR amplification was carried out with an 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 TLR4, TLR9 and β-actin; 56°C for TLR2 and TLR22) 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, sequencing and analysis.

2.3 TLR expression analysis by semi-quantitative RT-PCR

The relative levels of expression of each TLR gene were analyzed by densitometry using quantity one ^{TM} image acquisition software (BioRad INC., USA). The expression levels of the different TLRs were expressed as arbitrary units [9]. Optimization of PCR cycle numbers was done by amplifying TLRs and β-actin genes using a series of cycle numbers (25-35) and the cycle number that gave the clear products (30 cycles) was followed in the study under the above conditions. PCR products were resolved in a 2% agarose gel and stained with ethidium

bromide. The band intensities of PCR products were analyzed by quantity one ™ image acquisition software and the difference in the intensities in the background gel and that of each TLR amplicon was taken as the corrected intensity values which were then normalized with the corresponding β-actin mRNA expression. The expression levels of the different TLR mRNA in the different tissue of *P.pangasius* were calculated. Arbitrary units of 0.5 and below showed very low or no expression.

2.4 Nucleotide sequencing

The PCR amplified TLR amplicons were sequenced using commercial sequencing services (MWG, Bangalore, India). The sequence information was subjected to BLASTn analysis (www.ncbi.nlm.in) to confirm their identity and homology.

Results

PCR amplification of TLRs resulted in the amplicons of expected sizes (base pairs). Semi-quantitative analyses of the expression levels in terms of arbitrary units showed varied levels of expression of TLRs in the tissue samples studied. The expression levels of TLRs (TLR 2,4,9 and 22) recorded in skin, gill, brain, liver, intestine, kidney and spleen are shown in Fig.1. TLR4 showed relatively higher level of expressions in many of the tissues of *P. pangasius.* The highest expression was observed with TLR4 in gill, intestine and liver. TLR22 was the second highly expressed TLR followed by TLR2 and TLR9 in the tissues of *P. pangasius* . The sequences of the TLRs (TLR2,4,9 and 22) amplified from the tissues of *P. pangasius* were confirmed as they showed homology with the TLR sequences in the Genbank database (Data not shown). The sequence information TLRs amplified from *P. pangasius* were submitted in the Genbank database (NCBI). The Accession numbers of the sequences are JN126250 (TLR2), JN867637 (TLR4), JN648713 (TLR9) and JN867638 (TLR22).

Discussion

Expression of TLRs have been reported in various species of fishes *viz.,* Fugu [8] Japanese flounder [10], trout [11], seabream [12], common carp [13], gold fish [14], rohu [15] and channel catfish, [16]. Ubiquitous expression of TLR2, TLR5 and TLR22 in various tissues of puffer fish (Fugu) have been documented [8]. Tissue–specific expression of TLR22 has been recorded in anterior kidney and peripheral blood leukocyte and TLR2 in trunk kidney, spleen, gill, intestine, liver and brain of Japanese flounder [17]. Tissue–specific expression of TLR9 in has been recorded in kidney, digestive gland, skin and heart and gill of puffer fish [8]. In gold fish, tissue specific TLR expression was observed in kidney and spleen [14]. Use of

semi-quantitative PCR based on arbitrary units by densitometry have been followed by various researchers for gene expression studies [9],[6],[18]. Similar densitometry based semi-quantitative expression analysis have been carried out in this study to understand the expression profiles of TLR2, TLR4, TLR9 and TLR22 in the tissues of *P.pangasius*. Our observations showed that the expression levels varied with the TLRs and the tissue. In the gill tissue, the expression of TLR9 was insignificant where as TLR4 showed the highest expression. TLR22 expression was significantly higher in gill, intestine and liver compared to other TLRs studied. TLR22 has been reported to be mainly expressed in liver, digestive organ, gonads and brain of Japanese flounder [18] and was absent in gill tissue of fugu [13]. TLR22 is widely conserved among teleost and amphibians and its required for vertebrates living in water or wet conditions, but not in animals living on land [19]. In this study, the basal expression of various TLRs have been documented in various tissue of *P.pangasius viz.,* skin, gill, brain, liver, intestine, kidney and spleen and a comparatively higher expression levels have been observed in intestine, liver, kidney and spleen, which are known to play a immunological role in fish.

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Table 1 Sequences of primers used for the amplification of TLRs

Target gene	Primer code	Primer sequence 5'-3'	Amplicon size (bp)	Reference accession no.
TLR2	TLR2F TLR2R	TGC GGA TGT TTA ATG GGA AT TGG CTT GTA TCC ATG CTT TG	350	GU134618
TLR4	TLR4 F TLR4 F	TCA CCT GGA CAG CAA GAA TG AGG ACT TCC CTG CTT GAA A	158	GU321982
TLR9	TLR9 F TLR9 F	ATT GGA GAA CCG AGG GAG AT TGG TCC AAC AGG TGC ATT AG	709	GU809229
TLR22	TLR22 F TLR22 R	GGA CTG GAG ATT GTG CCT TC TGA TCA GGC AGA TGG TCT TG	130	GU459061
β- actin	BA F BA R	GAT TTG GCT GGT CGT GAT CT GCC CAT CTC CTG CTC GAA GT	150	BC067566

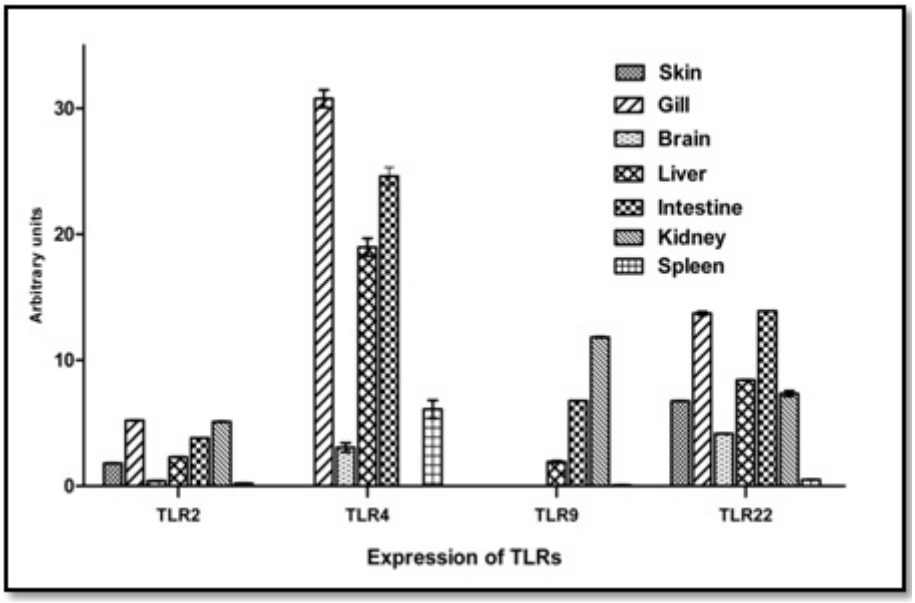

Fig 1. Expression levels of various TLRs in tissues of catfish, *P. pangasius*

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