Indian Journal of Animal Sciences **83** (9): 983–986, September 2013/Article

Differential expression profiling of toll like receptors 3, 4 and 9 genes in major tissues of Indian major carp *Catla catla*

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Received: 30 January 2013; Accepted: 14 May 2013

ABSTRACT

In the present study, differential expression of TLRs 3, 4 and 9 genes in liver, kidney and spleen tissues of Indian major carp *Catla catla*, were profiled using real time PCR TaqMan chemistry. Expression was highest in liver for TLR3 and TLR4, followed by spleen and kidney. However, for TLR 9, expression was highest in spleen, followed by liver and kidney. In conclusion, the work has generated novel findings on expression profiling of TLR 3, 4 and 9 genes in Indian major carp *Catla catla*. Moreover, the expression profile of TLRs in liver, head-kidney and spleen tissues are being reported for the first time in *Catla catla*. The TLR4 gene is non-responsive to LPS in many species of fishes. The hypothesis of TLR functionality can be tested in catla using the TLR4 sequences reported in this project.

Key words: Catla, Expression profiling, Innate immunity, TLRs

In the recent years, Indian aquaculture has gained tremendous importance because of its contribution to nutritional security, employment opportunity to millions of people and as a source of foreign exchange. Fishes have a relatively well-developed specific immune system and are able to produce both circulating antibodies (humoral response) and a cell-mediated response to antigenic stimuli. These humoral and cellular arms of the immune system are required to respond in differing degrees for various viral, bacterial, protozoan and fungal pathogens. The adaptive, acquired or specific immune system characterized by humoral immune response through the production of antibodies is defective in fish because fish has only IgM. Considering these data together, where biological system of fish is deficient in adaptive immune responses, lack immunologic memory and they mostly depend on innate or nonspecific immune responses (Magnadottir 2006), the role of toll like receptors are expected to be very important. TLRs 3, 4 and 9 recognize the viral double-stranded RNA, lipopolysachharide of the gram-negative bacteria, and CpG islands of the microbial genome, respectively. To understand the role of TLRs in the innate immunity of fishes, the present study was undertaken to study the tissue specific expression profile of TLR 3, 4 and 9 genes in catla fish, where no information is available on the presence of these TLRs and their expression profiles in different tissues in the Indian major carp *Catla catla*.

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MATERIALS AND METHODS

Collection of tissue samples: Tissue samples (spleen, head-kidney and liver) were collected aseptically using the standard protocol, in a sterile eppendorf tube containing Trizol reagent after sacrificing the fish. The tissue was immediately kept in dry ice and brought to laboratory.

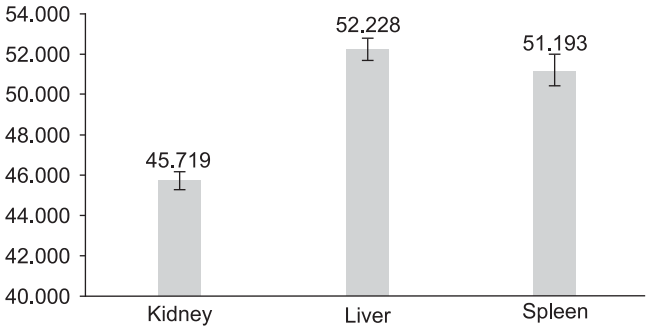

Fig. 1. Differential expression profiling of TLR3 gene in various tissues (liver, kidney and spleen) of the Indian major carp *Catla catla* (catla).

Table 1. Detail of the sequences (5'to 3') of the assay by design (ABD) for the probe-primer mix for TaqMan Assay of TLR 3, 4 and 9 genes

	TLR 3	TLR 4	TLR 9
Primer-F	CGC TGC ACG AGG AGA CA	GGC CCC AAG CAC TCT GA	GAA GAT CCT TGA TGT GCA GTC TCT
Primer-R	GCC TGC CTT TTT TCC ACA GAA	AGA TGC CAC TGT GGT TGA AGT	CAG CAG GAC AGA AAG TGT AAC CTT
Probe	ACTC GGA GGA AGA ACA A	CTG CCT GGC CTG CCT T	TCC CTT GGC GTT TCA T

Total RNA extraction and first strand cDNA synthesis: Total RNA was isolated from different tissue samples using Trizol reagent as per the mentioned protocol. RNA templates having absorbance ratio (260/280) between 1.9 and 2 were subjected to first strand cDNA synthesis, using reverse transcription kit, according to the manufacturer's instruction.

Real time PCR: The tissue samples (liver, head kidney and spleen), approximately 2 g weight were collected from catla fish (*Catla catla*) of age approximately 6 months (length approximately 25cm and weight approximately 700g) which were being reared in fishery farms of College of Fisheries, GADVASU, Ludhiana. The tissue samples were collected aseptically containing 1 ml of Trizol in 2 ml eppendorf, homogenized and further total RNA was isolated from different tissue samples using the standard protocol, and were then subjected to first strand cDNA synthesis, using reverse transcription kit. Quantitative real-time PCR was performed by TaqMan assay using biosystems. The real-time PCR reaction was carried out in 20 µl final volume containing 1X TaqMan master mix, 900nM of each primer and 200nM of probe and template cDNA for the genes TLR-3, 4 and 9 in spleen, head-kidney and liver (2 µl equivalent to 100 ng) in duplicates (Table 1). ß-actin was used as endogenous control. The conditions used for uracil inactivation phase (50°C for 2 min), initial denaturation (95°C for 10 min) followed by 40 cycles of denaturation (95°C for 15 sec), annealing + extension (60°C for 1 min). Cycle threshold (Ct) values were calculated using the SDS software v.2.3 with automatic baseline settings at threshold of 0.2.

RESULTS AND DISCUSSION

The expression profile (mean ΔΔCT ± SEM for the calibrated value expressed as 40 subtracted from the ΔCT value) of the TLR3 and TLR4 genes was the highest in liver, followed by spleen and kidney (Figs 1, 2). However, in TLR 9, the expression was the highest in spleen, followed by liver and kidney (Fig. 3). Paired t-test between the expression profile revealed that the expression level in liver was significantly high than that of kidney for TLR 3 ($P<0.005$), however not for TLR 4. While, for TLR 9, the expression levels in liver and spleen were significantly high than that of kidney ($P<0.05$).

Samanta *et al*. (2013) recently detected the constitutive expression of TLR 3 gene in various organs/tissues of Indian carp rohu (*Labeo rohita*) by quantitative real-time PCR and found a significant relationship of TLR3 induction and type I IFN. Mx (myxovirus-resistant protein), IL-1ß and TNF-α gene expression were observed in majority of the treated (polyinosinic-polycytidylic acid (poly I:C), a synthetic analogue of viral dsRNA) fish tissues, as compared to their control. The above study showed the important role of TLR3 in recognizing dsRNA, and in augmenting the innate immunity in fish in response to be viral infections. The differential expression has been found to the highest in liver for TLR3 ($P<0.05$) and TLR4 and in spleen for TLR9 ($P<0.05$) in katla (*Catla catla*). Similar studies on other Indian fishes were reported for TLR2 and TLR 22-like in Rohu (*Labeo rohita*) fish (Saurabh *et al*. 2011). Differential expression profiling of TLR genes has been studied in some fish species—rainbow trout (Su *et al*. 2008), cat-fish (Pridgeon *et al*. 2010), grass carp for TLR 7 (Yang *et al*. 2012), TLR1 and 2 in estuary cod (Wei *et al*. 2011), however,

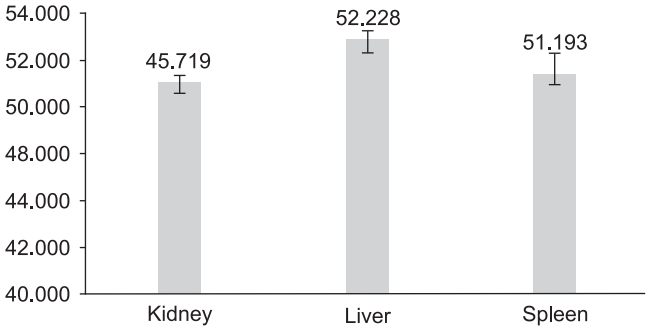

Fig. 2. Differential expression profiling of TLR4 gene in various tissues (liver, kidney and spleen) of the Indian major carp *Catla catla* (catla).

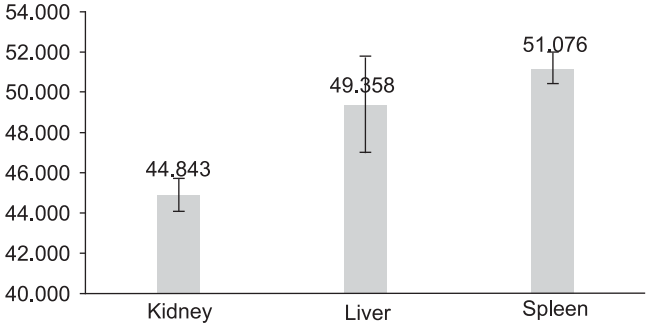

Fig. 3. Differential expression profiling of TLR9 gene in various tissues (liver, kidney and spleen) of the Indian major carp *Catla catla* (catla).

to the best of our knowledge, till date no report is available on differential expression profiling of TLR genes in catla. Tissue specific expression analysis of TLR5 genes by quantitative real-time PCR in Indian major carp mrigal (*Cirrhinus mrigala*) revealed wide distribution of the PAMPs in various organs and tissues. The highest expression of TLR5 and MyD88 was found in liver and of TRAF6 in kidney (Basu *et al*. 2012).

Liver is the site for detoxification of many compounds and substrates in the body. TLR3 recognizes exogenous and endogenous dsRNA and synthetic dsRNA polyriboinosinic: polyribocytidylic acid (poly I:C), thus playing an important role in elimination of viral agents which are intracellular in nature. Recent studies showed that TLR3 is expressed in the parenchymal and non-parenchymal cells of liver as well as on several types of immune cells. However, over-expression of TLR 3 has also been reported in liver diseases (Xiao *et al*. 2009). TLR3 is likely to be a mediator of innate activation and inflammation in the liver. In a similar kind of study on sweet water fish rohu, Saurabh *et al*. (2011) demonstrated the expression profiles of immune related genes in three major immunocompetent organs, viz. kidney, skin and liver during experimental infection with freshwater lice *Argulus siamensis*. Results showed that the expression of TLR 22 like, lysozyme-G and ß2-microglobulin genes in kidney was significantly ($P \leq 0.05$) down-regulated in lice-infected fish. The expression of TLR 22 like and TNFα genes in the skin were significantly up-regulated whereas that of C3 was significantly ($P \leq 0.05$) down-regulated in lice-infected fish with respect to control fish. However, no reports are available on the TLR 3, 4 and 9 genes in the *Catla catla*, which is an important carp in our sub-continent.

The TLR 4 gene is associated with defense from gram-negative bacteria by recognizing the evolutionarily conserved molecular pattern called lipopolysaccharide (LPS) on the membrane of such microbes. In our study, the difference between the expression profiles was not significantly different among the organs, though the expression was the highest in the liver. TLR4 gene was lost from the genomes of most fishes. The TLR4 genes in zebra-fish do not recognize the mammalian agonist LPS and are likely paralogous and not orthologous to mammalian TLR4 genes (Palti 2011). Reports are available on TLR4 expression profile in other species of fish. The zebra-fish TLR4 orthologs negatively regulates the MyD88-dependent signaling pathway which has been established from the lack of a TLR4 ortholog in some fish species and the lack of the essential co-stimulatory molecules for LPS activation via TLR4 (i.e., myeloid differentiation protein 2 (MD-2 and CD14) in all the fish genomes and expressed sequence tag databases available (Sepulcre *et al*. 2009). The TLR4 receptor complex for LPS recognition arose after the divergence of fish and tetrapods. TLR4 has been lost in most species, with the exception of the order Cypriniformes, where loss-of-function mutation(s) of the TILR domain allowed its retention as a negative regulator of the TLR-signaling pathway, which was duplicated later in these fish. It has been shown that extremely high concentrations of LPS (µg/ml) are required to activate *in vitro* isolated leukocytes from several phylogenetically distant teleost species. This may explain the patterns obtained for TLR4 in the present study. Thus, the results obtained in the present study are open to further validation and investigation about the ability of catla fish to express TLR4 under healthy condition.

TLR9 mRNA has been detected in lymphocytes as the main cell-source. The effects of synthetic un-methylated oligo-deoxynucleotides (ODN) containing CpG motifs (GACGTT, GTCGTT, AACGTT) has been evaluated on the immune response in the teleost fish gilthead-seabream (*Sparus aurata*) (Cuesta *et al*. 2008). They reported that these motifs are the most potent inducers of seabream immunity; however the involvement of TLR 9 warrants further investigations. In an another study on TLR 9 cDNA in salmon, conducted by Skjaeveland *et al*. (2008), it was demonstrated that the inferred protein sequence revealed several functionally important mammalian amino acid residues conserved in salmon. Furthermore, TLR 9 expression was elevated in head-kidney and leukocytes after *in vitro* treatment with CpG ODNs and recombinant trout interferon (IFN)-γ. The results indicated that the structure, expression and possibly the function of TLR9 are conserved across the teleost and mammalian lineages. The leucine-rich domain (LRD) and the Toll/interleukin-1 receptor (TIR) domain found in other vertebrate TLR9s were conserved in Japanese flounder TLR9 (Takano *et al*. 2007). The flounder TLR9 gene was highly expressed in epithelial and lymphoid organs, such as gills, intestines, kidney, spleen and stomach in an apparently healthy fish. The mRNA copy numbers of Japanese flounder TLR9 and its adapter protein, the myeloid differentiation factor 88 (MYD88) were increased in some organs including blood, gill, kidney and spleen after *Edwardsiella tarda* challenge. Immunohistochemical analysis revealed that TLR9 and MYD88 were expressed in the same cells of kidney. Our result also showed that the TLR9 gene expression was significantly high ($P<0.09$) in spleen, followed by liver and kidney in healthy catla fish, without any challenge.

ACKNOWLEDGEMENT

The work has been carried out by the grants funded by the Department of Biotechnology, Government of India, under the scheme "Aquatic and Marine Biotechnology". We thankfully acknowledge the support from the College of Fishery, GADVASU, Ludhiana, for providing the tissue samples.

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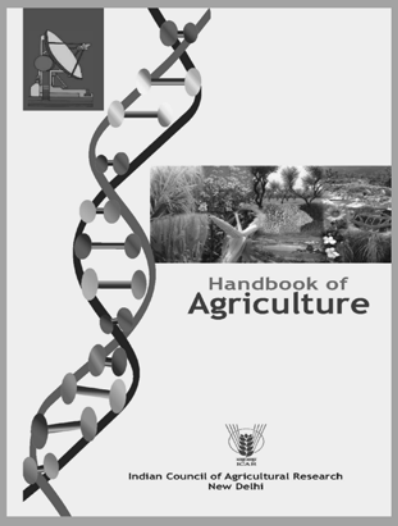

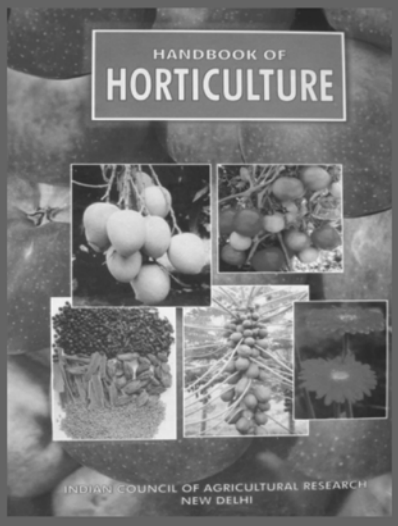

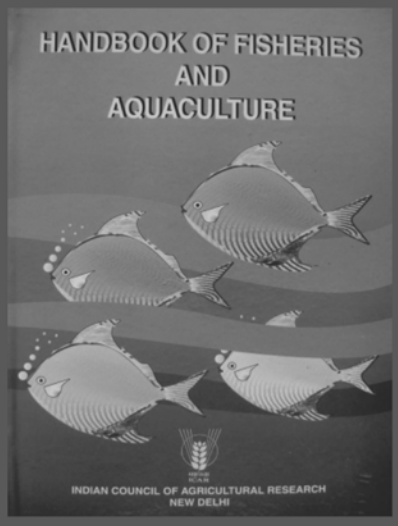

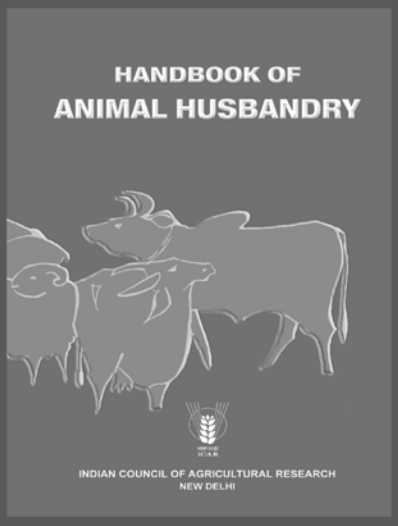

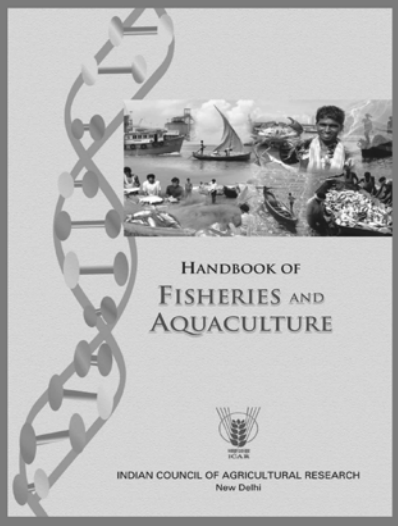

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