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Research Article

IN VIVO EFFECT OF CPG OLIGODEOXYNUCLEOTIDES (CPG ODNS) ON THE EXPRESSION OF TOLL-LIKE RECEPTOR9 (TLR9) IN CATLA CATLA

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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. Unmethylated cytidine-phosphate-guanosine (CpG) dinucleotides of bacterial DNA are known to induce innate and adaptive responses *via* TLR pathway. TLR9 has been identified to specifically recognize CpG motifs and trigger the immune responses during bacterial invasion. In fish, studies on the expression of TLRs due to CpG-ODN have not been reported. The present study was carried out to assess the expression of TLR9 at various time intervals (2h, 4h, 6h, 12h and 24h) in the important immune organs *viz.*, kidney and spleen of *Catla catla* upon induction with CpG ODNs (B and C). TLR9 was differentially expressed in kidney and spleen tissue of *C. catla* at 2h, 4h, 6h, 12h and 24h when induced with CpG ODNs (B and C) *in vivo*. Significantly ($P<0.001$) higher TLR9 expression was observed in kidney than spleen in both CpG ODN-B and ODN-C induced treatment groups. CpG ODN-B resulted in significantly higher ($P<0.001$) TLR9 expression in kidney and spleen tissues compare to CpG ODN-C.

Key Words: *Toll -Like Receptors (TLRs), Catla Catla, CpG ODNs, Semi-Quantitative RT-PCR*

INTRODUCTION
Innate immunity is the body's first line of defense which is involved in the earliest detection and elicitation of immune response against pathogens. Innate immune system relies on a set of pattern recognition receptors (PRRs) that detect the pathogen's molecular structures. Toll-like receptors (TLRs) are PRRs of type-1 transmembrane proteins present in neutrophils, macrophages, dendritic cells, vascular endothelial cells and intestinal epithelial cells (Phelan *et al.,* 2005 and Baoprasertkul *et al.,* 2006). PRRs recognize the bacterial pathogens based on the unmethylated cytidine-phosphate-guanosine (CpG) dinucleotide flanked by specific bases present in the bacterial DNA (Krieg *et al.,* 2000). CpG DNA is taken up by immune cells *via* receptor mediated endocytosis which interacts with TLR9 present in endocytic vesicles (Hemmi *et al.,* 2000). It stimulates TLR based pro-inflammatory immune response characterized by cytokine release and activation of both the innate and adaptive immune systems there by conferring protection against the invading pathogens. *C. catla* is one among the commercially important indigenous carp species of India. Bacterial diseases pose a major threat to the farming of various species of carps in India (Sahoo *et al.,* 2011). The objective of this study was to assess the *in vivo* effect of CpG oligodeoxynucleotides (CpG ODNs), a bacterial ligand on the expression of TLR9 in the tissues of kidney and spleen which are the important immune organs in *Catla catla.*

MATERIALS AND METHODS
Experimental Design
C.catla juveniles of approximately 30g±5g were procured from a local fish rearing unit in Thiruvallur, Tamilnadu, India. Fishes were maintained in tanks with fresh water, fed daily to satiation with feed pellets and acclimatized to lab conditions for a week prior to use in experiments. Fishes of 20 nos. each were used in control and treatment groups of the experiment that were maintained in triplicates. The fishes in

the treatment groups were intraperitoneally injected with 100 µl (30µg) of CpG- ODNs (B and C) separately (Tassakka and Sakai 2003) and the fishes in control group were injected with PBS (100 µl).

Tissue Samples

Tissue samples of kidney and spleen were collected aseptically from fishes of both control and treatment groups at various time intervals (2h, 4h, 6h, 12h, and 24h) post-induction with CpGs or PBS. Total RNA was extracted from the tissue samples using one step RNA reagent kit (BioBasic Inc., Canada) following manufacturer's instructions. About 2µg of the total RNA from each sample was used as template for reverse transcriptase using high capacity cDNA synthesis kit (*Applied Bio Systems* Inc., USA).

RT-PCR Analysis

Expression of TLR9 mRNA was analyzed by reverse transcriptase PCR (RT-PCR). cDNA from samples were used as templates for the PCR amplifications that were carried out using TLR9- specific self-designed PCR primers (SDDLTLR9/F and SDDLTLR9/R) and fish β-actin internal control following a published PCR protocol (Oshiumi *et al.,* 2003). The details of the primers used in the study are presented in Table 1. PCR was performed in a PCR thermal cycler (Eppendorf, Germany) in a total volume of 25µl with 22 µl of master mix, 1 µl (30 pmoles) forward primer, 1 µl (30 pmoles) reverse primer, and 1µl cDNA template. The PCR conditions followed were, initial denaturation at 94°C for 5min; followed by 30 cycles of denaturation at 94°C for 45s; annealing at 56°C for 1min and extension at 72°C for 45 sec. PCR products were resolved in a 2% agarose gel stained with ethidium bromide. The band intensities of PCR products were analyzed by quantity one^{TM} image acquisition software (BioRad INC., USA). The difference in the band intensities in the background gel and that of each TLR amplicon was considered as the corrected intensity values. These values were then normalized with the corresponding β-actin mRNA expression values. The expression levels of TLR9 mRNA in tissue samples of treatment and control fishes were calculated. Arbitrary units of 0.5 and below were considered as low or no expression. The values were expressed as mean$\pm$ SD. The data were analyzed statistically using 2way ANOVA test.

Table 1: Primers used for the amplification of TLR9 in our study

Primer Code	Primer Sequence	Accession No. of the Sequence Used for Primer Design
SDDLTLR9/F	5' ATT GGA GAA CCG AGG GAG AT 3'	GU809229
SDDLTLR9/R	5' TGG TCC AAC AGG TGC ATT AG 3'	

RESULTS AND DISCUSSION

Tissues of kidney and spleen collected from *C.catla* fishes in control group showed basal expression of TLR9. Significantly higher ($P<0.001$) expression of TLR9 was observed in the samples of kidney and spleen tissues collected from the treatment groups induced with CpG ODNs (B and C) than in the control groups (Figure 1 and 2).

In ODN-B induced treatment group, significant ($P<0.001$) up regulation in TLR9 expression was observed in the kidney tissue at 2h,4h,6h,12h and 24h post-induction. In spleen tissue, although there was upregulation of TLR9 expression at 2h,4h,12h and 24h post-induction, the expression was significantly ($P<0.001$) higher compared to control group only at 4h (Figure 1).

In ODN-C induced treatment fishes, kidney tissue showed upregulation in 2h, 6h, 12 and 24h, with significant ($P<0.01$) increased expression at 24h post-induction. Significant downregulation of TLR9 expression was observed in kidney tissue at 4h post-induction. In spleen tissue, upregulation in the TLR9 expression observed at 2h, 4h, 6h and 24h post-induction with the significant upregulation levels of TLR9 expression ($P<0.001$) at 6h and 24h ($P<0.05$). Significant downregulation of TLR9 expression was observed in spleen tissue at 12h post-induction (Figure 2).

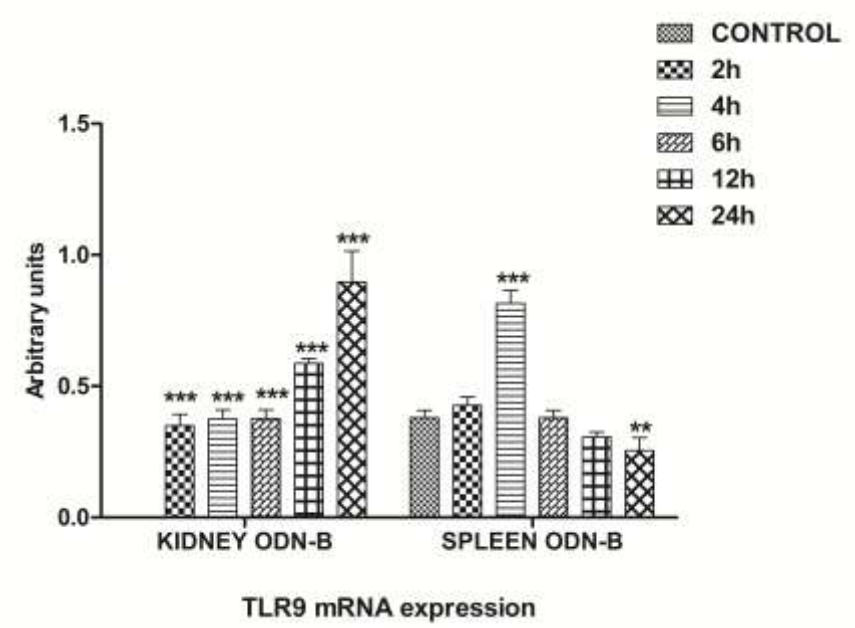

Figure 1: TLR9 mrna expression in *Catla catla* induced with ODN-B. Data presented as the mean± SD. ***denotes (P<0.001) and ** denotes (P<0.01).

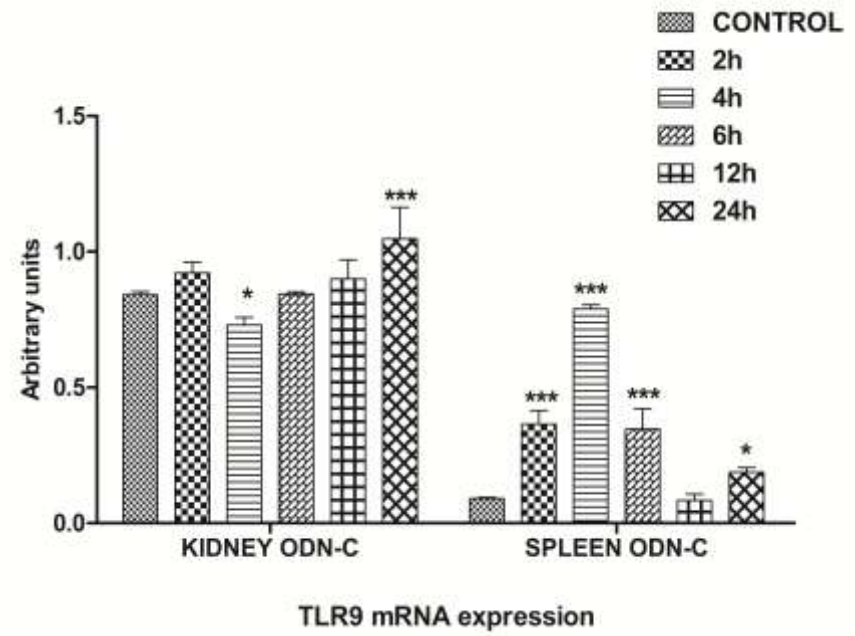

Figure 2: TLR9 mRNA expression in *Catla Catla* induced with ODN-C. Data presented as the mean± SD. ***denotes (P<0.001) and * denotes (P<0.05).

Unmethylated CpG motifs present in the bacterial DNA are selectively recognized by the vertebrate immune system resulting in non-specific immune responses (Tassakka and Sakai 2003). Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs are known to mimic the activity of bacterial DNA (Krieg *et al.,* 1995). CpGs have been reported to act as an immune adjuvant in various murine disease

models to augment both humoral and cell mediated immune responses (Jorgensen *et al.,* 2001). In murine and mammalian hosts, activation of TLR9 by CpG ODNs initiated an immunostimulatory cascade that resulted in maturation, differentiation and proliferation of natural killer (NK) cells, B cell proliferation, T cells and monocytes/macrophages resulting in the secretion of cytokines and chemokines (Krieg *et al.,* 1995 and Klinman *et al.,* 2004). It has been demonstrated that the expression of cytokines in fish could be stimulated by CpG-ODNs (Tassakka and Sakai 2004). CpG DNA/ODNs are taken up by immune cells *via* receptor-mediated endocytosis and it interacts with TLR9 present in endocytic vesicles (Hemmi *et al.,* 2000). Swelling and acidification of the endocytic vesicles and the generation of reactive oxygen species is effected by the interaction of TLR9 with CpG-ODNs (Yi *et al.,* 1998; Takeshita and Klinman 2000; Takeshita *et al.,* 2001). Although studies on immune response with reference to cytokine and chemokines secretion in CpG-ODNs induced fishes have been reported (Tassakka and Sakai 2003; Tassakka *et al.,* 2006), there is no report on the TLR expression in response to induction with CpG-ODNs in fishes. Synthetic ODN types A, B, C, D, and E that vary in the nucleotide sequences flanking the CpG dinucleotides and in immunostimulatory activity have been used to study the *invivo* and *invitro* immune responses in fishes (Tassakka and Sakai 2003). As CpG ODNs-B (GACGTT) and CpG-ODN-C (AACGTT) have shown high immunostimulatory effects (Tassakka and Sakai 2003) in fishes, CpG ODNs- B and C were used in our experiment.

Varying concentrations of CpG-ODNs ranging from 10ng/fish to 10µg/fish have been used to study the immune responses in fishes (Tassakka and Sakai 2003; 2004). As there is no report on the effective concentration CpG ODN to be used for the *in vivo* studies in fish, CpG ODN concentration of 30µg/fish CpG ODNs-B and C was used in our study. Comparison of the effectiveness of ODNs (B and C) in inducing *C.catla* to express TLR9 showed that ODN-B induces the kidney and spleen more effectively than ODN-C to express TLR9. Also, kidney of *C.catla* showed comparatively higher immunological response than spleen when induced with ODNs (B and C). The expression levels were higher in kidney tissue in each of the treatment at various time intervals of post-induction.

Induction with CpG ODNs in kidney cells of common carp have resulted in immune responses as evidenced by the increased expression of IL-1β, CXC, CC and LyC with significantly ($P<0.05$) higher expression of IL-1β and LyC at 24h post induction and the expression levels also varied in their study at various days post-induction (Tassakka and Sakai 2004). Similar observation of upregulation in TLR9 expression at 24h post-induction was recorded in our study.

In conclusion, our results showed that CpG-ODNs are effective in inducing TLR9 based innate immune responses in *C.catla*. CpG ODN-B elicits stronger immune responses than CpG ODN-C. Hence, CpG ODNs could be used as a potential immunostimulant/adjuvant to induce TLR9 expression and to improve protective immunity against bacterial diseases.

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