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Nucleotide Binding and Oligomerization Domain 1 (NOD1) Receptor in Catla (*Catla catla*) : Inductive Expression and Down-Stream Signaling in Ligand Stimulation and Bacterial Infections

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Abstract

The nucleotide binding oligomerization domain 1 (NOD1) is a cytoplasmic pattern recognition receptor (PRR), and is a member of the NOD-like receptor (NLR) family. It senses various pathogens or their products, and plays an important role in inducing innate immunity. This report describes the identification of NOD1 and its down-stream signaling in the Indian major carp, catla (Catla catla), one of the highly commercially important and extensively cultured freshwater fish species in the Indian subcontinent. The basal expressions of NOD1and its downstream signaling molecule RICK (receptor interacting serine-threonine kinase) were analyzed in gill, liver, kidney, intestine, heart, spleen, brain, eye, muscle, skin and blood of healthy catla fingerlings by quantitative real-time PCR (qRT-PCR) assay, and it showed their wide expression in all tested tissues. Among the tested tissues, highest expression of NOD1 was observed in liver, and RICK in gill. Stimulation with iE-DAP, LPS and poly I:C activated NOD1 receptor signaling resulting in significant ($p < 0.05$) induction of downstream signaling molecule RICK, and the effector molecules IL-1β in the treated fish group as compared to their control. Infection with Aeromonas hydrophila also activated NOD1 receptors signaling. These findings suggest the important role of NOD1 receptor as innate immune gene in fish.

Keywords: Indian major carp. *Catla catla*. NOD1, RICK, Innate immunity.

Introduction

Innate or non-specific immunity is an evolutionary ancient part of the defense mechanism in all multi-cellular organisms. It is the first line of defense against infection and is regarded as the primeval and hence the universal form of host defense[1]. A major component of the innate immune system is contributed by the pattern-recognizing receptors (PRRs) that recognize conserved microbial structures like peptidoglycan (PGN), lipoteichoic acid (LTA), zymosan, flagellin, lipopolysaccharides (LPS), lipoproteins, heat shock protein (hsp), CpG-DNA, and nucleic acids of the micro-organisms (pathogen/microbes- associated molecular patterns, PAMPs/MAMPs)[2,3]. NOD-like receptors (NLRs), Toll -like receptors (TLRs) and RIG-I like receptors (RLRs) are important PRRs that recognize various PAMPs and activate their signaling cascades to induce innate immunity[4,5].

The NLRs are large multi-domain protein with tripartite architecture with a C-terminal leucine rich repeat (LRR) domain, a central nucleotide oligomerization (NACHT) domain and a N-terminal protein-protein interaction domain composed of a CARD (caspase activation and recruitment domain) or PYD (pyrin domain) or BIR (baculovirus inhibitor of apoptosis repeat) domain. The LRR domain recognizes PAMPs, NACHT domain mediates self-regulation and oligomerization and the CARD/PYD/BIR domain transmits downstream signals[6,7]. NOD1 is a cytosolic protein that senses meso-DAP (meso-diaminopimelic acid) containing peptidoglycan of Gram-negative bacteria[8-10]. Following ligand recognition, the activated NOD starts self-oligomerization and recruit the down-stream interacting protein, RICK via CARD-CARD-interaction. Recruitment of RICK causes activation of Iκκ [IκB (inhibitor of NF-κB) kinase] complex, and stimulates NF-κB (nuclear factor κB) activation by ubiquitinylation of Iκκ-γ subunit of Iκκ complex, and MAPK (mitogen-activated protein kinase) resulting in ativation of specific transcription factors like AP-1 (activator protein 1), and induction of pro-inflammatory cytokines and chemokines *viz.,* IL (interleukin)-1β, IL-6, IL-8, TNF (tumor necrosis factor)-α and IFN (interferon)-γ[11-13].

Various NOD receptors were previously reported in human, mouse and porcine and recently in zebrafish, channel catfish, grass carp, rainbow trout, rohu, orange-spotted grouper, olive flounder and goldfish[14-25].

Catla (*Catla catla*) is one of the important freshwater carp and is commercially cultivated in the wide region of the Indian subcontinent. However, no study yet describes the role of any NOD receptors in catla. We therefore, carried out this study to identify NOD1 receptor, and its downstream signaling in catla following ligand stimulation and bacterial infections.

Material and Methods

Fish: Catla fingerling weighing ~30 g, was obtained from the Central Institute of Freshwater Aquaculture (CIFA) and was

stocked in 500 L aerated FRP tanks, and each tank contained 50 fishes. Before we start the experiment, fishes were acclimatized for 3-weeks in these tanks with daily two-third water exchange and were fed with commercial carp diet at twice a day. During the experiment, the water temperature varied between 26 to 29 ^{0}C and pH ranged between 7.4 to 7.6.

Bacteria: *Aeromonas hydrophila* (ATCC-35654) were cultured in LB broth (USB, USA) at 37^{o}C for 16 h with constant shaking, and the live bacterial count was determined as colony forming unit (CFU) following 10-fold serial dilutions in PBS (phosphate buffered saline) and plating on nutrient agar medium.

Cloning of NOD1 and RICK genes in catla: To clone NOD1 and RICK in catla, PCR primers were designed from the heterologous fish species, table-1. For NOD1, primers were designed from the conserved nucleotide domain of NOD1 in grass carp (GenBank ID: FJ937972.1) and zebrafish (GenBank ID: XM_002665060.2) and for RICK from zebrafish (GenBank ID: AF487540.1) only. Total RNA was extracted from catla gill, cDNA was prepared and PCR was carried out with 1 µl of cDNA in a 50 µl reaction volume under the conditions of one cycle of initial denaturation at 94°C for 2 min followed by 45 cycles of 94ºC for 30 sec, 60ºC for 30 sec, 72ºC for 1 min and a final extension at 72ºC for 5 min. One-fifth of the PCR product was analyzed in 2% agarose gel, and the single specific band was purified with agarose gel purification kit (Roche, Germany). The purified DNA was cloned in pGEM-T Easy vector (Promega, Madison, USA) and both strand sequencing were carried out with T7 and SP6 primer (ABI prism 3000). BLAST search was carried out for their confirmation[26].

Table-1
Primers used for cloning

Primer	Sequence (5'→3')
NOD1 FW	TGGCTGCAACAACATCACTGGT
NOD1 RV	CCTGAAGGTGTCCGCAAAGTC
RICK FW	GCCACTTCTACACCACGATCTCAA
RICK RV	CTCGGAGAACGCTGAACATGATC

***In- vivo* expression of NOD1 and RICK genes** : To study tissue specific expression of NOD1 and RICK genes, gill, liver, kidney, intestine, blood, heart, brain, skin, muscle, eye and spleen were collected separately from catla fingerlings in TRIzol reagent (Invitrogen, USA). Total RNA was extracted from each sample following the standard protocol of TRIzol reagent, and cDNA was prepared following the protocol as described later. Quantitative real-time PCR was carried out to examine NOD1 and RICK gene expression in various tissues keeping β-actin as an internal control. To eliminate individual variations, samples from three fish were collected and analyzed separately by qRT-PCR and their mean value was considered.

***In- vivo* ligand exposure and bacterial challenge:** Healthy catla fingerlings were divided into control and treated groups keeping three fish in each group. D-glutamyl-meso-diaminopimelic acid (iE-DAP) (InvivoGen, USA) was diluted in endotoxin free water at 10 mg/ml and 100 µl of endotoxin free water containing 200 µg iE-DAP was intra-venously (i.v.) injected, For LPS (lipopolysaccharide) treated group, purified LPS of *Escherichia coli* (serotype O55: B5) (SIGMA, Germany) was diluted in endotoxin free water at 5 mg/ml, and endotoxin free water (100 µl) containing 20 µg of diluted LPS was injected through i.v. route. The control fish group was i.v. injected with 100 µl of endotoxin free water. For poly I:C - treated fish group, purified poly I:C (Sigma, USA) was diluted in DEPC treated water at 10 mg/ml, and 100 µl of DEPC-treated water containing 300 µg of diluted poly I:C was i.v. injected. Under the same condition, control fishes were i.v. injected with 100 µl of DEPC-treated water. After 4 and 24 h of ligand treatment, blood was collected in TRIzol reagent. For bacterial infection, fish were intra-peritoneally (i.p.) injected with 100 µl of PBS containing *A. hydrophila* (1×10^6 CFU/fish). The control fish group was i.p. injected with 100 µl of PBS only, and kept separately in the aerated tank. After 24 h of *A. hydrophila* infection, control and treated group of fish were sacrificed and tissues were collected separately in TRIzol reagent.

RNA isolation and 1st strand cDNA synthesis : Total cellular RNA from different organs/tissues of fish was extracted with TRIzol reagent (Invitrogen, USA). From each sample, RNA concentration was measured by UV-spectrophotometer and the integrity of the RNA was assessed by observing the band intensity of 28 and 18S ribosomal RNA on 1 % agarose gel. For cDNA (complementary DNA) synthesis, total RNA (1 µg) was treated with DNase I (1 unit) (MBI, Fermentas, USA) and the reverse transcription (RT) was carried out using oligo-dT primer and RevertAid 1st strand cDNA synthesis kit (MBI, Fermentas, USA).

Real-time PCR analysis : Quantitative real-time PCR (qRT-PCR) of the target genes *viz;* NOD1, RICK , IL-1β and the reference gene β-actin were performed in LightCycler®480 II-real time PCR detection system (Roche, Germany). Amplifications were carried out in 10 µl reaction volume, containing 1 µl of cDNA, 0.25 µl of FW and RV primer (2.5 µM each), 5 µl of 2X lightCycler® 480 SYBR Green I master mix (Roche, Germany) and 3.5 µl of PCR grade water. The real-time PCR was carried out in triplicate wells under the following conditions: initial denaturation at 95ºC for 10 min followed by 45 cycles of 94 ºC/10 s, 56 ºC (for NOD1) or 58 ^{0}C (for RICK, IL-1β, β-actin) for 10s and 72ºC/10 s. The specificity of the RT-PCR was regulated using no-template controls. Nucleotide sequences and GenBank accession numbers of Primers used in real-time PCR assay have been given in table-2.

The PCR efficiencies were determined by analysis of serial dilutions of cDNA, and efficiencies were ~ 100 % which allowed the use of $2^{-\Delta\Delta CT}$ method for calculation of relative gene expression of the target gene NOD, RICK and IL-1β with that of the reference gene, β-actin[27]. We confirmed the correct size and single band amplification by analyzing qRT-PCR

products in ethidium bromide-stained 2% agarose gel. The data obtained from qRT-PCR analysis was expressed as the mean of three individual experiment ± standard error (s.e.), and the significant difference between the control and treated groups at each time point was determined by the Student's *t*-test using Microsoft Excel 2010 with $p < 0.05$ as significance level.

Table-2
Primers used for real-time PCR analysis

Primer	Sequence (5'→3')	GenBank Acc.No
NOD1 FW	GTTGGTGGGAAATACCTTGCC	KC542884
NOD1 RV	TGCTTTCGCCAGACTTCTTCC	
RICK FW	GGCGCCAGCTCTCTATCACTAA	KC542886
RICK RV	CCTCTTCAAATGGTATCCGTCTT	
IL-1β FW	ACCCCACAAAACATCGGCCAACC	AM932525
IL-1β RV	TCTTCTCCATTTCCACCCTCTC	
β-actin FW	AGACCACCTTCAACTCCATCATG	EU184877
β-actin RV	TCCGATCCAGACAGAGTATTTACGC	

Results and Discussion

In catla, NOD1 and RICK genes were cloned from gill, and were submitted to the GenBank with the accession numbers KC542884 and KC542886 respectively. The Indian major carps, catla and rohu (*Labeo rohita*), belong to the cyprinid family. Therefore, as expected, NOD1 and RICK in catla showed significantly higher (~ 98 %) similarity and identity in nucleotide sequence with rohu NOD1 and RICK[22].

In healthy catla fingerlings, tissue specific expression of NOD1 gene in muscle, eye, blood, intestine, brain, heart, kidney, liver, spleen, gill and skin was evaluated by qRT-PCR assay and the expression of NOD1 gene in various organs/tissues was represented as relative fold changes from the lowest NOD1 expressing tissue, eye, chosen as calibrator (1). NOD1 was constitutively expressed in all the tissues examined. Among the tested tissues, highest level of NOD1 expression was detected in liver (~ 5 fold), followed by intestine, blood, muscle, heart, brain, skin, spleen, kidney, gill and eye, figure-1A. The tissue specific expression of NOD1 in catla was markedly different from the previously reported fish species *viz.,* zebrafish, catfish, grass carp, orange spotted grouper, olive flounder and rohu[17-19, 22-24]. As shown in figure-1B, the distribution of RICK in various tissues of catla revealed its constitutive expression across the organs. Among the tested organs, RICK expression was lowest in the blood and was highest in gill (~ 6.5 fold) and was considerably different from the expression pattern in rohu[22]. Wide expression of NOD1 and RICK in various organs may indicate the important role of NOD1 as an innate immune gene in regulating anti-microbial activity in fish.

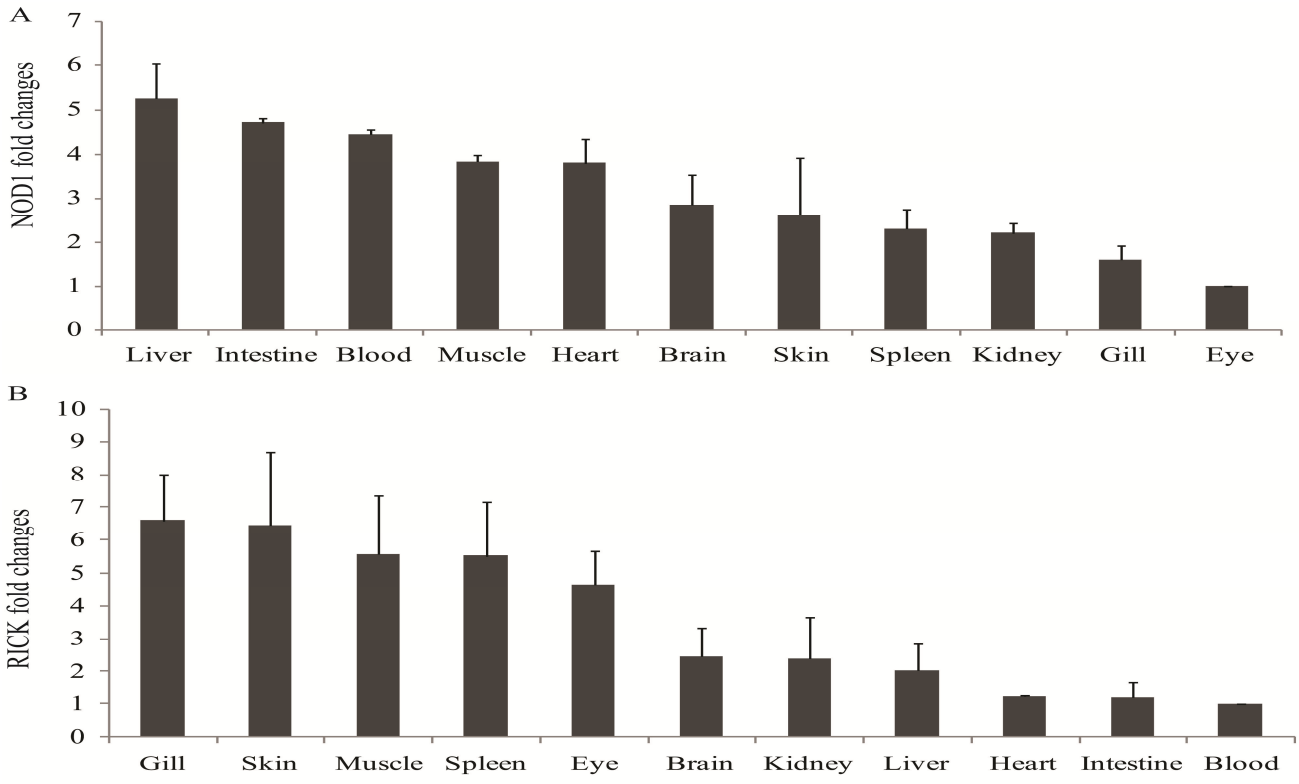

Figure-1
Basal expression of NOD1 and RICK genes in various tissues of catla (A) NOD1 and (B) RICK gene expression

In catla, activation of NOD1 signaling by potential ligands were investigated by analyzing the expression of NOD1, RICK and IL-1β in blood by qRT-PCR assay after 4 and 24 h of iE-DAP, LPS and poly I:C treatment. As shown in figure-2A, NOD1 expression was significantly ($p < 0.05$) up-regulated at both 4 and 24 h of ligands treatment. In iE-DAP treatment, NOD1 was up-regulated ~ 2.2 fold and ~ 2 fold at 4 and 24 h respectively. In LPS treatment, at 4 h, it was ~ 2 fold and at 24 h ~ 1.7 fold of up-regulation of NOD1 was observed. In case of poly I:C treatment, NOD1 was increased ~ 1.5 fold at both 4 and 24 h.

Then, we analyzed the expression of RICK, which is the downstream adaptor molecule of NOD1 receptor signaling pathway. The qRT-PCR result indicated that the RICK expression was significantly ($p < 0.05$) up-regulated in the treated fish as compared to the control fish, figure-2B. In NOD-signaling pathway, IL-1β is one of the downstream effector molecules. We therefore, analyzed the expression of IL-1β in blood of iE-DAP, LPS and polyI:C treated fish at 4 and 24 h. As shown in figure-2C, IL-1β expression was significantly induced in all above mentioned ligands treatment. However, the expression was maximum at 4 h in ligands treated fish and this result was almost correlated with the NOD1 and RICK expression indicating its expression through NOD1 signaling pathway.

NOD1 is a member of NLR family that functions as a cytoplasmic sensor of detecting pathogen associated molecular pattern (PAMPs), and upon recognition of PAMPS, they interact with a common adaptor protein RICK which activates the intra-cellular signaling cascades resulting in the induction of innate immune genes. Recruitment of RICK is expected to activate NF-κB and MAPK pathway resulting in the expression of pro-inflammatory cytokines[12,]. We investigated NOD1 signaling in catla after treating with iE-DAP, LPS and poly I:C. These ligands treatment activated NOD1 signaling with the inductive expression of downstream signaling molecules *viz.,* RICK and IL-1β. These data obtained in catla supported the previous reports of higher vertebrates signaling and suggested the conservation of innate immune signaling pathway from lower to higher vertebrates[28].

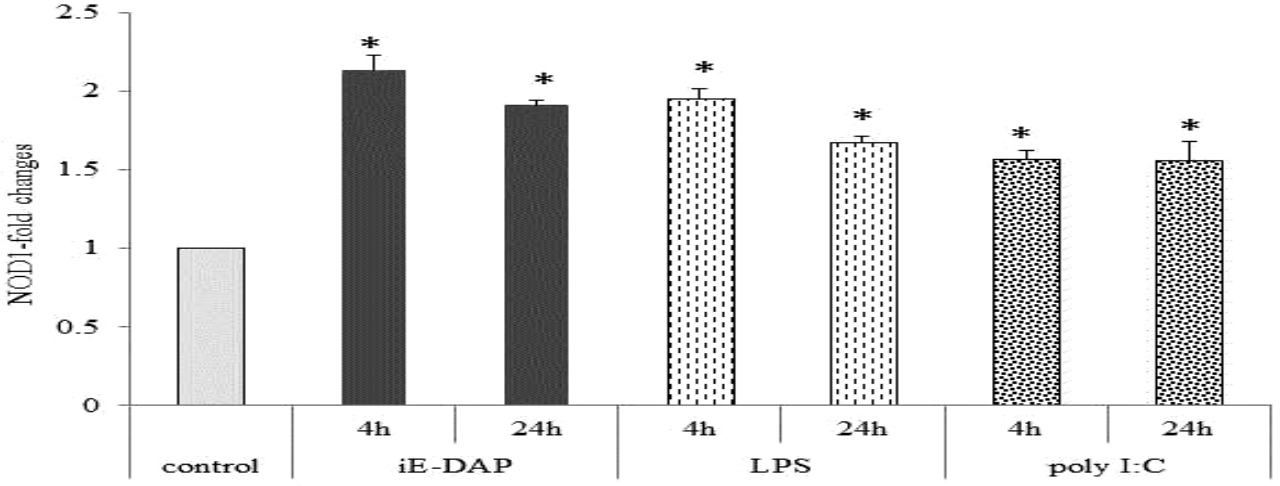

Figure-2(A)

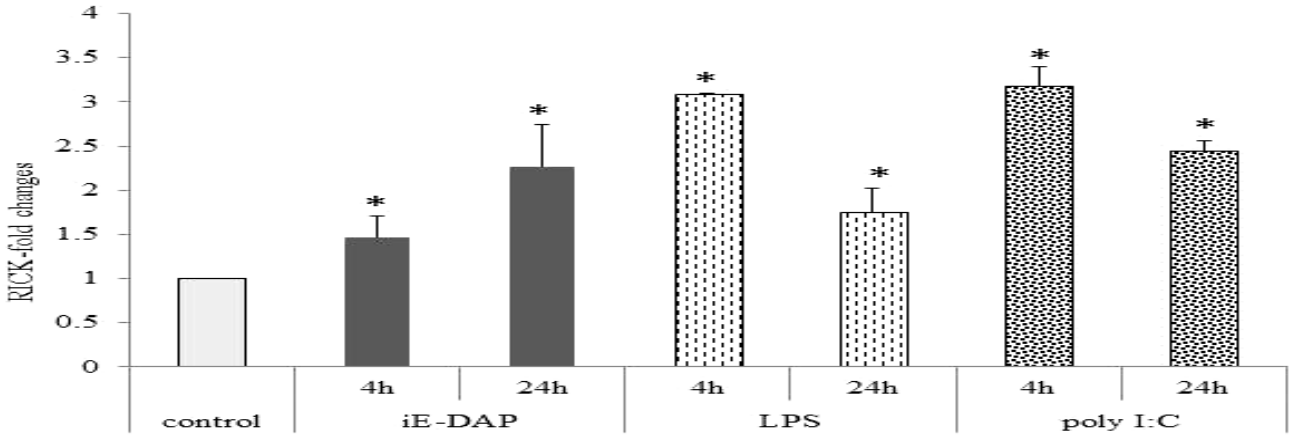

Figure-2(B)

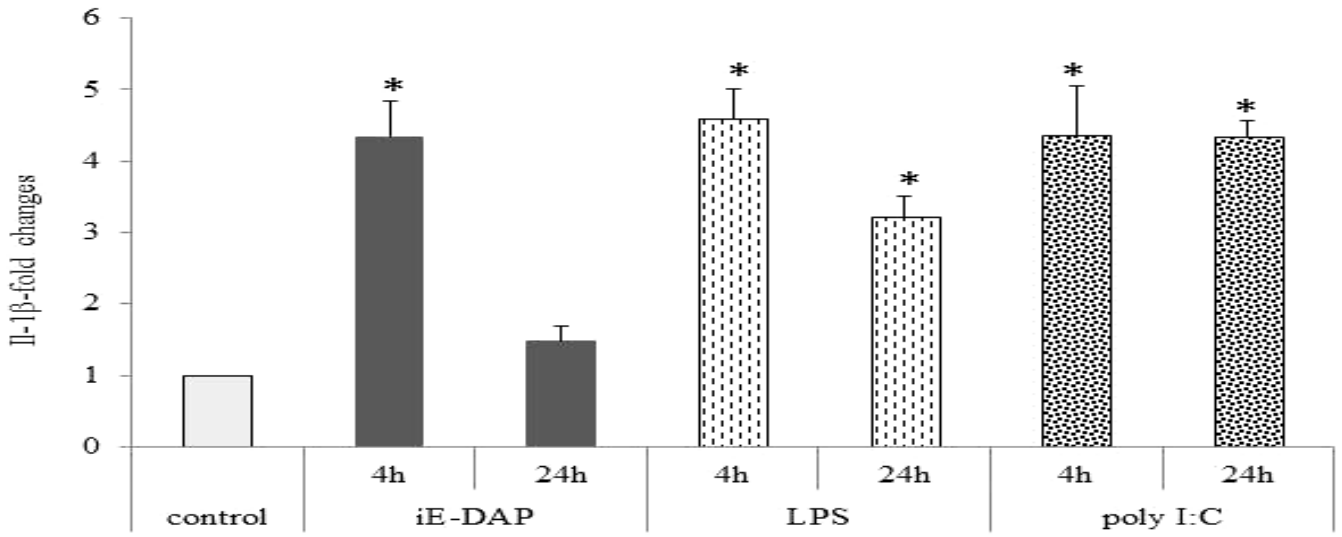

Figure-2(C)
Expression of NOD1, RICK and IL-1β in blood following ligands stimulation (A) NOD1 (B) RICK and (C) IL-1β gene expression, Significant difference ($p < 0.05$) between the control and treated fish group was indicated with asterisks (*)

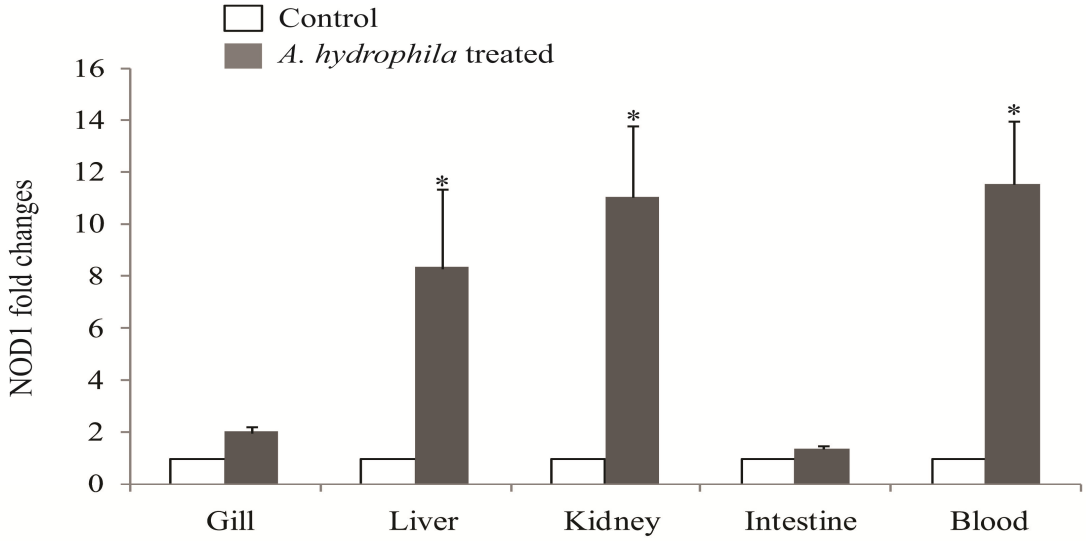

Figure-3
Modulation of NOD1 gene expression following *A. hydrophila* infection, Significant difference ($p < 0.05$) between the control and treated fish group was indicated with asterisks (*)

Fish farmers in India, encounter considerable economic losses due to fish diseases caused by a variety of pathogens belonging to the genus *Aeromonas*, *Edwardsiella*, *Flavobacterium*[29]. The innate immune system is the major defense mechanism in lower vertebrates particularly in fishes. Therefore, innate immune receptor such as NOD1 is expected to play significant role in host defense against bacterial infection[12].

To investigate whether NOD1 expression could be modulated in bacterial infection, we infected catla fingerlings with *A. hydrophila*, and examined NOD1 expression in gill, liver, kidney, intestine, and in blood by qRT-PCR assay at 24 h post infection. The results showed significant activation ($p < 0.05$) of NOD1 receptors in all tested organs (gill. liver, kidney and blood) at 24 h of *A. hydrophila* infection, figure-3. Among the tissues, maximum induction of NOD1 was observed in the infected fish blood (~ 11.5 fold) followed by kidney (~ 11 fold) and intestine (~ 1.3 fold) as compared to the control group. In mammals, NOD1 is shown to be involved in sensing Gram-negative bacteria invasion[30]. In catfish and rohu, NOD1 was activated during Gram-negative bacterial infections[18, 22].

Together, these data in various fish species strongly suggest the important role of NOD1 receptors sensing in pathogenic invasion and the induction of innate immunity. Further works are required to understand the detail mechanisms of NOD1 signaling in catla.

Conclusion

In conclusion, NOD1 receptor and its downstream adaptor molecule RICK have been identified in catla, a commercially important freshwater carp in the Indian subcontinent. Treatment with various ligands, i.e. iE-DAP, LPS and poly I:C activated NOD1 receptor signaling with the inductive expression of adaptor molecule RICK and effector molecules IL-1β. A similar finding of NOD1 activation was also noted following bacterial infection. The present findings demonstrate that NOD1 receptor in catla may play an important role in inhibition of bacterial and viral infection and functionally might be conserved among the other fish species.

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