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Pathogen recognition receptors in channel catfish: I. Identification, phylogeny and expression of NOD-like receptors

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ABSTRACT

Innate immune system plays a significant role in all multicellular organisms. The key feature of the system is its ability to recognize and respond to invading microorganisms. Vertebrates including teleost fish have evolved an array of pathogen recognition receptors (PRRs) for detecting and responding to various pathogen-associated molecular patterns (PAMPs), including Toll-like receptors (TLRs), nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs), and the retinoic acid inducible gene I (RIG-I) like receptors (RLRs). In this study, we identified 22 NLRs including six members of the NLR-A subfamily (NODs), two members of the NLR-B subfamily, 11 members of the NLR-C subfamily, and three genes that do not belong to any of these three subfamilies: Apaf1, CIITA, and NACHT-P1. Phylogenetic analysis indicated that orthologs of the mammalian NOD1, NOD2, NOD3, NOD4, and NOD5 were all identified in catfish. In addition, an additional truncated NOD3-like gene was also identified in catfish. While the identities of subfamily A NLRs could be established, the identities of the NLR-B and NLR-C subfamilies were inconclusive at present. Expression of representative NLR genes was analyzed using RT-PCR and qRT-PCR. In healthy catfish tissues, all the tested NLR genes were found to be ubiquitously expressed in all 11 tested catfish tissues. Analysis of expression of these representative NLR genes after bacterial infection with *Edwardsiella ictaluri* revealed a significant up-regulation of all tested genes in the spleen and liver, but a significant down-regulation in the intestine and head kidney, suggesting their involvement in the immune responses of catfish against the intracellular bacterial pathogen in a tissue-specific manner. The up-regulation and down-regulation of the tested genes exhibited an amazing similarity of expression profiles after infection, suggesting the co-regulation of these genes.

1. Introduction

The innate immune system is the primary defense mechanism among invertebrate animals, and plays a key role in vertebrate animals as well, particularly in lower vertebrates such as fishes where adaptive immunity is relatively less developed. It recognizes and senses potentially harmful pathogens and rapidly triggers appropriate defense mechanisms that prevent or minimize tissue damage. Primarily, the innate response is mediated by germ-line encoded pathogen/pattern-recognition receptors (PRRs) that recognize the conserved molecular signatures associated with pathogens termed pathogen-associated molecular patterns (PAMPs). After sensing the PAMPs, host innate immune cells initiate a broad spectrum of defense responses that result in the development of inflammation and host defense against infection (Akira et al., 2006). PRRs comprise an array of sensors and are found in the extracellular space, membrane-associated variant cell types or in the cytosol. Three major classes of PRRs have been identified: (1) The Toll-like receptors (TLRs) that recognize ligands on either extracellular surface or within the endosome, (2) the nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) that are cytoplasmic receptors, and (3) retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), a group of virus recognizing intracellular receptors (Akira et al., 2006; Meylan et al., 2006; Chen et al., 2009; Franchi et al., 2010; Hansen et al., 2011). Of these PRRs, TLRs were the earliest characterized and are the most extensively studied PRRs in both vertebrates and

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invertebrates (EwaSnaar-Jagalska et al., 2004; Jault et al., 2004; Hansen et al., 2011), while RLRs are a recently characterized small group of PRRs that are key cytosolic pattern recognition receptors for detecting nucleotide PAMPs of invading viruses and they are crucial for the RNA virus-triggered interferon response (Takeuchi and Akira, 2008; Zou et al., 2009).

Unlike TLRs, NLRs are a recently identified large group of intracellular PRR family characterized by the presence of a nucleotide-binding domain, the NACHT domain (Damiano et al., 2004; Koonin and Aravind, 2000; Hansen et al., 2011) or closely associated NB-ARC domain, and established as a set of proteins capable of inducing inflammation and apoptosis in animals and plants (van der Biezen and Jones, 1998; Philpott et al., 2000; Girardin et al., 2001). The typical characteristics of the NLR family include the presence of three structural domains: (a) An N-terminal protein–protein binding or effector domain, (b) A central nucleotide oligomerization (NACHT) domain (named because of the presence of the domain in NAIP, CIITA, HET-E and TP-1 proteins), and (c) A C-terminal leucine-rich repeat (LRR) domain (Benko et al., 2008; Chen et al., 2009). However, the NLRs do not possess a signal peptide or transmembrane domain, which indicate their locations in the cytosol. The C-terminal LRR domain is the potential ligand recognition site and the NACHT domain mediates self-regulation and oligomerization. The N-terminus is responsible for protein–protein interaction, signal transduction and initiation of the downstream immune cascade (Rosenstiel et al., 2008; Jin and Flavell, 2010). The N-terminus of NLR contains different effector domains such as the pyrin domain (PYD), caspase recruitment domain (CARD) or baculovirus inhibitor of apoptosis repeat domain (BIR).

Based on the different N-terminal domains, NLRs are divided into three subfamilies: The CARD containing NODs (nucleotide-binding oligomerization domain) and IPAF (CE protease-activating factor), the BIR containing NAIPs (neuronal apoptosis inhibitory proteins), and the PYD containing NALPs (NACHT, LRR and PYD containing proteins) (Carneiro et al., 2007; Rosenstiel et al., 2008), plus a few NLRs that do not fall within these three subfamilies. Nonetheless, these classifications are quite theoretical, and members of these families from various organisms do not always fit into such a hierarchical description.

The NLR family is known to share a distinct structural motif similarity to the disease resistance superfamily of proteins (R-proteins) in plants (Jones and Dangal, 2006). Apart from the structural similarities, they also possess some functional and regulatory similarities, suggesting a common evolutionary route in the NLR pathway (da Silva Correia et al., 2007). The evolutionary conservation is also reflected by the presence of orthologs in fish for most of these genes in mammals (Stein et al., 2007). In addition, teleost fish possess an additional group of NLRs (Stein et al., 2007; Laing et al., 2008; Hansen et al., 2011), e.g., the zebrafish genome contains three distinct NLR subfamilies: the first subfamily (NLR-A) resembling mammalian NODs, the second (NLR-B) resembling mammalian NALPs and the third one, a unique subfamily of genes in teleost fish with portions resembling mammalian NOD3 and NALPs (Laing et al., 2008). The initial study of Laing et al. (2008) provided the foundational framework for analysis of these receptors in teleost fish.

Among cultured fish, channel catfish (*Ictalurus punctatus*), is the primary aquaculture species in the United States. A number of innate immune genes have been characterized in catfish including chemokines (Peatman et al., 2006; Peatman and Liu, 2007; Bao et al., 2006) antimicrobial peptides (Bao et al., 2005, 2006; Xu et al., 2005; Wang et al., 2006), TLRs (Bilodeau and Waldbieser, 2005; Baoprasertkul et al., 2006, 2007a,b), members of the lectin family of proteins (Takano et al., 2007; Zhang et al., 2011) and a few NLRs (Sha et al., 2009). Against this background, the objective of this work was to systematically identify NLR genes from channel catfish and conduct phylogenetic and expression analysis in the context of comparative analysis. Here we report a complete set of NLR genes from channel catfish and their expression in normal tissues and after infection with *Edwardsiella ictaluri*.

2. Materials and methods

2.1. Database mining and sequence analysis

To identify the NLR genes, RNA-seq results (Liu et al., 2011, 2012) and the whole genome database of catfish (unpublished data) were searched using available zebrafish (*Danio rerio*) and human NLRs as queries. The retrieved reconstructed transcripts were translated using ORF Finder (http://www.ncbi.nlm.nih.gov) and GENSCAN (Burge and Karlin, 1997). The predicted ORFs were verified by BLASTP against NCBI non-redundant protein sequence database. The NLR genes from other organisms were retrieved from the NCBI database for analysis. The simple modular architecture research tool (SMART) was used to predict the conserved domains based on sequence homology and further confirmed by conserved domain prediction from BLAST. The full-length amino acid sequences as well as the partial sequences coding for the conserved domains were used in the phylogenetic analysis. Multiple protein sequence alignments were done using the ClustalW program. Phylogenetic and molecular evolutionary analyses were conducted using MEGA4 (Tamura et al., 2007).

2.2. Expression analysis of catfish NLRs

Reverse-transcriptase PCR (RT-PCR) and quantitative real-time PCR (qRT-PCR) were employed to study the mRNA expression of selected NLR genes. To study the normal expression of these genes in healthy fish, blood, skin, muscle, gill, heart, liver, spleen, intestine, head kidney, trunk kidney and brain were collected from five individual fish and pooled. Three such pools were used in the present study. The tissues were snap-frozen in liquid nitrogen and immediately subjected to RNA extraction using RNeasy Mini Kit (Qiagen, USA) following the manufacturer's protocol. The extracted total RNA was quantified using a UV-spectrophotometer and an aliquot (1 μg) of RNA was treated with 1 unit of RNase-free DNase (Qiagen) prior to reverse transcription. A uniform quantity of DNA-free RNA was reverse-transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) following manufacturer's protocol.

PCR was carried out using Platinum Taq DNA polymerase, 10× buffer and 50 mM $MgCl_2$ (Invitrogen). The 20 μl PCR reaction mixture contained 2.0 μl 10× buffer 1.0 μl of $MgCl_2$ (25 mM), 1.0 μl of dNTP (10 mM), 0.4 μl of Taq polymerase (1 U), 1 μl (10 pmol/μl) of each primers, 2 μl cDNA and 12.6 μl PCR-grade water. Gene-specific primers and internal reference 18SrRNA-specific primers were used separately in the PCR amplification. Amplification was performed on a Bio-Rad PCR system for 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min. The PCR products were resolved on a 2% agarose gel.

2.3. Bacterial challenge and quantitative real-time PCR

Healthy channel catfish (Marion strain) was exposed to a Gram negative bacterium, *E. ictaluri* via immersion challenge following the procedure reported by Peatman et al. (2007). Briefly, one hundred fish (9.3 cm, mean length and 11.2 g, mean weight) were acclimatized in the laboratory for 3 days with water temperature maintained at 27 °C. A virulent strain of *E. ictaluri* was cultured in a brain heart infusion (BHI) medium by incubating in shaking incubator at 28 °C overnight. The experimental fishes were immersed in 15 L aerated freshwater mixed with bacterial culture

added to a concentration of 4×10^6 CFU/ml. During the immersion, water circulation was turned off for 2 h followed by continuous water flow-through. Another set of unexposed fish were maintained as control group. Fifteen fishes were sacrificed at different time-points [(4 h, 24 h, 4 day and 6 day post-infection (p.i)] from both experimental and control groups. Same tissue from five individual fishes was pooled, and RNA and cDNA were prepared as described above.

Three replicate RNA samples derived from both normal and infected fish at different time points were analyzed for gene expression profiles using qRT-PCR. After master mix was prepared, each sample was divided into three PCR replicates. Each qRT-PCR reaction consisted of a total volume of 10 μl containing 5.0 μl SsoFast EvaGreen Supermix (Bio-Rad, USA), 0.5 μl of each primer (5 pmol/μl), 2 μl cDNA and 2 μl PCR-grade water. The thermal cycling was carried out on a C1000 Thermal Cycler (Bio-Rad, USA) using the cycling conditions: denaturation, 95 °C/30 s, 40 cycles of 95 °C/5 s, 57 °C/5 s, and 72 °C/5 s followed by dissociation curve analysis to verify the specificity of amplified products. The qRT-PCR data were exported into a Microsoft Excel Sheet for analysis. The relative expression ratio of target gene in experimental group and the control group was calculated using the $2^{-\Delta\Delta}$CT method. The data generated were further analyzed statistically using one-way ANOVA.

3. Results

A total of 22 NLRs were identified from channel catfish including six NLRs from subfamily A, two from subfamily B, 11 from subfamily C, and three NLRs that do not fall within the three subfamilies (Table 1). Initially, the sequences were obtained from our assembled full length cDNAs (Liu et al., 2012), and these were validated by aligning their sequences against the partial draft genome sequences (not published data). All these sequences were deposited to the NCBI transcriptome shortgun assembly (TSA) database with continuous accession numbers of JP593145-JP593163 (Table 1).

3.1. Identification of NOD-like receptors (subfamily A NLRs)

Teleost fish possess orthologs for all five members of the mammalian NOD subfamily (Laing et al., 2008). Previously, full coding sequences of NOD1, NOD4 and NOD5, and partial coding sequences of NOD2 and NOD3 were reported from channel catfish (Sha et al., 2009). In the present study, we identified full-length coding regions for NOD1, NOD2, NOD3, NOD4, and NOD5 encoding 946, 981, 1136, 1726, and 1001 amino acids, respectively (Table 1). One additional NOD receptor most similar to NOD3 was also identified. This newly identified NOD3-like receptor (880 amino acids) harbored sequences with very high identity to the previously identified NOD3, but was truncated at the 3′ end with only 4 LRRs as compared to 15 LRRs in the previously identified NOD3. Given the high levels of identity, we speculate that this gene is probably a duplicated gene of NOD3 although the orthology relationship is not clear at this time. Herein they will be referred to as NOD3a for the previously identified NOD3, and NOD3b for the newly identified NOD3-like receptor.

Although the subfamily A NLR receptors are characterized by the CARD domain at the N-terminus, they harbor different numbers of CARD domains: NOD1, NOD3a, NOD3b all possess just one CARD domain, NOD2 has two CARD domains, whereas NOD4 and NOD5 do not have any CARD domains (Table 2 and Fig. 1). All six NOD receptors have a NACHT domain and variable numbers of LRR domains, with NOD3b and NOD5 carrying only 4 and 5 LRRs, respectively, and NOD3a carrying 15 LRRs (Table 2, also see Fig. 1).

3.2. Identification of NALP-like receptors (subfamily B NLRs)

Mammalian genomes such as the human genome have 14 NALPs. Using sequence similarity searches, Laing et al. (2008) identified

Table 1
A list of NOD-like receptor genes characterized from channel catfish (*Ictalurus punctatus*).

Gene	Accession No.	Length (Nucleotide)	Length (Amino acids)	Domains identified	References
Subfamily-A					
NOD1	FJ004844	4290	946	CARD-NACHT-LRR	Sha et al. (2009)
NOD2	JP593145	4655	981	CARD-NACHT-LRR	Sha et al. (2009); this work
NOD3a	JP593146	5679	1136	CARD-NACHT-LRR	Sha et al. (2009); this work
NOD3b	JP593147	3104	880	CARD-NACHT-LRR	This work
NOD4	FJ004847	5970	1726	NACHT-LRR	Sha et al. (2009)
NOD5	FJ004848	4007	1001	NACHT-LRR	Sha et al. (2009)
Subfamily-B					
NLR-B1	JP593148	2872	859	CARD-NACHT	This work
NLR-B2	JP593149	4344	1218	NACHT	This work
Subfamily-C					
NLR-C1	JP593150	5150	1102	NACHT-LRR-PRY/SPRY	This work
NLR-C2	JP593151	3520	1016	NACHT-LRR-PRY/SPRY	This work
NLR-C3	JP593152	4031	1121	NACHT-LRR-PRY/SPRY	This work
NLR-C4	JP593153	4046	606	NACHT-LRR	This work
NLR-C5	JP593154	4431	897	NACHT-LRR	This work
NLR-C6	JP593155	4067	1131	NACHT-LRR	This work
NLR-C7	JP593156	4019	1054	NACHT-LRR	This work
NLR-C8	JP593157	2235	651	NACHT-LRR	This work
NLR-C9	JP593158	2183	726	NACHT-LRR	This work
NLR-C10	JP593159	3030	967	NACHT-LRR	This work
NLR-C11	JP593160	3009	738	PAAD_DAPIN-NACHT	This work
Other NLRs					
Apaf1	JP593161	5175	1262	CARD-NB/ARC-WD40	This work
CIITA	JP593162	5288	1072	NACHT-LRR	This work
NACHT-P1	JP593163	4408	1459	AAA-WD40	This work

Table 2
Characteristics of subfamily A NLRs (NOD) receptors.

Genes	CARD domain	NACHT domain	LRR domain
NOD1	1 CARD	Yes	7 LRRs
NOD2	2 CARD	Yes	6 LRRs
NOD3a	1CARD	Yes	15 LRRs
NOD3b	1CARD	Yes	4 LRRs
NOD4	No	Yes	13 LRRs
NOD5	No	Yes	5 LRRs

six NALP-like sequences in the zebrafish genome assembly Zv6. However, only two of the six gene sequences can be retrieved now from the zebrafish genome assembly Zv9, suggesting some recent changes in the assembly of the zebrafish genome sequences. When the 14 human and the two zebrafish NALP-like receptors were used to query the catfish cDNAs, only two NALP-like genes were identified, which are referred to as NLR-B1 and NLR-B2, respectively (see Fig. 1, subfamily B NLRs). NLR-B1 encodes 859 amino acids and NLR-B2 encodes 1218 amino acids. NLR-B1 has

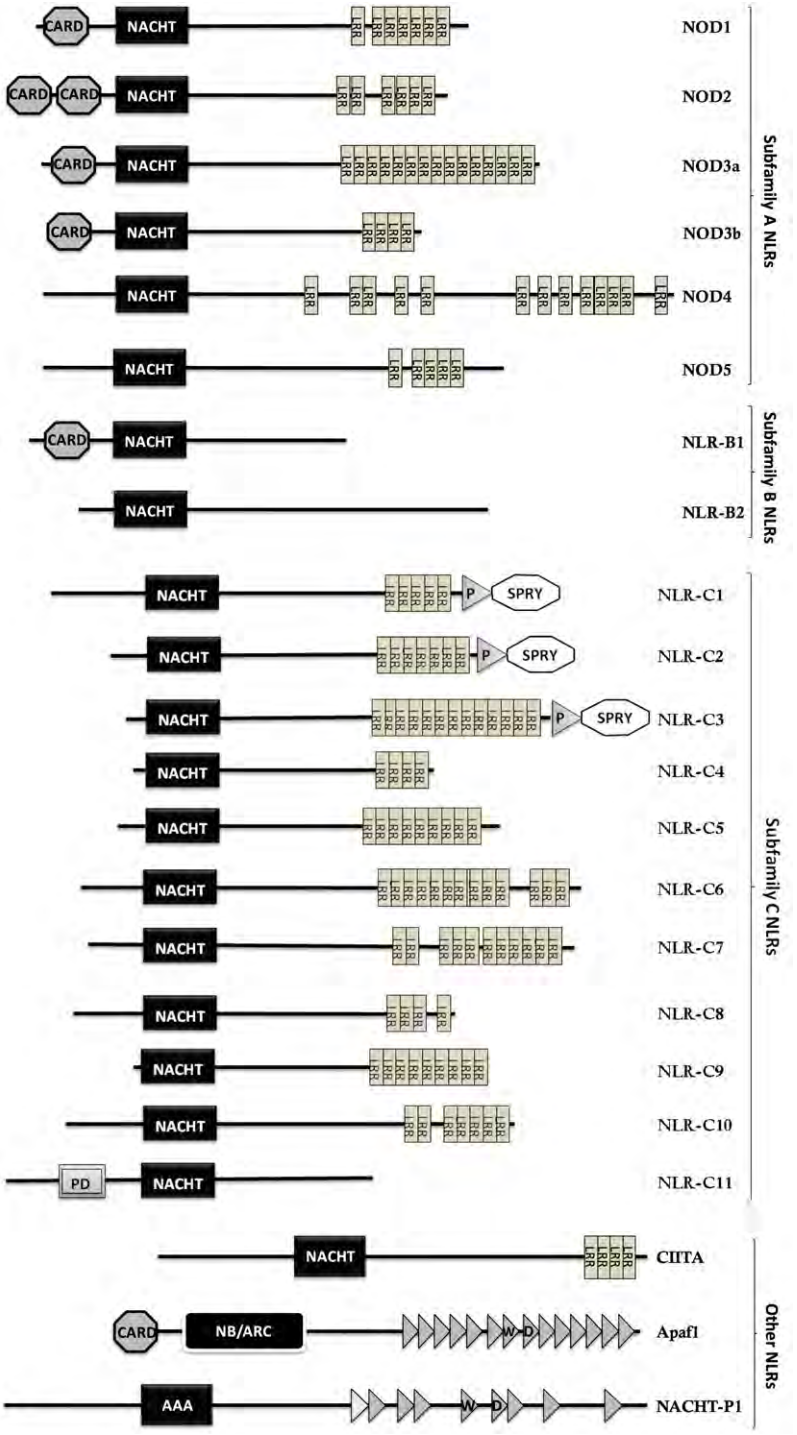

Fig. 1. Schematic representation of the domain architecture of NLRs in channel catfish. Domains were determined using SMART. *Abbreviations*: CARD; caspase recruitment domain, NACHT: nucleotide binding/oligomerization domain, LRR: leucine rich repeats, PD: pyrin domain (PAAD_DAPIN), P SPRY: B30.2 or PRY/SPRY domain, NB/ARC: NB/ARC domain, AAA: ATPases, Filament: filament domain, WD: WD40 repeats.

a CARD domain, but NLR-B2 does not. Both NLR-B1 and NLR-B2 have the NACHT domain (Fig. 1).

3.3. Identification of subfamily C NLR receptors

Teleost-specific subfamily C NLR receptors possess NACHT domains and share significant sequence similarity with human NOD3, and yet are different from zebrafish NOD3 (Laing et al., 2008). This subfamily is characterized by the presence of a NACHT domain and a LRR domain. In zebrafish, subfamily C members may possess an N-terminal effector domain such as Pyrin and/or a C-terminal B30.2 domain (PRY/SPRY). Using a set of 201 NLR subfamily C sequences identified from the zebrafish genome (Stein et al., 2007) as queries, a total of 281 distinct sequence contigs were identified to be homologous to the queries. However, when examined against the criterion for the presence of both a NACHT and a LRR domain, only 10 contigs fit the criteria. Additionally a single contig containing both a NACHT and an N-terminal Pyrin domain was also identified. The coding sequences of these 11 cDNAs were further characterized. As detailed under the section of Phylogenetic analysis, the nomenclature (numbering) of this subfamily of NLRs is quite arbitrary at this time.

As shown in Table 1, NRL-C1, NRL-C2, and NLR-C3 possess NACHT-LRR-PRY-SPRY domains, while NLR-C4,NLR-C5, NLR-C6, NLR-C7, NLR-C8, NLR-C9, NLR-C10 have only NACHT-LRR domains. NLR-C11 has a NACHT domain at the N-terminus, but lacks an LRR domain at the C-terminus. In contrast to the situation described for zebrafish with N-terminal effector domains such as the pyrin domain, an effector domain at the N-terminus was not found within the vast majority of catfish NLR-C receptors. Only in NLR-C11, a pyrin domain was found at the N-terminus (Fig. 1, subfamily C NLRs).

3.4. Identification of additional members of NLRs

Apart from the genes classified under the three subfamilies, three additional genes: Apaf1 (apoptotic protease activating factor 1), CIITA (major histocompatibility complex class II, transactivator) and NACHT-P1 were identified in catfish. Catfish Apaf1 encodes1282 amino acids with three predicted domains: an N-terminal CARD, a central NB-ARC and C-terminal WD_{40} repeats (here 14 WD repeats in the catfish Apaf1). The catfish CIITA gene encodes 1072 amino acids characterized by a distinct NACHT and four LRR domains (Fig. 1, other NLRs). No effector domain at the N-terminus could be identified in this gene. In a way, NACHT-P1 is similar to Apaf1, as both harboring WD domains (Fig. 1, other NLRs).

3.5. Phylogenetic analysis of NLRs

Phylogenetic analyses indicated that the identities of the subfamily A members can be clearly established. As shown in Fig. 2, catfish NOD1, NOD2, NOD3, NOD4, and NOD5 were placed within their corresponding clades containing both teleost and mammalian equivalents, suggesting orthologous relationships across the analyzed vertebrate sequences. The newly identified NOD3b NLR receptor was included in the NOD3 clade, albeit with moderate bootstrapping support.

While the phylogenetic analysis of subfamily A NOD-like receptors provide good information for the identification of these receptors, phylogenetic analysis of subfamily B and C receptors was less conclusive because of the lack of relevant sequences from other teleosts and apparent rapid diversification of these NLR subfamily members with teleost species. Two members of the subfamily B NLR receptors were found in catfish with one being placed into a subclade containing zebrafish NLRB1 and NLRB2, and clustered with human NALP genes. However, catfish NLRB1 was distantly related to either of the zebrafish genes and fell into an unsupported position on the tree (Fig. 3).

As subfamily C NLR receptors are teleost-specific, the sequences available for phylogenetic analysis are scarce. With few exceptions, catfish and zebrafish NLRC receptors were placed into clades exclusive of the other species, a pattern indicative of rapid duplication and divergence and fairly typical of innate immune gene families in teleosts (Fig. 3). Additional analysis of orthologous relationships between catfish and zebrafish may be strengthened by examination of syntenic regions when catfish genome scaffolds become available in the near future.

Phylogenetic analysis of NLR receptors that did not belong to the three families suggested proper identification of catfish Apaf1, CIITA and NACHT-P1. They each were placed within their relevant clades with strong bootstrapping support (Fig. 4).

3.6. Expression of NLR genes

We selected a representative member from each of the three standard NLR subfamilies, and three members of the non-canonical NLR subfamily for downstream expression analyses. Given our previous examination of the other catfish NOD genes (Sha et al., 2009), we chose the novel NOD3b gene here, as well as CARD-domain containing NLRB1, NLRC1, CIITA, Apaf1 and NACHT-P1. Homeostatic expression profiles were determined in eleven tissues (blood, skin, muscle, gill, heart, liver, spleen, intestine, head kidney, trunk kidney and brain) of pooled samples with five fish in each pool to understand distribution of expression throughout catfish. As shown in Fig. 5, the selected NLR genes were ubiquitously expressed in all the tissues tested.

3.7. Expression of selected NLR genes after bacterial infection

To examine the expression pattern of the selected NLR genes after bacterial infection, expression of the representative NLR genes was determined in intestine, head kidney, spleen, and liver collected at various time points: 4 h, 24 h, 4 days and 6 days after infection with *E. ictaluri*. Strikingly, all six genes (NOD3b, NLR-B1, NLR-C1, CIITA, Apaf1 and NACHT-P1) showed similar patterns of expression in all the four tissues (Fig. 6). However, the magnitude of up-regulation or down-regulation varied among tissues. In intestine and head kidney, expression of all six genes was down-regulated. In contrast, expression of all the six genes was up-regulated in spleen and liver (Fig. 6). The induction of expression of these genes in spleen and liver was generally transitory, with up-regulation observed at 4 h after infection generally returning to basal levels by 4 day after infection. However, in the liver, the induced expression of these genes lasted at high levels with the exception of the NACHT-P1 whose expression returned to normal level 4 days after infection. In spite of the similarity of the general patterns of up-regulation of the six genes, the kinetics of up-regulation varied with each of these genes (Fig. 6).

4. Discussion

NLRs are a recently characterized group of pathogen recognition receptors. They are believed to be intracellular bacteria-recognizing receptors. As such, NLRs has been a subject of interest in the area of innate immunity. Here for the first time, we have systematically identified a total of 22 NLRs from channel catfish including six NOD-like receptors (subfamily A), two NALP-like receptors (subfamily B), 11 subfamily C NLRs (teleost-specific), and three additional NLRs that do not fall within the three subfamilies.

Mammalian genomes have five NOD-like receptor genes, and the orthologs of all five NOD-like receptor genes were found in

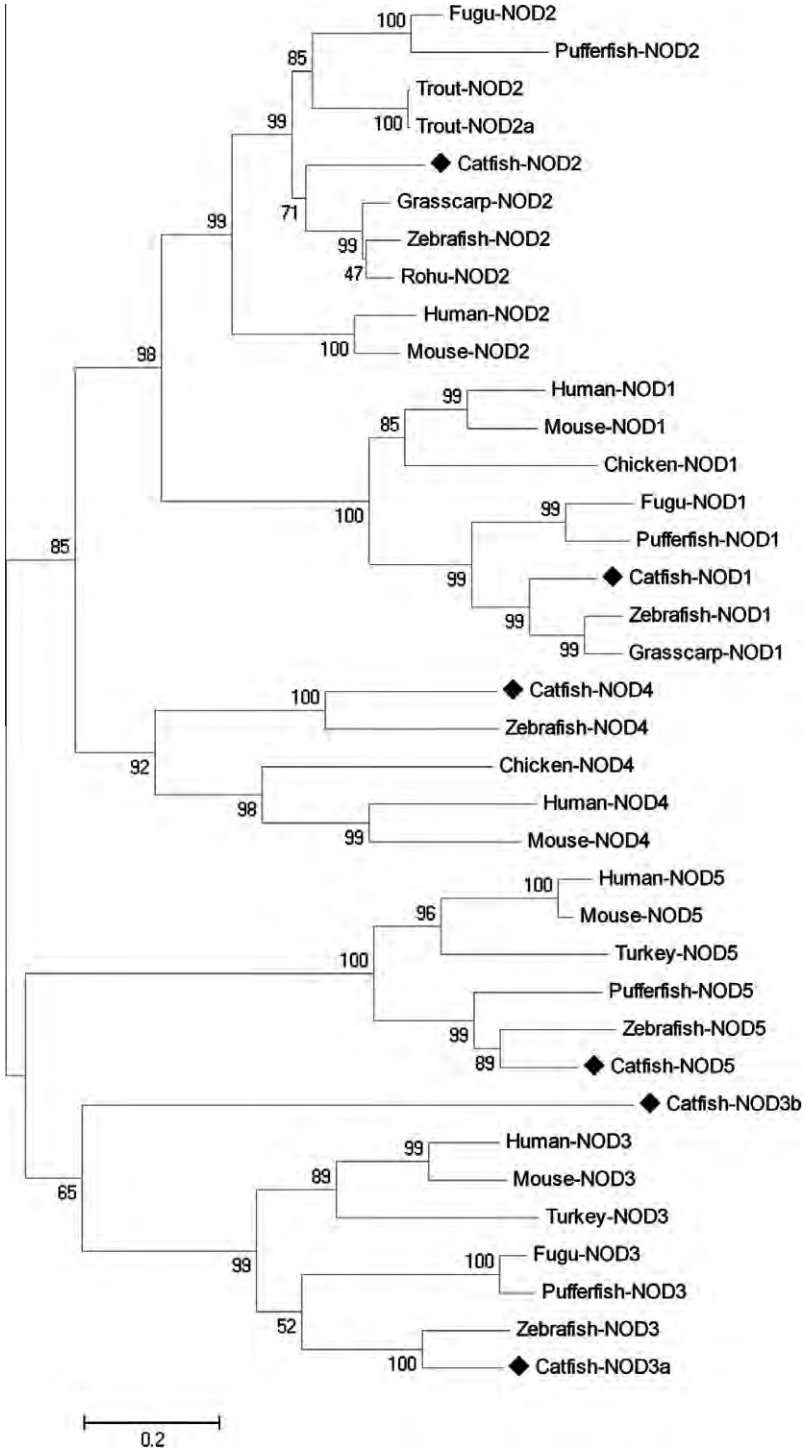

Fig. 2. Phylogenetic relationship of catfish and other vertebrate NODs. ClustalW alignments of all the amino acid sequences were used to generate a Neighbor joining tree using MEGA4 software. The tree is shown with 10,000 replicates from the bootstrap test and the percentage of bootstrap values were given next to the branches.

the zebrafish genome (Laing et al., 2008). In catfish, all five NOD-like receptors were previously identified, but the coding sequences of NOD2 and NOD3 were incomplete (Sha et al., 2009). Here we obtained complete coding sequences for all five NOD-like receptors in catfish. In addition, we found one additional transcript that shares high levels of sequence similarity with NOD3, but was truncated at the C-terminus. Based on structural and sequence similarity to NOD3, we speculated that NOD3b may represent a duplicate of NOD3 in which the C-terminus portion of the gene was subsequently lost.

A large number of subfamily B NLRs (NALP-like) has been identified in mammals. In the human genome, 14 NALPs were identified (Hughes, 2006). Laing et al. (2008) reported six NALP-like receptor sequences in the zebrafish genome. However, using all 14 human and two zebrafish NALP-like receptors as queries to search the catfish genome sequences database identified only two NALP-like receptors. Of these, phylogenetic analysis only supported a homologous relationship between catfish NLR-B2, zebrafish NALP-like genes, and human NALPs. However, both putative catfish NLR-B genes lack the pyrin and LRR domains typical of

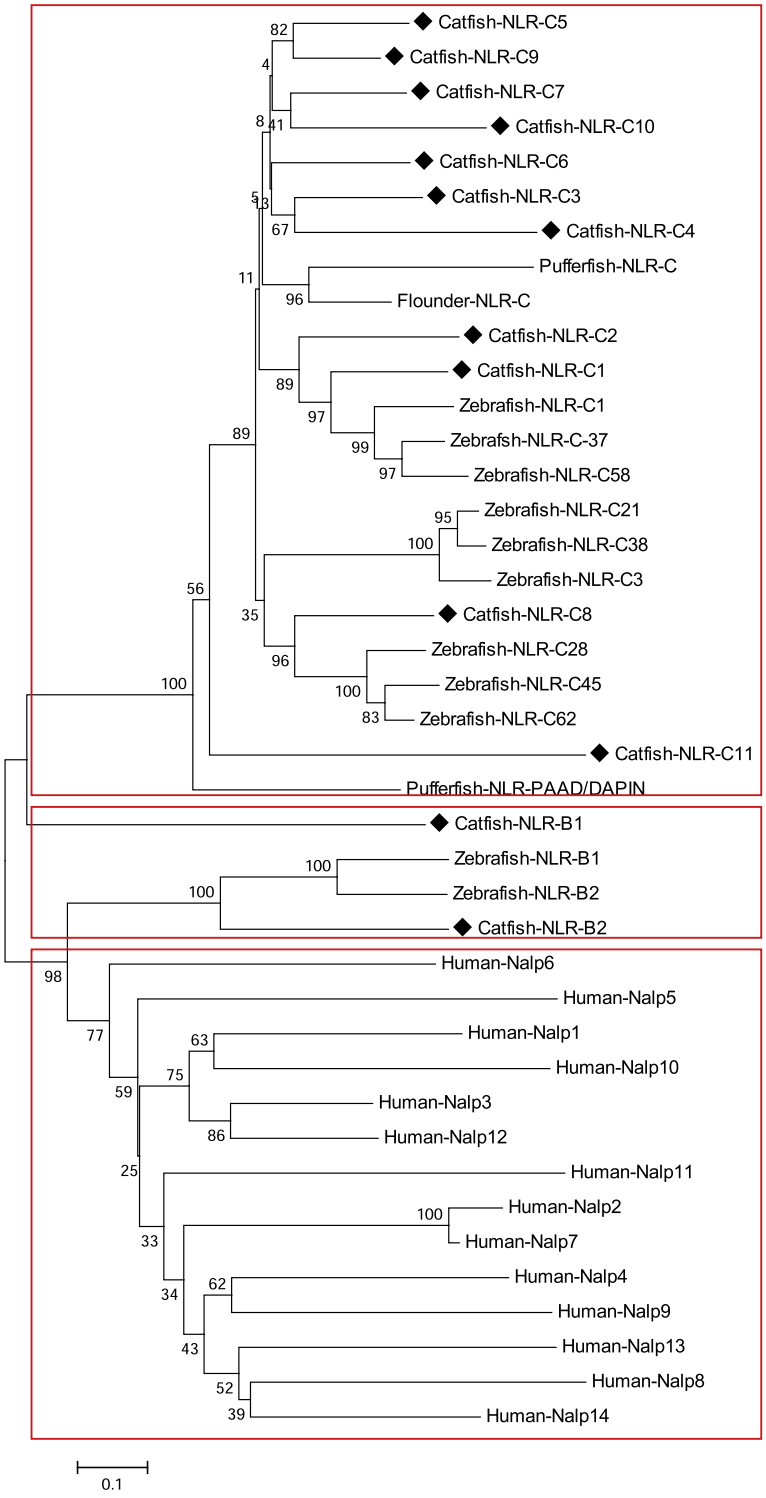

Fig. 3. Phylogenetic relationship of catfish and other vertebrate NLR-B and NLR-C receptors. ClustalW alignments of all the amino acid sequences were used to generate a Neighbor joining tree using MEGA4 software. The tree is shown with 10,000 replicates from the bootstrap test and the percentage of bootstrap values were given next to the branches.

canonical NALP genes, making it difficult to support the notion that these genes are functional equivalents of the human NALP subfamily despite some indications of a shared evolutionary history. Laing et al. (2008) raised doubts as to whether NALP-like genes encode functional PRRs in poikilotherms. Our expression analysis indicates that NLR-B genes do respond in a similar manner to other LRR-containing NLRs. However, additional work will be required to ascertain whether these genes retain the ability to recognize pathogen or rather serve as downstream immune mediators. In particular, comparative genome mapping analysis, upon the availability of the whole genome sequence of catfish in the near future, may provide additional insight into orthologies of these genes, especially in cases where the functional domains are not fully conserved.

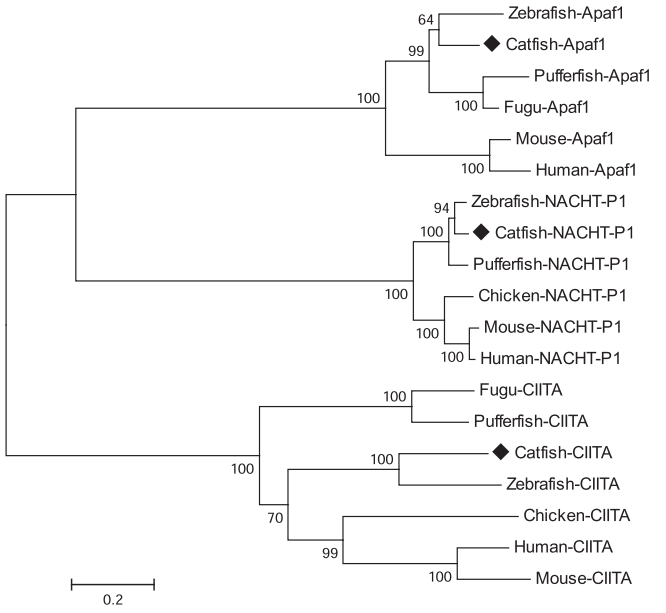

Fig. 4. Phylogenetic relationship of other members of NLRs of catfish and other vertebrates. ClustalW alignments of all the amino acid sequences were used to generate a Neighbor joining tree using MEGA4 software. The tree is shown with 10,000 replicates from the bootstrap test and the percentage of bootstrap values were given next to the branches.

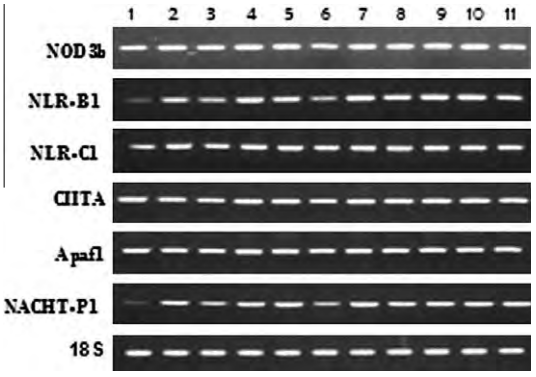

Fig. 5. RT-PCR-based expression analysis of selected members of NLRs in different tissues of healthy channel catfish. Tissues from five animals were pooled for RNA extraction. 1, blood; 2, skin; 3, muscle; 4, gill; 5, heart; 6, liver; 7, spleen; 8, intestine; 9, headkidney; 10, trunk kidney; 11, brain. 18SrRNA was used as an internal control and gene names are indicated on the left of the panel.

The NLR-C subfamily of NLR genes is teleost-specific and is currently not well defined. In zebrafish, members of the NLR-C subfamily were described as a group of multiple genes that possessed NACHT domains and shared significant homology with human NOD3, yet distinct from zebrafish NOD3 (Laing et al., 2008). Recently, NLR-C (NLRP) subfamily has been described as a mysterious group of genes (Hansen et al., 2011). It appears that this subfamily is only present in teleosts (Laing et al., 2008; Hughes, 2006; Zhang et al., 2010). According to Hansen et al. (2011), multiple NACHT domain-containing genes are evident in zebrafish genome, though they do not encode recognizable LRR or PYRIN domains. Numerous NLR-C related sequences were identified from the zebrafish genome (Laing et al., 2008) with predicted pyrin (PAAD_DAPIN) domains, but the pyrin domain was not found at the N-terminus of most of the catfish NLR-C members with the exception of NLR-C11. No other effector domain could be identified in any of the genes in the subfamily. However, the presence of an additional C-terminal B30.2 (PRY/SPRY) domain in many of the genes of this

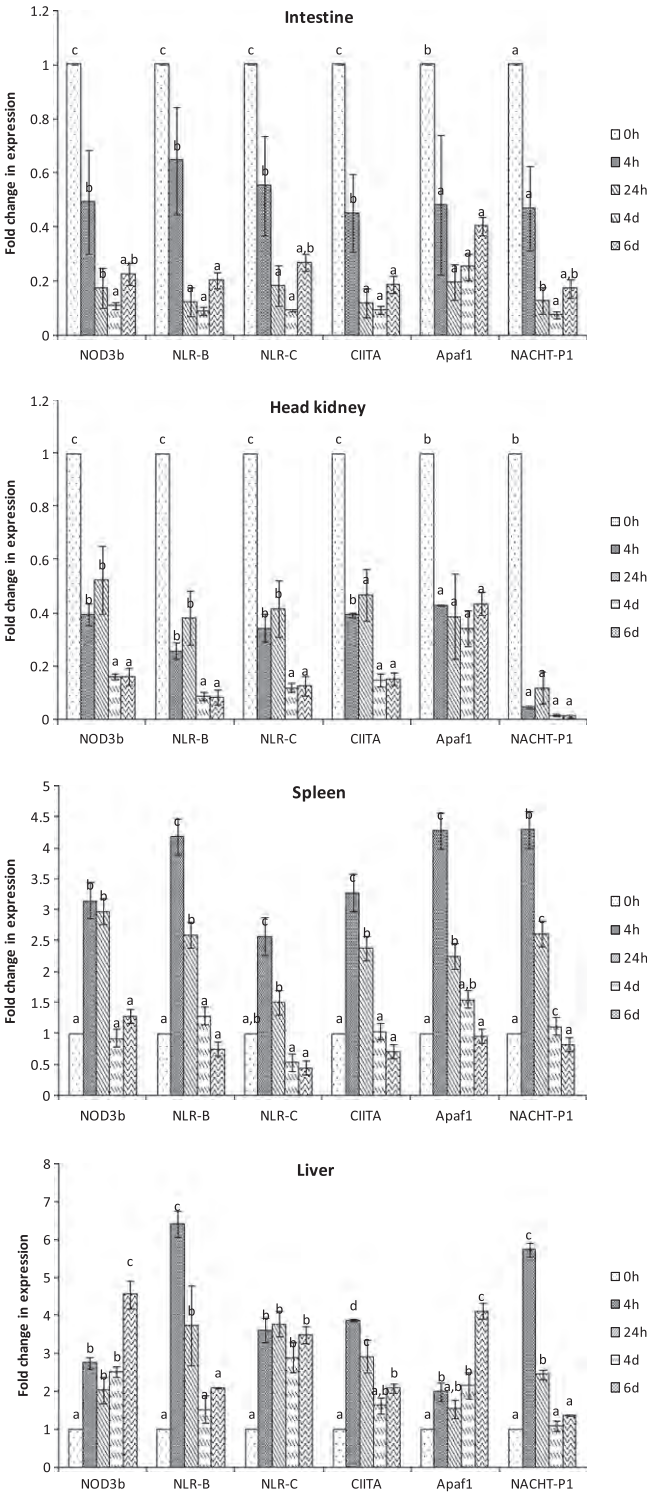

Fig. 6. qRT-PCR-based expression analysis of selected NLR genes in different tissues of channel catfish following bacterial infection. Healthy catfish were exposed to *E. ictaluri* through immersion. Tissues were collected at four different time points after bacterial challenge and RNA extracted from fifteen fishes (3 pools of 5 each) were used in the qRT-PCR. Six representative NLR genes were tested with 18SrRNA as internal reference. The results were expressed as mean ± standard error (bars) from three replicates. Significant difference ($p < 0.05$) among controls and various treatments are shown by a different letter, i.e., "a" is statistically different from "b", and "b" is different from "c" etc.

subfamily in catfish was noteworthy, as it has been reported that NLRPs containing the B30.2 domains appears to be unique to teleosts (Hansen et al., 2011). All the genes reported here, however,

showed variable numbers of LRR domains, suggesting varied PAMP specificities.

A search for NLR-C members resulted in identification of a large number (281) genome sequence contigs in catfish. However, the vast majority of these genes harbors only a NACHT domain or do not harbor any NLR-like domains, insufficient to be classified as members of the NLR-C subfamily. Of these 281 sequences, only 11 can be classified as members of NLR-C subfamily because they harbor NACHT and LRR domains, or pyrin and NACHT domains. Interestingly, all the 11 distinct NLR-C members were found from the cDNAs assembled from RNA-Seq data, suggesting all 11 NLR-C receptors were expressed. Apparently, future studies are warranted to characterize these large numbers of homologous genes.

Phylogenetic analysis of existing subfamily C NLR receptors did not yield informative conclusions concerning their identities because many of the members were most related to other members from the same species. This could indicate that subfamily C receptors include far larger gene numbers in catfish with many members yet to be discovered, or that these members were derived from recent lineage-specific gene duplication events.

Laing et al. (2008) have stated that many NLR-C genes may be non-functional genes or pseudogenes, although a small number may have some new functions. In the present study, although we have identified many contigs in the genome database, RNA-seq database searches revealed only a limited number of NLR-C genes. This observation might corroborate the predictions made by Laing et al. (2008) and others that most of the predicted NLR-C genes in the genome database may be non-functional. We have chosen one of the representative genes (NLR-C1) having B30.2 (PRY/SPRY) domain to study the expression of the gene in various tissues of catfish, and its expression was found in all tissues, similar to what was found in Japanese flounder (Unajak et al., 2011).

Three catfish genes were identified as NLR receptors that do not fall into the three known subfamilies. These included Apaf1, CIITA, and NACHT-P1. Apaf1 plays a role in apoptosis in mammals; CIITA controls the expression of both major histocompatability class I and class II molecules, and it is reported to play a crucial role in some human immune disorders (Fritz et al., 2006). NACHT-P1 was found in zebrafish, *Tetraodon* and fugu genomes with orthologs in human and mouse (Stein et al., 2007).

In humans, NAIP and IPAF are additional divergent members of NLR. Our search with NAIP and IPAF sequence of human did not identify any sequence in catfish RNA-seq or genome database. This is in accordance with the observation made in other fish genomes (Laing et al., 2008; Hansen et al., 2011). However, four sequences containing multiple baculovirus inhibitor of apoptosis protein repeat (BIR) domain were identified in catfish. One of the proteins identified matched completely with the baculoviral IAP repeat containing 2 (BIRC2) gene reported from catfish (Li et al., 2005). This protein has CARD and RING domains at the C-terminal and three BIR domains at the N-terminal. A similar observation has been made by Hansen et al. (2011) in which BIRC2 and BIRC3 orthologs with BIR-BIR-BIR-CARD-RING domains, likely regulators of apoptosis, were identified in the zebrafish genome, indicating the presence of triplicated BIR domains during early vertebrate evolution. Interestingly, NAIP has been identified in frog and reptiles, and according to Hansen et al. (2011), absence of NAIP in fish genome could be due to the fact that this gene has evolved after bony fish split from tetrapods or that this gene is lost in teleost lineage. The other three BIR repeat containing-sequences identified in the present study did not match with the reported sequence BIRC2 from catfish. Since these sequences do not have a NACHT domain, we did not consider the sequences as NAIP.

Analysis of expression of representative members of NLR receptors indicated their constitutive expression pattern in healthy catfish tissues. Due to the large numbers of genes, we analyzed expression of NOD3b, NLR-B1, NLR-C1, Apaf1, CIITA, and NACHT-P1. All the six genes were expressed in all the tested tissues. Not only were the expression patterns of these genes similar in healthy catfish tissues, their expression patterns are amazingly similar after infection, suggesting a certain level of co-regulation of these genes, in response to bacterial infection with a Gram negative intracellular pathogen, *E. ictaluri* (Fig. 6). Variations were observed at different times after infection within the same tissue. This was probably caused by variations of expression in different individual fish. At each time point, tissue samples were collected from five fish. While a general trend was clearly observed, it may be difficult to compare quantitatively the five fish at one time point with another five fish at a different time point.

While up-regulation of some NLR genes was reported when fish were exposed to bacterial and viral infection or viral mimicking substances (Chang et al., 2010; Chen et al., 2010; Sha et al., 2009), a significant observation in this study was that the induction of NLR genes is tissue-specific. Expression of all six tested genes were down-regulated in the intestine and head kidney, but up-regulated in spleen and liver, suggesting the involvement of these genes in immune function against the intracellular pathogen. A similar pattern of expression was reported for NOD2 in *Labeorohita* in which the gene expression was induced significantly in liver and blood at 12 h post-infection, but not in gill, kidney and intestine (Swain et al., 2012). In a similar study on catfish NOD genes in relation to *E. ictaluri* infection, Sha et al. (2009) also reported a similar trend wherein they observed notable up-regulation of only NOD1 among all the other NOD genes tested in intestine. It is interesting to note that among the tissues tested, the examined genes showed strongest induction in the liver after bacterial infection. The induction of NLR genes in liver could be due to the fact that liver is one of the organs directly involved in pathogenesis of ESC and liver necrosis and haemorrhage are characteristic of acute *E. ictaluri* infection in channel catfish (Newton et al., 1989). Accordingly, in an investigation on channel catfish TLR5, highest expression was noted in liver after the bacterial infection (Bilodeau and Waldbieser, 2005). Further, it has been established that liver plays a significant role in the host defense against invading microorganisms (Gao et al., 2008) and this has been supported by work on immune genes in fish showing that as in mammals liver might be an important innate immune organ (Su et al., 2010). Although exact ligand specificity and functional roles of various NLR genes with their diverse LRR domains are not elucidated conclusively, the present study on the representative receptors such as NOD3b, NLR-B1, NLR-C1, CIITA, Apaf1 and NACHT-P1 after bacterial infection revealed a clear induction of these genes in liver and spleen, and this indicates that they have the potential to play a crucial role in innate immune defense of catfish. Future research is warranted to conclusively prove the ligand-specificity, downstream signaling mechanism and the kinetics of individual receptor proteins in the event of a pathogen incursion or physiological stress.

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