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Short sequence report

Structural characterisation and expression analysis of Toll-like receptor 2 gene from catfish

Puttharat Baoprasertkul, Eric Peatman, Jason Abernathy, Zhanjiang Liu*

The Fish Molecular Genetics and Biotechnology Laboratory, Department of Fisheries and Allied Aquacultures and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, Auburn University, 203 Swingle Hall, Auburn, AL 36849, USA

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Abstract

Toll-like receptors (TLRs) are important components of innate immunity. They were found to recognise specific structures on pathogens termed pathogen-associated molecular patterns (PAMPs) and utilise conserved signaling pathways to activate pro-inflammatory cytokines and type-1 interferons. In spite of much understanding gained from the mammalian systems, many fish TLRs are unknown. Recent studies in Japanese flounder as well as in zebrafish suggested that the ligand binding and activation of inflammatory responses in fish may be different from and more complex than those found in mammals. In channel catfish, the major aquaculture species in the United States, only partial sequences of TLR3 and TLR5 were reported. As a part of efforts to characterise the innate immune components in channel catfish, here we cloned and sequenced both the cDNA and the gene for TLR2, a receptor believed mostly responsible for recognition of lipopeptides on the surface of most Gram-positive bacteria. However, expression analysis after infection with a Gram-negative bacterium, *Edwardsiella ictaluri* indicated that TLR2 was modestly down-regulated in the head kidney tissue of blue catfish, and with a similar pattern in the head kidney of channel catfish though the down-regulation in channel catfish was not statistically significant. In the spleen, an insignificant down-regulation was initially observed early after infection, with an increase of TLR expression later after infection. These results suggest the involvement of TLR2 in the responses after the bacterial infection. As LPS is believed to be the major PAMP for Gram-negative bacteria, additional research is warranted to determine the functions and mechanisms of TLR2 in infections of Gram-negative bacteria.

Keywords: Catfish; Fish; TLR2; Immunity; Toll-like receptor; Gene expression; Disease

Innate immunity has long been regarded as a host organism's initial line of defence against pathogen invasion. The roles of pro-inflammatory cytokines, chemokines, and complement factors as immune activation signals and the roles of NK cells, phagocytes, antimicrobial peptides, and the membrane attack complex as immune effectors have been well characterised for some time in mammalian species. The molecular components involved during initial contact between host and pathogen, however, were poorly understood until recently. To detect non-specific antigens, a fast-acting, non-clonal mechanism of pathogen recognition, differing significantly from that of adaptive immunity, was clearly required. The discovery of a family of receptors homologous to the *Drosophila* receptor Toll provided

* Corresponding author. Tel.: +1 334 844 4054; fax: +1 334 844 9208.
E-mail address: zliu@acesag.auburn.edu (Z. Liu).

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molecular evidence of such a mechanism in vertebrate species [1,2]. These Toll-like receptors (TLRs) were found to recognise specific structures on pathogens termed pathogen-associated molecular patterns (PAMPs) [3] and utilise conserved signaling pathways to activate pro-inflammatory cytokines and type-1 interferons [4,5]. TLRs are defined by the presence of a Toll/IL-1 receptor (TIR) domain in their cytoplasmic portion and by leucine-rich repeats (LRR) in their extracellular domain [6]. At least 11 TLRs have been identified to-date from mammals. Functional studies have revealed that these receptors recognise and respond to a wide range of exogenous as well as endogenous ligands. Roach et al. [7], in their study of the evolution of vertebrate TLRs, identified six major subfamilies, each recognising a general class of PAMPs. Under this classification, individual TLRs recognise lipopeptides (TLR2), dsRNA (TLR3), lipopolysaccharide (LPS, TLR4), flagellin (TLR5), and nucleic acid and heme motifs (TLR7-9) [7]. The number of reported TLR ligands, however, continues to increase rapidly, frustrating attempts to characterise TLR specificity. Additionally, reports of multiple TLRs acting together in pathogen recognition and signaling indicate that much work is still needed to complete our understanding of the TLR family [4,8].

TLR2 is best known as a receptor recognising conserved components of Gram-positive bacteria such as lipoteichoic acid (LTA), peptidoglycans (PGN), and lipoproteins [9,10]. However, TLR2 interacts with a wide range of additional ligand types including zymosan, derived from yeast, glycosylphosphatidylinositols (GPIs) from protozoan parasites, LPS of Gram-negative bacterium *Porphyromonas gingivalis*, and the LPS of zoonotic pathogen *Leptospira interrogans* [11–14]. While TLR4 is traditionally considered the receptor for PAMPs of Gram-negative bacteria, recent studies have shown that TLR2 may function together with TLR4 [15] or independently in this role [16]. Additionally, TLR2 can form functional pairs with TLR1 or TLR6, capable of recognising and specifically responding to a variety of PAMPs [17,18].

TLR2 structure [19,20] and functional patterns (above) have been investigated extensively in mammalian species. More recently in teleost fish, the structures and expression patterns of TLR2 have been reported in fugu (*Takifugu rubripes*), zebrafish (*Danio rerio*) and Japanese flounder (*Paralichthys olivaceus*) [21–24]. As part of our efforts to gain a better understanding of the innate immune response of channel catfish (*Ictalurus punctatus*), the primary aquaculture species in the US, here the complete cDNA of catfish TLR2 was sequenced, and the TLR2 gene was identified, sequenced, and characterized from the catfish BAC library, the TLR2 genomic structure determined, catfish TLR2 conservation assessed through sequence and phylogenetic analysis, the expression patterns of catfish TLR2 in various healthy tissues examined, and TLR2 expression after infection with *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), compared in susceptible (channel) and resistant (blue) catfish (*Ictalurus furcatus*).

A channel catfish partial cDNA sharing highest BLASTX identity with TLR2 sequences from fish and mammalian species was identified through analysis of catfish ESTs [25–27]. High-density filters of a channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA). Each set of filters contained 10× genome coverage of the channel catfish BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). As part of ongoing efforts to physically map important genes, an overgo probe was designed based on the partial TLR2 cDNA sequence and hybridised to the catfish BAC library. Sequences of the overgo primers are: Overgo A: GCAGCATGATACAGTTAGCGTTTC, and Overgo B: GTGAGGTGCTTCAACAGAAACGCT. Overgo hybridisation was conducted as described [28–30]. Positive clones were identified according to the clone distribution instructions from CHORI, and one clone, BAC_153_N05, was picked out for sequencing analysis. The catfish TLR2 gene was sequenced by primer walking and its sequence has been deposited in GenBank with accession number DQ372072.

BLAST searches were conducted to determine gene identities and the full open reading frame of catfish TLR2, and the DNASTAR software package was used for sequencing analysis [31]. The MegAlign program of the DNASTAR package was used for TLR2 TIR domain sequence alignment using ClustalW. The receptor structure was characterised based on the amino acid sequence using the simple modular architecture research tool (SMART) [32]; http://smart.embl-heidelberg.de/ and TMHMM programs [33].

The full-length of amino acid sequences from known TLR2 genes was retrieved from GenBank for phylogenetic analysis. ClustalW was used for multiple alignments of amino acid sequences.

Challenge experiments of Marion select strain from channel (*I. punctatus*), and blue catfish (*I. furcatus*) were conducted at the hatchery of the Auburn University Fish Genetics Research Unit as previously described [34] with modifications [28,35]. Briefly, catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared cultured from a single colony of ESC bacteria, *E. ictaluri*, from a natural outbreak in Alabama (the outbreak number ALG-02-414).

Eleven tissues were collected from healthy channel catfish including head kidney, spleen, intestine, stomach, skin, muscle, liver, trunk kidney, ovary, brain and gill. Head kidney and spleen were collected from challenged channel catfish and blue catfish. Samples were collected from 10 fish at each time point including control (0 h), 4 h, 24 h, 72 h after challenge, and moribund fish. Samples of each tissue from 10 fish were pooled. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powder and thoroughly mixed. A fraction of the tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [36] using the Trizol reagents kit from Invitrogen following manufacturer's instructions. Extracted RNA was stored in a −80 °C freezer until used as a template for reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR.

RT-PCR was used to study an expression of catfish TLR2 in various normal tissues. RT-PCR reactions were conducted by two step RT-PCR using M-MuLV reverse transcriptase (New England Biolabs, Ipswich, MA). RT reactions were conducted in 40 μl containing 4 μg DNase I-treated RNA, 4 μl (40 μM) oligo dT primers, 8 μl (2.5 mM each) dNTPs, 1 μl RNase inhibitor, 1× RT reaction buffer, and 200 units of RT. Detailed procedures followed the instructions of the manufacturer. The sequences of primers for RT-PCR of TLR2 are: TLR2 upper primer: ACAGGCTT ACGTCGCTGGAC, TLR2 lower primer: TCCAGAACGGTCAGGCTCAC. After RT reaction, 1 μl of the RT products was used as templates for PCR using JumpStart Taq polymerase (Sigma, St. Louis, MO). The reactions also included the gene specific primers and primers of β-actin serving as an internal control. The sequences of the RT-PCR primers for the beta-actin internal control are: beta-actin upper primer: AGAGAGAAATTGTCCGTGACATC, beta-actin lower primer: CTCCGATCCAGACAGAGTATTTG. The reactions, for both healthy and challenged tissues, were completed in a thermocycler with the following thermo-profiles: denaturation at 94 °C for 2 min followed by 38 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min. Upon the completion of PCR, the reaction was incubated at 72 °C for an additional 10 min. The RT-PCR products were analysed by electrophoresis on a 1.0% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

Quantitative real-time RT-PCR using a LightCycler (Roche) was carried out to characterise catfish TLR2 expression after ESC infection. Concentration and quality of total RNA from spleen and head kidney were determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers for TLR2 and internal control used in quantitative real-time PCR were the same as RT-PCR. One-step quantitative real-time PCR was carried out in the LightCycler using a Fast Start RNA Master SYBR Green I reagents kit (Roche) following manufacturer's instructions with modifications as previously described [34]. Relative expression ratios were obtained by normalising the expression of the target gene, as determined by mean crossing point deviation, by that of a non-regulated reference gene, β-actin, using the Relative Expression Software Tool 384 v. 1 (REST) developed by Pfaffl et al. ([37]; http://www.wzw.tum.de/gene-quantification/). Each reaction was carried out in triplicate. Expression ratio results were tested for significance by a randomisation test built into the software. All ratios are relative to expression of the gene in 0 h (control) RNA samples.

Analysis of the domains of the catfish TLR2 gene using the SMART program revealed that it encodes the two motifs characteristic of TLR genes, several leucine-rich repeats (LRRs) in the N-terminal region and a Toll-interleukin-1 receptor (TIR) domain at the C-terminal end (Fig. 1). Two microsatellites were detected in the upstream and downstream genomic regions, an $(AT)_9$ repeat at 632 bp and an $(AC)_{21}$ repeat at 3606 bp.

A striking feature of the catfish TLR2 gene is that it does not contain any introns and is, therefore, an intronless gene. Structurally, catfish TLR2 consists of one exon with an open reading frame of 2373 bp, encoding a protein of 790 amino acid residues (Fig. 1). Catfish TLR2 has a 5′-untranslated region (UTR) of 54 bp and a 3′-UTR of 200 bp, as determined by comparing the catfish cDNA and gene sequences. The gene structure of one coding exon in catfish TLR2 is shared by zebrafish and human TLR2 genes, but not by fugu or Japanese flounder TLR2 genes which are encoded by 11 and 12 exons, respectively [21,24]. Before the structure of the zebrafish TLR2 gene was available, it was hypothesised that a large number of introns were acquired in fish TLR2 genes after the divergence of vertebrates [21,24]. The present results, coupled with those from zebrafish [23], clearly suggest that in catfish and zebrafish TLR2 genes are intronless. Both catfish and zebrafish belong to Ostariophysi and are more closely related to each other phylogenetically than to fugu. This raises interesting questions about the timing of intron invasion and possible functional/evolutionary reasons behind the striking structural differences in TLR2 genes.

A ClustalW-generated multiple sequence alignment using all available TLR2 sequences from fish species as well as those of other vertebrates indicated high levels of amino acid sequence conservation within the TIR domain (Fig. 2).

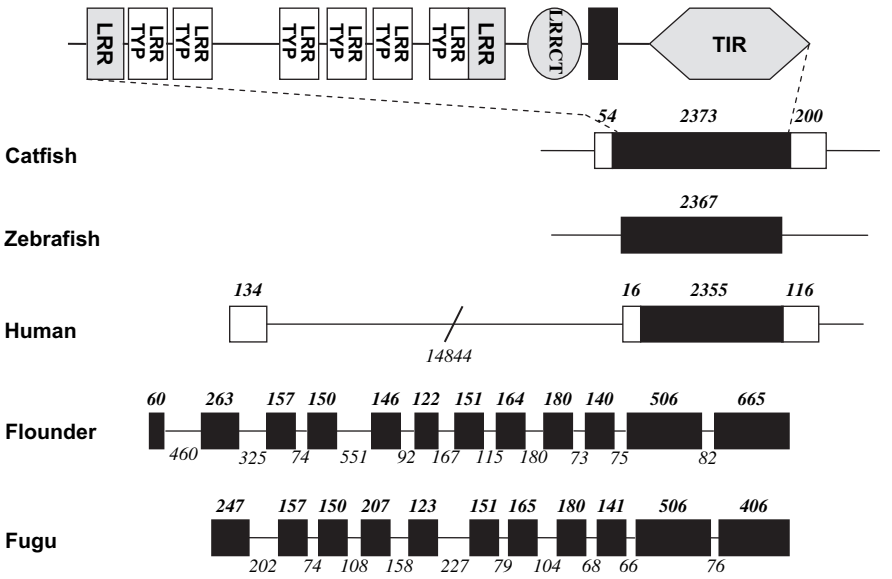

Fig. 1. Schematic representation of catfish TLR2 domains, predicted by SMART and TMHMM programs. Domains include the leucine-rich repeats (rectangles) and C-terminal leucine-rich repeats (oval), followed by a putative transmembrane region (black rectangle), and a TIR domain (hexagon). A comparison of TLR2 genomic structure and organisation from catfish, zebrafish, human, flounder and fugu is also presented. Exons are represented by boxes; solid boxes represent coding region of the gene; white boxes represent 5′-untranslated region (UTR) and 3′-UTR. Their sizes in base pairs are shown on the top of the boxes. Introns are represented by a line and the size is shown below the line.

Initially sequence alignment was performed without using the rainbow trout sequences (Accession number AJ628348 and AJ878915). Catfish TLR2 shared highest full-length amino acid sequence similarity with zebrafish (60.2%), and the lowest similarity was found in mouse and human (41.1%) (Table 1). As expected, higher amino acid identities were found among the conserved TIR domain, ranging from 82.2% in zebrafish to 65.1% in flounder (Table 1). Sequence alignment was then conducted with the rainbow trout sequences designated as TLR2 in the GenBank (AJ878915), but the similarities between the catfish and the trout sequences were low. The overall similarities were approximately 20% with the entire amino acid sequences, and the similarities within the TIR domain were 43%. Further BLAST searches suggested that the trout sequence AJ878915 was most similar to flounder TLR3. As Toll-like receptors share some level of similarities among them, especially within the TIR domains, the establishment of orthologies among all teleost TLR genes requires availability of additional TLR gene sequences from various species.

RT-PCR analysis using total RNA from various healthy tissues of channel catfish indicated that TLR2 is ubiquitously expressed albeit at different levels. High levels of TLR2 expression were detected in liver, brain, and gill while only low TLR2 expression was detected in skin and muscle (Fig. 3). Similar ubiquitous patterns of TLR2 expression were found in various healthy tissues of fugu and flounder [21,24], whereas expression was not detected in several non-immunological zebrafish tissues such as testis and heart [22].

In order to assess potential roles of TLR2 during bacterial infection in catfish, quantitative real-time PCR was conducted using RNA isolated from the head kidney and spleen of blue and channel catfish at several time points after challenge with *E. ictaluri*, the causative agent of ESC. Channel catfish are highly susceptible to ESC infection while blue catfish are more resistant [38]. TLR2 expression changes for both species in infected spleen samples were small and did not rise to a level of statistical significance (Fig. 4). Expression for both species in spleen was down-regulated approximately twofold at 4 h, and upregulated twofold at 24 h relative to the 0 h control. At 72 h post infection, channel catfish TLR2 expression in spleen increased threefold, while a twofold down-regulation was observed in blue catfish. In infected head kidney samples, a consistent pattern of down-regulation was observed from both species over all time points. Expression changes in blue catfish, however, were larger (up to eightfold) and statistically significant, while smaller, insignificant changes were recorded from channel catfish. Catfish TLR2 appears to respond to ESC infection by down-regulation post infection, although further characterisation is needed in the future. In contrast,

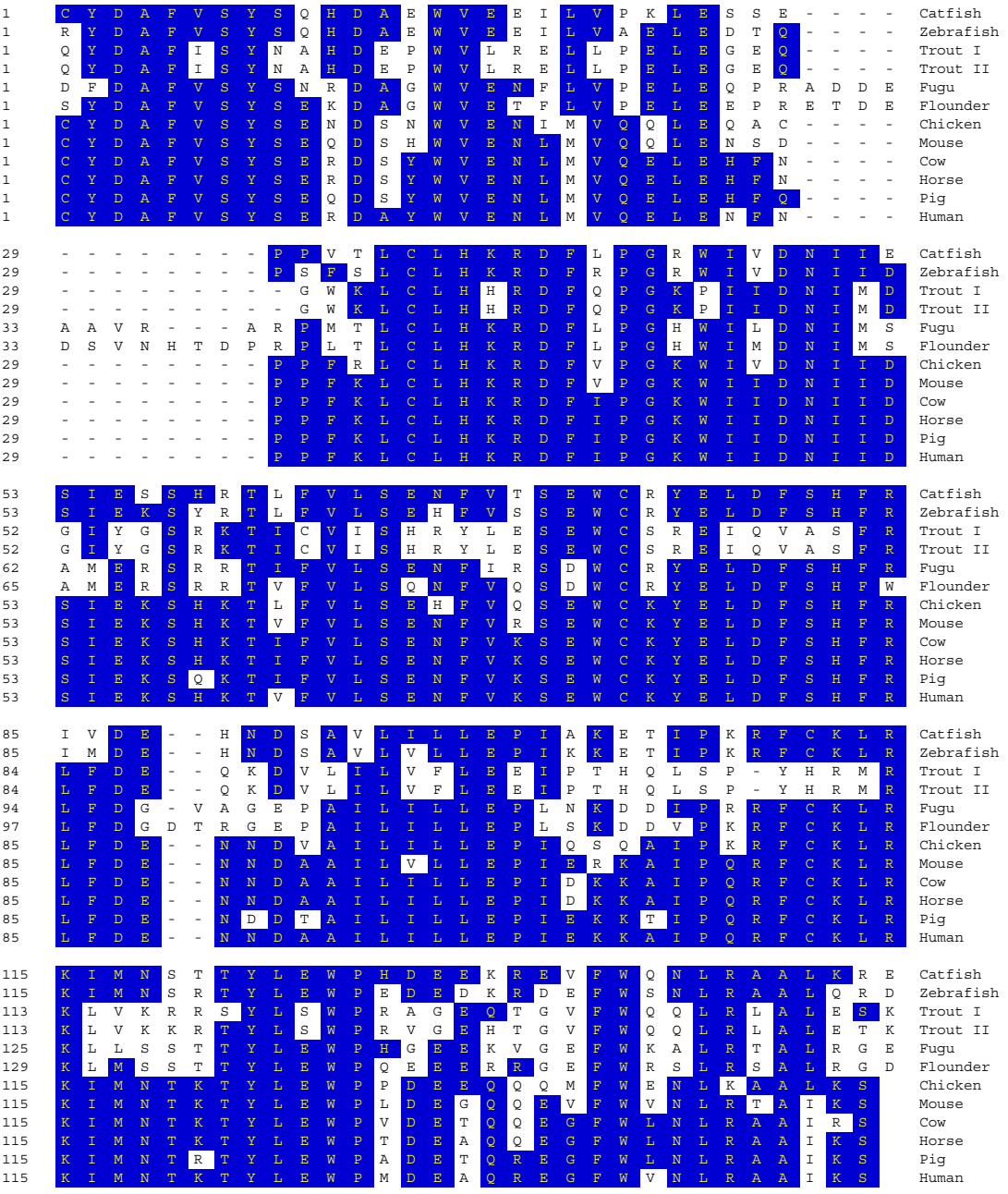

Fig. 2. Alignment of the catfish (DQ372072) TLR2 TIR domain with those of zebrafish (NP_997977), Rainbow trout I (AJ628348), Rainbow trout II (AJ878915), fugu (AAW69370), Japanese flounder (BAD01044), chicken (BAB16113), cow (NP_776622), horse (AAR08196), mouse (NP_036035), pig (BAD91799), and human (NP_003255) TLR2 genes.

a pattern of quick up-regulation of expression after ESC infection has been characteristic of many other genes involved in the catfish innate immune response including CC and CXC chemokines [28,35,36,39,40], antimicrobial peptides [29,41–43], and pro-inflammatory cytokines [44].

Induction of TLR2 expression has also been reported in Japanese flounder peripheral blood leukocytes (PBLs) at 1 h and 3 h after PGN and polyI:C stimulation, respectively [24]. Meijer et al. [23] reported that zebrafish TLR2 expression was induced in fish infected with *Mycobacterium marinum*. Both previous studies in fish utilised

Table 1
Percentages of amino acid identities between catfish TLR2 and those from other species using ClustalW

Catfish	Full sequence (%)	TIR domains (%)
Zebrafish	60.2	82.2
Rainbow Trout I (AJ628348)	20.1	43.1
Rainbow Trout II (AJ878915)	19.6	43.8
Fugu	46.6	65.8
Flounder	47.7	65.1
Chicken	41.3	72.4
Pig	42.3	71
Cow	42.5	70.3
Horse	41.2	71
Mouse	41.1	70.3
Human	41.1	72.4

Percentages were compared based on full-length and TIR domain amino acids sequences.

Gram-positive bacteria or their components as stimulants or infectious agents. However, the present results with Gram-negative bacteria *E. ictaluri* combined with the observed gene induction after stimulation with dsRNA polyI:C [24] indicate that TLR2 may function more widely in fish than previously assumed. Similar studies of TLR3, classically considered a receptor for viral dsRNA, in catfish after infection with *E. ictaluri* revealed induced expression [45,46]. All these observations suggest that Toll-like receptors may be involved in a more complex patterns of ligand binding and recognition than previously thought [7]. Further work is needed to determine the pathogen specificities of TLRs in fish species.

All vertebrate species studied to-date possess a functional, well-conserved TLR2 gene, indicating strong selective pressure for the gene's ability to recognise important classes of PAMPs. Conservation of microbial PAMPs has likely led to the maintenance of a highly conserved TLR family across vertebrate species. The interspecies conservation of the TLR multigene family differs significantly from other immune families of genes under less selective restraints [7,28,47].

Much work remains in assessing the potential of TLRs in immune research and their applications in mammalian and fish species. TLR2 research in mammals continues to uncover polymorphisms associated with infectious and inflammatory diseases [20,48,49]. Two microsatellites revealed by genomic sequencing of the TLR2 gene environ should allow mapping of the TLR2 gene and future QTL analysis in catfish. Additionally, understanding the function of fish TLRs and their pathogen specificities may lead to the development of better immunostimulants for use in commercial aquaculture [50]. The future identification and characterisation of additional TLR family members and the components of their signaling pathways [51] in aquaculture species should further our knowledge of the teleost innate immune response.

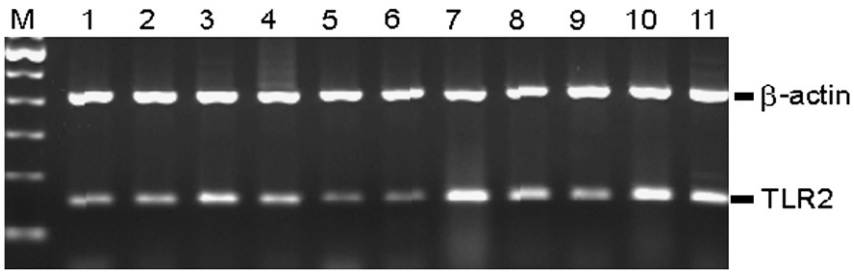

Fig. 3. RT-PCR analysis of catfish TLR2 gene expression in various healthy tissues. RT-PCR products were analysed on an agarose gel. M, 100 bp molecular weight; 1, head kidney; 2, spleen; 3, intestine; 4, stomach; 5, skin; 6, muscle; 7, liver; 8, trunk kidney; 9, ovary; 10, brain; 11, gill. The positions of the RT-PCR amplified bands of beta-actin and TLR2 are indicated on the right margin.

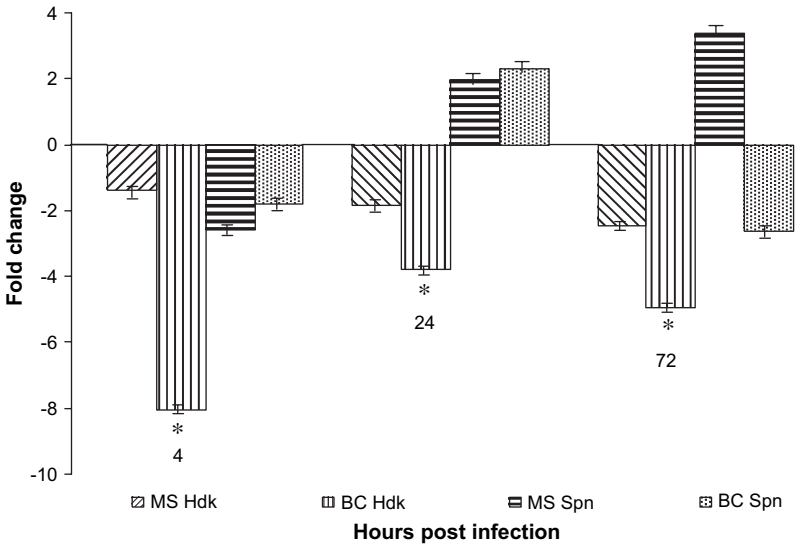

Fig. 4. Real-time PCR analysis of catfish TLR2 gene expression in head kidney and spleen following infection with *Edwardsiella ictaluri* at different time points (4, 24 and 72 h). Fold change indicates the ratio of expression at the specified time after ESC exposure to that at 0 h before ESC infection, as normalised with an internal reference gene of catfish beta-actin. Three independent Lightcyler reactions were run for each sample and statistical analysis was conducted using the software REST. Error bars indicate standard error and an asterisk indicates statistical significance. MS and BC signify Marion select strain of channel catfish and blue catfish, respectively.

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