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Molecular cloning and expression of toll-like receptor 4 (*tlr4*) in the blunt snout bream (*Megalobrama amblycephala*)

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ABSTRACT

Toll-like receptors (TLRs) play a pivotal role in teleost innate immune system. In this study, *Megalobrama amblycephala* (*ma*) *tlr4* gene was cloned, its putative polypeptide product characterized, and expression analysed. *Matlr4* cDNA is 2862 bp long, with an open reading frame of 2364 bp encoding 787 amino acids. MaTlr4 is a typical TLR protein, including the extracellular part with nine leucine-rich repeat motifs, a transmembrane region and a cytoplasmic Toll/interleukin-1 receptor domain. MaTlr4 has the highest level of identity (94%) and similarity (97%) with the grass carp Tlr4.2 homolog. This was also corroborated by the phylogenetic analysis, which placed MaTlr4 in a cluster with other cyprinid homologs. *Matlr4* mRNA was ubiquitously expressed in all examined tissues and during all sampled developmental stages. The observed peak in *matlr4* mRNA expression during gastrula and somite stages is in good agreement with its proposed role in the development of the neural system. Temporal expression patterns of *matlr4* and *maMyD88* mRNAs and proteins were analyzed in liver, spleen, head kidney, trunk kidney and intestine after *Aeromonas hydrophila* infection. And mRNA expression varied between different time-points. Both MaTlr4 and MaMyD88 protein expressions at 12 hpi were significantly enhanced in head kidney and intestine. These results indicate that *matlr4* is involved in the immune response in *M. amblycephala*, and that it is indeed a functional homologue of *tlr4*s described in other animal species.

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1. Introduction

Innate immune system is a fundamental defense mechanism against pathogen invasion in lower vertebrates (including fish and amphibians), faster, but less specific than adaptive immunity (Akira et al., 2006; Magnadottir, 2006). Toll-like receptors (TLRs) are among the key pattern recognition receptor (PRR) classes, which specifically recognize conserved microbial pathogen structures known as pathogen-associated molecular patterns (PAMPs). These include lipoproteins, polysaccharides, polyinosinic:polycytidylic acid (poly I:C), lipopolysaccharides (LPS), flagellin, peptidoglycans, nucleic acids, CpG-DNA, double-stranded and single-stranded viral RNA, etc. (Takeda and Akira, 2005; Medzhitov, 2001). TLRs are a type I transmembrane proteins, comprised of an N-terminal extracellular domain, containing a varying number of leucine-rich repeats (LRRs) responsible for recognizing PAMPs, a transmembrane domain (TM) and a C-terminal intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domain involved in binding downstream adaptor molecules that are important for the activation of signal transduction (Akira and Takeda, 2004).

TLR4 was the first member of the TLR family to be identified and recognized as an important player in the innate immunity (Takeda and Akira, 2003; Medzhitov et al., 1997). Mammalian TLR4 recognizes LPS, which are a major component of the outer membrane of Gram-negative bacteria, then transmits the signal through the TIR domain, which is involved in binding the adaptor molecules, such as myeloid differentiation factor 88 (MyD88), that initiate the expression of downstream pro-inflammatory genes (O'Neill and

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Table 1
Primers for *M. amblycephala* tlr4 cloning and expression analyses.

Target gene	Primer name	Primer sequence (5′-3′)
Partial *matlr4* cDNA primers	p*tlr4*-F	TCGTGTCCTTACCGCTTCA
	p*tlr4*-R	GTTCTCCAGATTCTCCAT
matlr4-specific primers for 3′ RACE	*tlr4*-3′-outer	ATTCTAATGAGAGGCTAC
	tlr4-3′-inner	CTATGATGAAGCCTGGGT
matlr4-specific primers for 5′ RACE	*tlr4*-5′-outer	TTATTTGTTCCAACTCTA
	tlr4-5′-inner	GCGGTAAGGACACGAGAC
Vector primers for colony PCR	Vector-F	CGTTGTAAAACGACGGCCAG
	Vector-R	ACACAGGAAACAGCTATGAC
matlr4 primers for qPCR	*tlr4*-F	TGGTGCGCTTTGAGTTTGA
	tlr4-R	AAGGTTCCCTGCTCCCACTTC
maMyD88 primers for qPCR	*MyD88*-F	GACAACAGGGATTAGACG
	MyD88-R	TGGAACAGACTGAATACAAC
β-actin primers for qPCR	*β-actin*-F	ACCCACACCGTGCCCATCTA
	β-actin-R	CGGACAATTTCTCTTTCGGCTG

Bowie, 2007; Vogel et al., 2003). After TLR4 recognizes the LPS, downstream signaling forks into MyD88-dependent and MyD88-independent pathway: the former leads to the activation of nuclear factor—kappaB (NF-κB) and the expression of proinflammatory genes, such as tumour necrosis factor (TNF) and IL, while the latter gives rise to interferon 3-mediated expression of Type I interferons (IFN) and IFN-inducible genes (Medzhitov et al., 1998; Janeway and Medzhitov, 2002; Lu et al., 2008; Kawasaki and Kawai, 2014; Liu et al., 2014). Studies using knockout mice have revealed that MyD88 is a critical adaptor molecule in TLR4 signaling: MyD88-deficient mice were resistant to LPS-induced septic shock, and MyD88-deficient macrophages failed to produce proinflammatory cytokines after LPS stimulation, despite the ability to activate NF-κB. However, the expression of Type I interferons and interferon-inducible genes was not impaired in MyD88-deficient macrophages (Adachi et al., 1998; Kawai et al., 1999, 2001). This demonstrated that MyD88-dependent pathway is crucial for defense against pathogens in TLRs/IL-1R signaling pathway (apart from TLR3, which is not mediated through MyD88-dependent pathway, but through TRIF-dependent pathway) (Kawasaki and Kawai, 2014).

To date, in fish, *tlr4* genes have been identified only in ictalurids, like channel catfish (*Ictalurus punctatus*) (Quiniou et al., 2013), and cyprinids, including zebrafish (*Danio rerio*) (Jault et al., 2004; Meijer et al., 2004), grass carp (*Ctenopharyngodon idella*) (Huang et al., 2012), rare minnow (*Gobiocypris rarus*) (Su et al., 2009), common carp (*Cyprinus carpio*) (Kongchum et al., 2010), mrigal (*Cirrhinus mrigala*) (Madhubanti et al., 2013) and naked carp (*Gymnocypris przewalskii*) (Tong et al., 2015). *Tlr4* gene has not been found in genomes of three-spined stickleback (*Gasterosteus aculeatus*), green-spotted pufferfish (*Tetraodon nigroviridis*) and Japanese pufferfish (*Takifugu rubripes*) (Oshiumi et al., 2003; Baoprasertkul et al., 2007).

Due to fast growth and popularity among consumers, blunt snout bream (*Megalobrama amblycephala*), endemic to China and commonly known as Wuchang fish, has rapidly become one of the most economically important freshwater polyculture fish species in China during the last few decades. Its production is still growing: from 625,789 tons in 2009 to 730,962 tons in 2013, ranking sixth in Chinese freshwater fish production (Liu and Liu, 2009; Bureau, 2013). However, along with the increased production, it has also become increasingly vulnerable to various pathogens, especially *Aeromonas hydrophila*, which often results in catastrophic economic losses (Nielsen et al., 2001).

Hence, identification of *M. amblycephala* genes involved in disease resistance and immunity is of great importance for the aquaculture industry. Apart from this, structural and functional studies of *tlr4* in fish are needed to understand why it seems to have been lost during the evolution in the majority of fish species, while being retained in Cyprinidae and Ictaluridae (Oshiumi et al., 2003; Baoprasertkul et al., 2007). *Tlr4* expression has been shown to be responsive to *A. hydrophila* bacterium or LPS stimulation in some cyprinid species (Su et al., 2009; Madhubanti et al., 2013; Pei et al., 2015), however, *tlr4a* and *tlr4b* expression was unchanged in response to *Escherichia coli*, *Legionella pneumophila* and LPS challenge in *D. rerio* (Sepulcre et al., 2009; Sullivan et al., 2009). Similarly, studies have demonstrated that MyD88 expression was upregulated in fish following stimulation with *A. hydrophila*, *Edwardsiella tarda*, *Streptococcus uberis*, *Vibrio parahaemolyticus*, poly I:C or LPS (Yao et al., 2009; Wei et al., 2011; Basu et al., 2012a, 2012b). Hence, the objectives of this study were to identify and characterise *tlr4* in *M. amblycephala*, and then investigate its expression patterns at different developmental stages, in different tissues, and after a challenge with *A. hydrophila*, in order to indirectly infer its functional role. In order to infer a better perspective of the functional significance of *matlr4*, we have also experimentally determined the activation of MyD88, which is a crucial adaptor molecule of tlr4-mediated signal transduction pathway; and compared the expression patterns between the two genes. The ultimate goal is to contribute to a better understanding of the evolution of the fish TLR family and the activation of MyD88-dependent signaling pathway in fish innate immune system. The results imply that *M. amblycephala* (*ma*) *tlr4* participates in anti-*A. hydrophila* immune processes, which will help further elucidation of regulation mechanisms in the innate immune response of *M. amblycephala* to bacterial infection.

2. Materials and methods

2.1. Fish sampling and challenge experiment

Healthy *M. amblycephala* specimens (n = 120) for the challenge experiments (mean body weight = 150 ± 10 g; mean body length = 20 ± 1 cm), as well as 3 female and 3 male adult specimens for obtaining embryos via artificial breeding, were obtained from the Tuanfeng fish farm (Huanggang city, Hubei province, China). The fish were acclimated in aerated freshwater tanks at 26 ± 2 °C and fed commercial pellet feed twice daily for seven days before the onset of the study. Healthy fish were divided into three groups: 20 "blank" (non-injected), 50 "challenge" (*A. hydrophila* injected) and 50 "control" [phosphate-buffered saline (PBS)-injected]. For full-length cDNA cloning and tissue-specific expression analyses, three healthy specimens from the blank group were anesthetized with 100 mg/L tricaine methanesulfonate (MS-222, Sigma—Aldrich

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