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EXPRESSION ANALYSIS OF INTERLEUKIN-1 RECEPTOR ASSOCIATED KINASE-4 (IRAK-4) GENE INMIDGUT OF LIPOPOLYSACCHARIDE INDUCED *PENAEUS MONODON*

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ABSTRACT : Interleukin-1 receptor associated kinase-4 (IRAK-4) has been identified as a central signal transduction mediator of the Toll-like receptor (TLR) and Toll/interleukin-1 receptor (TIR) pathways both in vertebrate and invertebrate innate immunity. To investigate the role of Toll-pathway in innate immune response of shrimp, *Penaeus monodon*, expression analysis of IRAK-4 gene in mid-gut of LPS induced shrimps wasdone at five time points. Responses of this gene to LPS by Quantitative real time PCR (qPCR) expression analysis of the IRAK-4 gene wasdone in control and LPS induced *P. monodon*. Mid-gut tissue expressions of theIRAK-4 gene was significantly up regulatedin the LPS induced group. The study indicatedthe critical role of IRAK-4 in gut immunityof *P. monodon*.

Key words : Toll-like receptor (TLR), IRAK-4, LPS, *P. monodon*, gut immunity.

INTRODUCTION

World production of the important species of cultivated shrimp, *Penaeus monodon* and *Litopenaeus vannamei*, has increased exponentially since the early 1970s. India occupies fifth position amongst the major shrimp farming countries in the world (Mishra *et al*, 2017). The production of shrimp from aquaculture in 2005 reached over 2.2 million MT (Tanticharoen *et al*, 2008). However, with the rapid increase of shrimp culture increased problems with serious bacterial disease outbreaks occur, which included luminescent bacterial disease, Vibriosis and Bacterial septicaemia mostly due to poor pond management.

The innate immune system is considered the most ancient and universal form of host defense, is an efûcient ûrst line of defense against invading microbes both in invertebrates and vertebrates. The crustaceans are usually devoid of highly evolved specific immune system unlike their vertebrate counterparts and depend very much on non-specific cellular and humoral factors as the first line of defense against invading pathogens (Karunasagar *et al*, 2014). Pattern recognition proteins (PRPs) present in the haemocytes of crustaceans recognize sugar moieties of microbial cell wall components such as lipopolysaccharide (LPS) or peptidoglycan (PG) from bacteria or β-1, 3-glucans from fungi (Soderhall *et al*, 1996; Amparyup *et al*, 2012). Pattern recognition receptors (PRRs), part of the ancient innate arm of the immune system, are conserved in invertebrate and vertebrate lineages. They recognize the conserved molecular structure of pathogens, known as pathogen-associated molecular patterns (PAMPs) and trigger the signaling pathways that activate immune cells in response to pathogen infection (Palti, 2011). Among various PRRs, Toll-like receptors (TLRs) are the ûrst and best characterized innate immune receptors. All TLRs are type Itransmembrane proteins that contain three parts: an extracellular N-terminus with leucine-rich repeat (LRR) domain, a transmembrane domain and an intracellular C-terminus with a Toll/IL-1 receptor (TIR) domain (Takeuchi *et al*, 2010; Zhu *et al*, 2013). TLR/TIR signal transduction contains several interleukin-1receptor-associated kinases (IRAKs) as downstream signaling mediators. All IRAK members have two typical domain structures, a N-terminal domain containing a death domain (DD) and a central kinase domain (Janssens *et al*, 2003). The kinase domain is required for the serine/ threonin

kinase activity during signal transduction. Among the four mammalian IRAKs (IRAK-1, IRAK-2, IRAK-M and IRAK-4), only IRAK-1 and IRAK-4 have been shown to have active kinase activity (Li *et al*, 2002; Wang *et al*, 2013). IRAK-1 and IRAK-4 are orthologs of the Pelle serine/threonin kinase molecules, in the Drosophila Toll signaling cascade, which is operating against Gram-positive bacteria and fungi (Towb *et al*, 2009; Suzuki *et al*, 2002). Among IRAK family members, IRAK-4 shares the highest sequence homology with the Drosophila Pelle (Wang *et al*, 2006). In contrast to Drosophila Pelle kinase, the mammalian IRAK-4 is also involved in response to Gram-negative bacteria infections (Swantek *et al*, 2000; Takeda *et al*, 2005).

IRAK-4 is present in several animal species from sponges to mammals and it is expressed in various tissues (Goodson *et al*, 2005; Phelan *et al*, 2005; Ge *et al*, 2011; Li *et al*, 2002). It is the central mediator in NF-kB activation and innate immunity signaling and its kinase activity is necessary for activation of IRAK-1 and perhaps other signal transducing substrates (Suzuki *et al*, 2002). The IRAK-4 is a key kinase in signal transduction by the lipoprotein receptor Toll-like receptor (TLR) 2 and the lipopolysaccharide (LPS) receptor TLR 4 (Dunne *et al*, 2010).

TLRs might be important to maintain the composition of the intestinal bacteria in the intestine epithelial cells IECs (Fukata *et al*, 2007). Being an aquatic animal, shrimp are constantly exposed to a variety of bacteria and viruses. The digestive system of shrimp is the main entry port for microbial infections. Hence, it is considered that intestine is one of the target sites for bacterial invasion. The gastrointestinal (GI) track of shrimp canbe separated into three mainparts consisting of foregut, midgut and hindgut lined from head to tail, respectively (Soonthornchai *et al*, 2010). Shrimp have several known first line of defense mechanisms to protect themselves against pathogen invasion. The external cuticle provides an effective physical and chemical barrier against the attachment and penetration of pathogens (Martin *et al.* 2004). Unlike foregut and hindgut the midgut (equivalent to intestine) is not lined by cuticle (Lovett *et al*, 1990) and therefore provides a favorable site for invasion of pathogens (Ruby *et al*, 1980; Jayabalan *et al*, 1982). The injection method always leads to the activation of an immuneresponse, but it bypasses the initial step of bacterial infection (Vodovar *et al*, 2004). Therefore, in the present study, we have elucidated *Penaeus monodon*, the black tiger shrimp with LPS through injection mean and evaluating the comparative analysis of an expression proûle of IRAK-4 genein midgut of GI track and its tissue mRNA expression in LPS induced as well as in the PBS injected in *P. monodon* shrimp.

MATERIALS AND METHODS

Experimental animals and sample collection

Healthy *P. monodon* weighing approximately 20 g were obtained from the local Panvel farm of Mumbai and acclimatized in aquarium tanks containing sea water with 15ppt salinity under continuous aeration for two weeks before the experiment. The animals were fed with pelleted commercial feed at the rate of 5% of their body weight.

Experimental design and LPS challenge

Healthy shrimps (n = 40) were divided into two groups for the experimental study. Each animal in group I (20 shrimps) was injected intramuscularly in the second abdominal segment with 10µg ofLPS. Each animal in Group II (20 shrimps) was injected with 100µl of sterile PBS and served as negative control.Prior to the experiment, 3 animals were collected for determining the expression level at zero hour (un-injected control). Post injection, three animals were sacrificed at time point of 2, 6, 12, 24h and 48h from both groups. Mid-gut tissue was dissected out and preserved at –80^{0}C (Sanyo, Japan) in RNA later for further studies.

RNA extraction and cDNA synthesis

Total RNA was extracted from mid-gut tissue using Trizolreagent (Invitrogen, USA) as per manufacturer's instructions. Complementary DNA (cDNA) was synthesized in a reaction mixture containing 5×first strand buffer (250mM tris-HCl, pH 8.3, 375mMKCl, 15mM $MgCl_2$), 10mM DTT, 100U of Superscript II Reverse Transcriptase (Invitrogen, USA), 20U RNase inhibitor (Invitrogen, USA), 200mM each of dATP, dCTP, dGTP and dTTP (HiMedia) and oligo (dT) using the protocol prescribed by the manufacturer (Invitrogen, USA).

Quantitative Real time PCR (qPCR) of IRAK-4 gene

mRNA expression of IRAK-4 gene in the experimental samples was estimated by real-time PCR in

Table 1 : Primers used for gene-specific RT-PCR and real-time PCR.

S. No.	Primer	Sequence (5′ to 3′)	Tm (°C)	Refrences	Size (bp)
1.	IRAK-4 Q-F/R	GGGTCGAGTGCCAAGTATGC GCGATCTCGAGCACATTCTT	60	Shreedharan *et al* (2017)	400
2.	EF-1α Q-F/R	GGTGCTGGACAAGCTGAAGGC CGTTCCGGTGATCATGTTCTTGATG	60	Amparyup *et al* (2009)	150

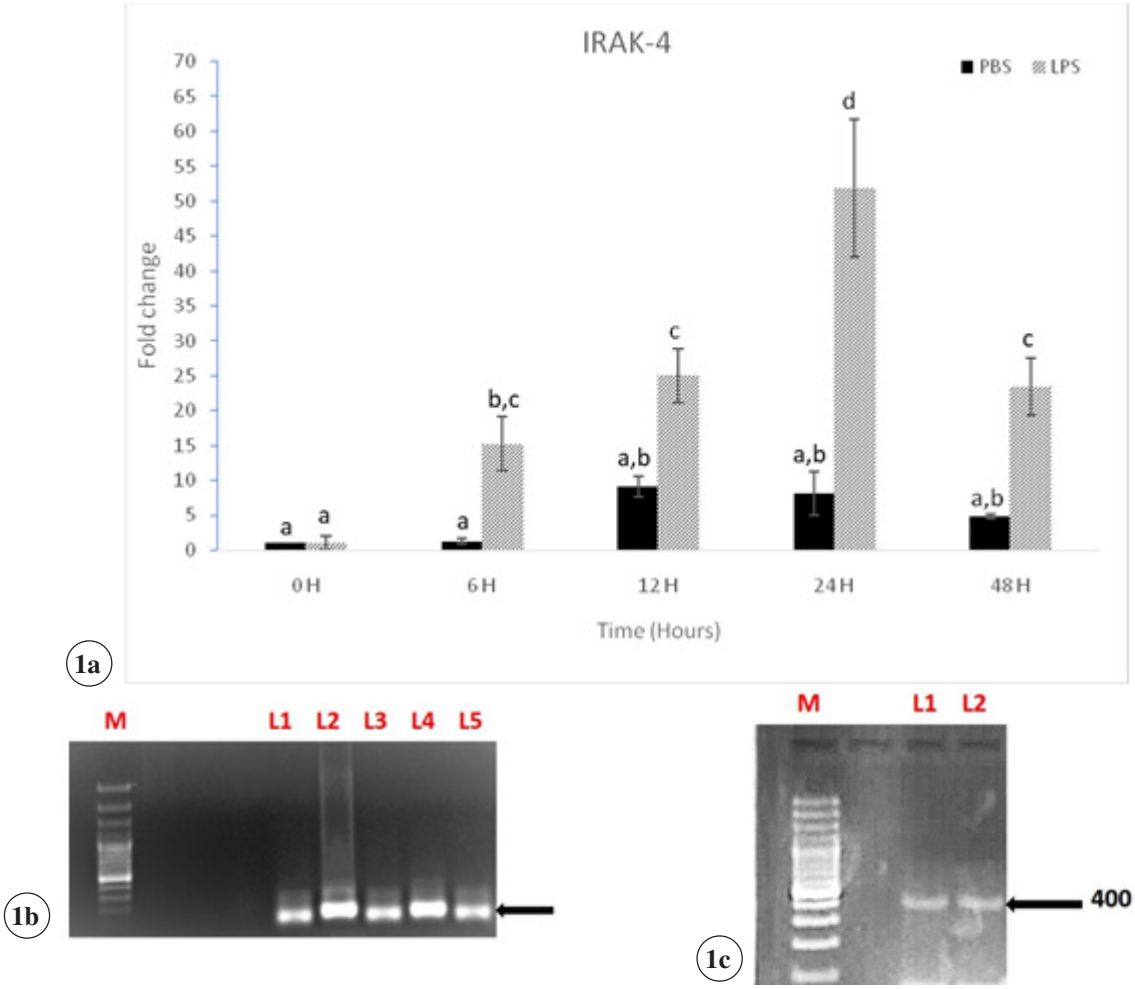

Fig. 1a : Relative fold expression of IRAK-4 gene in midgut of *P. monodon*

Fig. 1b : Semi-quantitative PCR expression of EF-1á (150 bp) gene at various time points in post LPS infection in mid-gut tissue, where Lane 1: 0 h, lane 2: 6 h, lane 3: 12 h, lane 4: 24 h, lane 5: 48 h.

Fig. 1c : Semi-quantitative PCR expression of IRAK-4(400 bp) gene in mid-gut tissue at 48 h post LPS infection, where Lane 1: PBS group; Lane 2: LPS group.

Fig. 1 : Relative qPCR expression of IRAK-4 gene at various time points in post LPS infection in mid-gut tissue, PBS treated shrimps served as control. Expression was calculated relative to EF-1α and the data shown as mean ± SE, where $P < 0.05$ was considered as statistically significant.

ABI 7500 Real-time PCR system (Applied Biosystems, USA) using forward and reverse primers given in Table 1. EF-1α was used as internal control with primers EF-1α-F and EF-1á-R (Table 1). The ampliûcations were performed in a 96 well plate using ABI 7500 real-time PCR system set at default thermal proûle. Equal quantity of cDNA (100ng), for all experimental and control samples, was ampliûed in a 25ìL qPCR mixture containing 12.5 ìL of 2× SYBR Green master mix (Takara Bio Inc., Japan), and 0.5 ìL of each gene-speciûc primer (25ìM). All reactions were performed in duplicates (each well containing 10 ìL of reaction mix). The relative fold-change was determined by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) using SPSS 22.0. The results were expressed as mean ± SEM (standard error of the mean). $P< 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

In order to understand the pathway of TLR system, the essential molecule involved in the transduction of Toll-pathway of *P. monodon*, IRAK-4 in mid-gut tissue was investigated by real-time PCR analysis. In mammals, pathogen recognition by Toll-like receptors (TLRs) is central to the activation of the innate immune response. TLRs can interact with distinct pathogen-associated molecular patterns (PAMPs) derived from viruses, bacteria and fungi. Stimulated by PAMPs, all of the TLRs,

except TLR3, recruit the adaptor protein myeloid differentiation primary response protein 88 (MyD88) through the Toll-IL-1R (TIR) domain, leading to the receptor complex formation of IL-1 receptor-associated kinase-4 (IRAK-4), IRAK1 and tumor necrosis factor receptor-associated factor 6 (TRAF6) (Qian *et al*, 2001; Lye *et al*, 2004; Takeuchi *et al*, 2010). The TLR-mediated NF-êBsignaling pathway is essential in antibacterial and antiviral defense in Drosophila and humans (Lemaitre *et al*, 2007; Valanne *et al*, 2011; Leulier *et al*, 2008). This pathway also seems to be an attractive target of bacterial and viral pathogens (Santoro *et al*, 2003; Hiscott *et al*, 2006). In invertebrates, however, this pathway has only been well-studied in Drosophila, and there are few reports of interactions between TLR signaling pathway of invertebrate hosts and viral pathogens. Here, we propose the existence of a potential TLR pathway in shrimp and report that IRAK-4 being a central molecule is elucidated at the induction with ligand LPS. IRAK-4 gene was found to be up regulated in mid-gut tissue obtained from LPS induced shrimp samples in comparison to the animals from control group injected with PBS. A 15 fold expression was observed at 6 h post LPS induction (Fig. 1) and the expression was gradually seen to increaseupto25 fold at 12 h post infection while the expression in the PBS group was 9.08 fold. Relative fold expression was highest at 24 h and then reduced to 23 fold at 48 h while in the PBS group, the maximum expression at 12 h and 24 h post injection. Between the LPS and PBS samples, it was observed that at all time points post infection, the IRAK-4 gene expression was significantly up regulated ($p \leq 0.005$) in midgut tissue (Fig. 1 a & b).

IRAK-4 is the central members of the TLR-mediated NF-κB pathway because they not only form receptor complex with MyD88 and Tube (or IRAK1) but also participate in downstream signal transduction with NF-κB family proteins (Janssens *et al*, 2003). To further test this hypothesis, the ability of LPS ligand induction and stimulation was evaluated *in-vivo* by injection mean. We found that IRAK-4 gene was up regulated in mid-gut tissue obtained from LPS induced shrimp samples in comparison to the animals from control group injected with PBS. This is in agreement to the studies involving IRAK-4-knockout mice indicate that IRAK-4 is critically required for the induction of many TLR/IL-1R-dependent immune responses (Suzuki *et al*, 2002). Mice lacking IRAK-4 were resistant to LPS-induced sepsis due to an absence of inflammatory cytokine production. NF-κB and MAPK activation was severely compromised in response to LPS in cells lacking IRAK-4 while these signals were absent in response to IL-1b. Additional studies have shown that IRAK-4 was also required for IL-18R-dependent responses (Suzuki *et al*, 2003). Reports have also found that the absence of IRAK-4 results in impaired T cell responses due to defects in T cell receptor (TCR) signaling (Suzuki *et al*, 2006), although this is in contrast to other recent findings of Kawagoe *et al* (2007). A bacterial defense function of this protein has been known for a long time, and for example IRAK-4 deficient mice have increased mortality upon a bacterial infection (Suzuki *et al*, 2002).

In present study, the critical role of IRAK-4 gene has been proved in mid-gut infections/inflammatory responses. The study revealed dynamic upregulation of IRAK-4 gene expression in mid-gut of *P. monodon* on LPS induction. This study will set a benchmark in gut immunity in shrimp against gram negative bacteria.

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REFERENCES

Amparyup P, Sutthangkul J, Charoensapsri W and Tassanakajon A (2012) Pattern recognition protein binds to lipopolysaccharide and β-1, 3-glucan and activates shrimp prophenoloxidase system. *J. Bio. Chem.* **287**(13), 10060-10069.

Dunne A, Carpenter S, Brikos C, Gray P, Strelow A, Wesche H, Morrice N and O'Neill L A (2010) IRAK1 and IRAK4 promote phosphorylation, ubiquitination and degradation of MyD88 adaptor-like (Mal). *J. Bio. Chem.***285**(24), 18276-18282.

Fukata M and Abreu M T (2007) TLR4 signalling in the intestine in health and disease. *Biochem. Soc. Trans.* **35**, 1473–1478.

Ge H, Wang G, Zhang L, Zhang Z, Wang S, Zou Z, Yan S and Wang Y (2011) Molecular cloning and expression of interleukin-1 receptor-associated kinase 4, an important mediator of Toll-like receptor signal pathway, from small abalone *Haliotis diversicolor*. *Fish and Shellfish Immunology* **30**(4-5), 1138-1146.

Goodson M S, Kojadinovic M, Troll J V, Scheetz T E, Casavant T L, Soares M B and McFall-Ngai M J (2005) Identifying components of the NF-κB pathway in the beneficial Euprymnascolopes-Vibrio fischeri light organ symbiosis. *Applied and Environmental Microbiology* **71**(11), 6934-6946.

Hiscott J, Nguyen T A, Arguello M, Nakhaei P and Paz S (2006) Manipulation of the nuclear factor-κB pathway and the innate immune response by viruses. *Oncogene* **25**(51), 6844.

Janssens S and Beyaert R (2003) Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. *Molecular Cell* **11**(2), 293-302.

Jayabalan N, Chandran R, Sivakumar V and Ramamoorthi K (1982) Occurrence of luminescent bacteria in sediments. *Curr. Sci.* **51**(14), 710-1.

Karunasagar I, Kumar S N, Maiti B and Rai P (2014)

Immunostimulation of Crustaceans. In: Gudding R, Lillehaug A and Evensen O (eds.). *Fish Vaccinations*. Wiley Blackwell Publications, U.K. 352–371.

Kawagoe T, Sato S, Jung A, Yamamoto M, Matsui K, Kato H, Uematsu S, Takeuchi O and Akira S (2007) Essential role of IRAK-4 protein and its kinase activity in Toll-like receptor–mediated immune responses but not in TCR signaling. *J. Exp. Medicine* **204**(5), 1013-1024.

Lemaitre B and Hoffmann J (2007) The host defense of Drosophila melanogaster. *Ann. Rev. Immunol.* **25**, 697-743.

Leulier F and Lemaitre B (2008) Toll-like receptors—taking an evolutionary approach. *Nature Reviews Genetics* **9**(3), 165.

Li S, Strelow A, Fontana E J and Wesche H (2002) IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. *Proc. Natl. Acad. Sci.* **99**(8), 5567-5572.

Livak K J and Schmittgen T D (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2–ΔΔCT method. *Methods* **25**(4), 402-408.

Lovett D L and Felder D L (1990) Ontogenetic changes in enzyme distribution and midgut function in developmental stages of *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). *The Biological Bulletin* **178**(2), 160-174.

Lye E, Mirtsos C, Suzuki N, Suzuki S and Yeh W C (2004) The role of interleukin 1 receptor-associated kinase-4 (IRAK-4) kinase activity in IRAK-4-mediated signaling. *Journal of Biological Chemistry* **279**(39), 40653-40658.

Martin G G, Rubin N and Swanson E (2004) *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. *Diseases of Aquatic Organisms* **60**(1), 21-29.

Palti Y (2011) Toll-like receptors in bony fish: from genomics to function. *Develop. Comp. Immunol.* **35**(12), 1263-1272.

Phelan P E, Mellon M T and Kim C H (2005) Functional characterization of full-length TLR3, IRAK-4 and TRAF6 in zebrafish (*Danio rerio*). *Molecular Immunology* **42**(9), 1057-1071.

Qian Y, Commane M, Ninomiya-Tsuji J, Matsumoto K and Li X (2001) IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkB. *J. Bio. Chem.* **276**(45), 41661-41667.

Ruby E G, Greenberg E P and Hastings J W (1980) Planktonic marine luminous bacteria: species distribution in the water column. *Appl. Environ. Microbiol.* **39**(2), 302-306.

Santoro M G, Rossi A and Amici C (2003) NF κB and virus infection: who controls whom. *The EMBO Journal* **22**(11), 2552-2560.

Soderhall K (1996) The prophenoloxidase activating system in invertebrates. *New Directions in Invertebrates Immunology* 229-53.

Soonthornchai W, Rungrassamee W, Karoonuthaisiri N, Jarayabhand P, Klinbunga S, Söderhäll K and Jiravanichpaisal P (2010) Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Develop. Comp. Immunol.* **34**(1), 19-28.

Mishra S S, Das R, Choudhary P, Debbarma J and Sahoo S N (2017) Present status of Fisheries and Impact of Emerging Diseases of Fish and Shellfish in Indian Aquaculture. *J. Aquatic Res. nd Marine Sci.* 5-26.

Suzuki N, Chen N J, Millar D G, Suzuki S, Horacek T, Hara H, Bouchard D, Nakanishi K, Penninger JM, Ohashi P S and Yeh W C (2003) IL-1 receptor-associated kinase 4 is essential for IL-18-mediated NK and Th1 cell responses. *The Journal of Immunology* **170**(8), 4031-4035.

Suzuki N, Suzuki S, Duncan G S, Millar D G, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S and Penninger J M (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* **416**(6882), 750.

Suzuki N, Suzuki S, Duncan G S, Millar D G, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S and Penninger J M (2002) Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. *Nature* **416**(6882), 750.

Suzuki N, Suzuki S, Millar D G, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen N J, Elford A R and Suzuki J I (2006) A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. *Science* **311**(5769), 1927-1932.

Suzuki N, Suzuki S and Yeh W C (2002) IRAK-4 as the central TIR signaling mediator in innate immunity. *Trends in Immunology* **23**(10), 503-506.

Swantek J L, Tsen M F, Cobb M H and Thomas J A (2000) IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. *The Journal of Immunology* **164**(8), 4301-4306.

Takeda K and Akira S (2005) Toll-like receptors in innate immunity. *Int. Immunol.* **17**(1), 1-4.

Takeuchi O and Akira S (2010) Pattern recognition receptors and inflammation. *Cell* **140**(6), 805-820.

Tanticharoen M, Flegel T W, Meerod W, Grudloyma U and Pisamai N (2008) Aquacultural biotechnology in Thailand: the case of the shrimp industry. *Int. J. Biotechnol.***10**(6), 588-603.

Towb P, Sun H and Wasserman S A (2009) Tube is an IRAK-4 homolog in a Toll pathway adapted for development and immunity. *J. Innate Immunity* **1**(4), 309-321.

Valanne S, Wang J H and Rämet M (2011) The Drosophila toll signaling pathway. *The Journal of Immunology* **186**(2), 649-656.

Vodovar N, Acosta C, Lemaitre B and Boccard F (2004) Drosophila: a polyvalent model to decipher host–pathogen interactions. *Trends in Microbiology* **12**(5), 235-242.

Wang X, Smith C and Yin H (2013) Targeting Toll-like receptors with small molecule agents. *Chemical Society Reviews* **42**(12), 4859-4866.

Wang Z, Liu J, Sudom A, Ayres M, Li S, Wesche H, Powers J P and Walker N P (2006) Crystal structures of IRAK-4 kinase in complex with inhibitors: a serine/threonine kinase with tyrosine as a gatekeeper. *Structure* **14**(12), 1835-1844.

Zhu L Y, Nie L, Zhu G, Xiang L X and Shao J Z (2013) Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Develop. Comp. Immunol.* **39**(1-2), 39-62