Pertanika J. Trop. Agric. Sc. 41 (3): 1479 - 1488 (2018)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

The Response of TLR3 and IL-1β Genes Following Exposure to LPS, Poly (I:C), Zymosan in Culture of Gouramy (*Osphronemus gouramy*) Kidney Cells

Diah Kusumawaty[1]*, Sony Suhandono[2], I Nyoman Pugeg Aryantha[2] and Adi Pancoro[2]

[1]Department of Biology Education, Indonesia University of Education, Jl. Dr. Setiabudi No 229, Bandung 40154, Indonesia
[2]School of Life Sciences and Technology, Bandung Institute of 14 Technology, Jl. Ganesha No. 10, Bandung 40132, Indonesia

ABSTRACT

The aims of the present study are to isolate and characterise structure TLR3 and IL1β gene and evaluate the potential and signaling mechanism following exposure of Polyinosinic: polycytidylic acid (poly (I:C)), Lipopolysaccharide (LPS) and Zymosan as antigens in gouramy (*Osphronemus gouramy*). Gouramy kidney cells were stimulated with LPS, Poly I:C and Zymosan. Following incubation at 28°C, relative expression levels of Toll Like Receptor 3 (TLR3) and interleukin-1β (IL-1β) were examined at one hour and six hour after treatment. A Real Time Polimerase Chain Reaction approach was utilised to search for the effects of Poly I:C, LPS, and Zymosan exposure to gouramy kidney cells between one hour to six hours after treatment, LPS in kidney cell increased expression of interleukin-1β and downregulated expression of TLR3. Poly (I:C) which is an antigen that responds to antiviral, induces an increase in the transcription both of Toll like receptor 3 and IL-1β. Zymosan in kidney cells increased expression of TLR3 but downregulated expression of IL1β. This study shows that TLR3 was activated not only by Poly I: C but also LPS and Zymosan. However, antigen Poly IC-induced labor IL-1β and TLR3 expressed higher than the antigen LPS and Zymosan, so, this research showed a TLR3 response to Poly I:C is more dominant than Zymosan and LPS. TLR3 in gouramy kidney cell

ARTICLE INFO

Article history:
Received: 20 October 2017
Accepted: 01 June 2018
Published: 29 August 2018

E-mail addresses:
diah.kusumawaty@upi.edu (Diah Kusumawaty)
sony@sith.ac.id (Sony Suhandono)
nyoman@sith.ac.id (I Nyoman Pugeg Aryantha)
adi@sith.ac.ids (Adi Pancoro)
* Corresponding author

ISSN: 1511-3701
e-ISSN: 2231-8542

transcript was identified in response to poly I:C, Zymosan, and LPS. These results indicate that TLR3 in gouramy also plays a role for defense against bacterial infection and virus.

Keywords: IL1β, Innate immune, LPS, Poly(IC), *Osphronemus gouramy,* TLR3, Zymosan

INTRODUCTION

Interleukin 1-beta (IL1-β) is a pro-inflammatory cytokine that is important and has a major role in the immune response specific system or non-specific activation (Oppenheim, Kovacs, Matsushima, & Durum, 1986). IL-1β is also known as major of cytokine because IL-1β may mediate some immune responses and may be synthesised as an inactive precursor, which is processed into biologically active IL-1β as a response to various proinflammatory stimuli. This is why IL-1β is an inflammatory cytokine that is most widely studied in fish (Rauta, Nayak, & Das, 2012). Studies on IL-1β have been done in many fish such as rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), carp (*Cyprinus carpio*), orange spotted grouper (*Epinephelus coioides*) and Japanese flounder (*Paralichthys olivaceus*) (Engelsma, Stet, Schipper, & Verburg-van Kemenade, 2001; Lu et al., 2008; Scapigliati et al., 2001; Taechavasonyoo, Kondo, Nozaki, Suzuki, & Hirono, 2013; Zou, Grabowski, Cunningham, & Secombes, 1999).

Other valuable information showed that stimulation of Toll Like Receptor 3 (TLR3) and Toll Like Receptor 4 (TLR4) are one of the several responses that can secrete IL-1β (Maelfait et al., 2008). Previous studies revealed that many Toll-Like Receptors (TLRs) immune signal pathway factors play a major role in immune responses in many teleost fish. One of the relevant TLRs in teleost is TLR3. TLR3 has been known to play a role in response to virus and bacteria. Zebrafish that is injected with *Streptococcus agalactiae* shows induce expression of TLR3 (Hsieh, Pan, & Chen, 2010), whereas in mammalian Toll Like Receptor 3 (TLR3) play role response to virus (Matsumoto, Oshiumi, & Seya, 2011). Gouramy is one of the largest freshwater cultured species in Indonesia, and is one of the important freshwater fish commodities in various regions in Indonesia. In addition, gouramy is also included in the 15 main commodities that can increase the production and income of farmers Gouramy has suffered serious diseases caused by the viral, bacterial and parasitic infections in recent years, which result in enormous economic losses.

However, information on gouramy immune gene is scarce, as the mechanism and regulation of their immune response is still not well understood. Research on gouramy fish associated with bacterial infections has been widely practised, such as to asses the endurance level or mortality rate against infection of *Aeromonas hydrophila* after giving extracts of natural ingredients such as *Phaleria macrocarpa* (Christien, Djayus, & Ezraneti, 2014). Research has also been carried out to determine the immunostimulatory potential of *Spirulina platensis* extract for histopathologic

changes in gouramy fish due to *Aeromonas hydrophila* infection. (Simanjuntak, Wibowo, & Indarmawan, 2016). However, in gouramy fish, molecular innate immune mechanisms has not been widely studied. Kusumawaty, Suhandono, Pancoro, and Aryantha (2017) have also conducted a study to see changes in the expression of TLR2, Myd88 and TRAF6 genes in liver, kidney and spleen of post-infection *Aeromonas hydrophila*.

Gene response information on stimulation of analogue compounds such as LPS (Gram negative), Zymosan (fungi and Gram positive bacteria) and Poly IC (DNA virus) by in vitro method can be used to see early descriptions of unknown gene functions, so it can be used as a model for initial pathway of gene response to microbes. In fish, in vitro stimulation research is generally performed on kidney head cells, because the kidneys in the fish function in settings and central organs for the interaction of the endocrine immune system. (Fierro-Castro et al., 2012; Taechavasonyoo et al., 2013). In addition, fish kidney is the main organ that functions in the setting and is the central organ for interaction of the endocrine immune system (Tort, Balasch, & Mackenzie, 2003). Although in vitro stimulation studies of head kidney cells with analogue compounds have been widely practised, studies on the gouramy have not been carried out as yet.

This study aims to isolate and characterise structure TLR3 and IL1β gene and evaluate the potential and the signaling mechanism following exposure of Polyinosinic: polycytidylic acid (poly (I:C)), Lipopolysaccharide (LPS) and Zymosan as antigens in gouramy (*Osphronemus gouramy*). Phylogenetic tree, BLAST and structure predictive analysis is characterised OgIL-1β and OgTLR3 gene. The temporal expression profiles of OgTLR3 and OgIL-1β genes after stimulation with polyI:C, LPS, and Zymosan were compared to better understand their potential role in gouramy immune responses using real time PCR. In this study, the partial cDNA and threatment kidney cell respon to LPS, poly IC and Zymosan of *Osphronemus gouramy* TLR3 (OgTLR3) and *Osphronemus gouramy* IL1β (OgIL1β) were first described in gouramy. Identified OgTLR3 and OgIL-1β are derived from kidney which is believed to be the core organ for immune-endocrine interaction (Tort et al., 2003).

MATERIALS AND METHODS

Partial cDNA Sequencing of IL-1β and TLR3

Partial cDNA OgIL-1β and OgTLR3 gene were isolated from the kidney of gouramy using miTotalTM RNA Column (Viogen) according to the instructions in the manual protocol. The primer design was performed using amino acid sequence of several species from the Perciformes or Teleostei order. Furthermore, the DNA sequence of the target cds is aligned using the Bioedit programme. The alignment results were then used to design the primers using the Primaclade programme (Gadberry, Malcomber, Doust, & Kellogg, 2004) to obtain the degenerate primer sequence. The subsequently obtained

degenerate primer was used to amplify the partial genes of TLR 3 and IL1B from the carp cDNA. The primers used to isolate IL-1β and TLR3 from gouramy are listed in Table 1. Amplification of gene using Dreamtaq enzyme mix (Thermoscientific) were according to the instructions of the manual protocol.

Synthesis of cDNA using Moloney Murine Leukemia Virus reverse transcriptase (MMLV-RT) (Thermoscientific) used one µg RNA from kidney cell following the handbook's protocol. The amplicon was sequenced at Macrogen Korea. The sequencing result of OgIL-1β and OgTLR3 genes was checked for identification

Table 1
Primer Listed to Isolated IL-1β and TLR3 from Gouramy

Gene	Sequence 5'-----3'	Size Nt	Ta °C
TLR3_F1	GACCACAGTGCCAGGCCTCA	680	55
TLR3_R1	AAGGCTGGCACCCTCTCCCT		
TLR3_nestedF	GACTGCACRTGYGAGAGCAT	549	60
TLR3_nestedR	TTTRAATCGTCTACACCAGGG		
IL1β_F	TAACACTGAGAGGACAACTG	700	55
IL1β_R	GAAGAGAAACCGCACCAT		

using Basic Local Alignment Search Tool (BLAST) method, aligned and compared with the data of DNA sequences of other OgIL-1β and OgTLR3 genes available at the GenBank database NCBI (National Center for Biotechnology Information). The DNA sequences from species with clear names were put into one group. The group of similar DNA sequences was analyzed using multiple sequence alignment method. The phylogenetic tree was developed using the tool program of Clustal X software and MEGA 5 software (Tamura et al., 2011). The target DNA outcome is also used to locate the target amino acid by predicting it using the OFR finder program on the NCBI as well as estimating the protein domain structure with the SMART program (http://smart.embl-heidelberg.de, 24 August 2015).

Identification of these genes was necessary to design and synthesise the specific primer pair of gouramy that were used to analyse gene expression by using real time PCR. The primers used for gene expression analysis had analysed the quality of the amplification results through electrophoresis and looked at the pattern of amplified graphs using real time PCR

Expression Analysis of IL-1β and TLR3 Genes

The isolated kidney cell from 50gr of gouramy was re-suspended in primary culture medium (RPMI1640 supplemented 100 mg/ml of streptomycin and 100 IU/ml of penicillin). Kidney cell approximately 10^7 cells was treated with 250 µg/ml LPS (from *Escherichia coli* 0127:B8, SigmaeAldrich,

USA), 50 µg/ml polyI:C (Sigma-Aldrich, USA), 10 µg/ml Zymosan, and PBS for control at 28°C. The cells were harvested at one and six hours after treatment. Total RNA was extracted from kidney cell using miTotalTM RNA Column (Viogen) according to instructions in the manual protocol. cDNA synthesis using MMLV reverse transcriptase (Thermoscientific) used one µg RNA from kidney cell following the manual protocol. The cDNA samples were diluted 5-times with deionized water. The changes of mRNA level of the IL-1βand TLR3 were determined by real-time PCR which was performed with SYBR green PCR master mix (First Base) using Biorad CFX 96 Real-time PCR system following the manufacturer's instructions. The expression levels of target genes were normalised to the expression level of elongation factor-1 (EF-1) and Gapdh (glyceraldehyde-3-phosphate dehydrogenase) as an internal control gene and were expressed relative to the average level in the groups at one hour and six hours post treatment.

RESULTS AND DISCUSSION

Structural Characterisation Predicted Partial cds of TLR3 Genes and IL-1β Genes

Sequence of DNA and amino acid of IL1-β and TLR3 gene of gouramy has been submitted to NCBI GenBank. Accession numbers of NCBI GenBank are KT884611.1 (IL1-β) and KT884606 (TLR3). Figure 1A shows the analysis of phylogenetic tree based on partial ORF region of TLR3 gene gouramy (178 amino acids) known to most homologous to part TIR2 domain superfamily of ORF TLR3 large yellow croaker fish (*Larimichthys crocea*). Based on BLAST method in NCBI database, the identity index is 82%. Figure 1B shows ORF partial IL1-β gene (204 amino acids) gouramy most homologous with domain area IL1 superfamily part of ORF IL1-β fish Japanese flounder (*Paralichthys olivaceus*). Based on BLAST method in NCBI database the identity index is 79%. Based on Figure 1A and Figure 1B gouramy lowest homology based on partial both of ORF TLR3 and partial ORF IL1-β is with zebrafish (*Danio rerio*). This shows that gouramy and *D. rerio* are not closely related based on amino acid sequence TLR3 and IL1-β.

Based on Figure 2A, analysis of prediction protein structure using SMART programme is known that 2-47 amino acid regions are part of areas LRRCT (Leucine Rich Repeat C-terminal domain), a trans membrane helix region 51-73 and 106-178 part of areas TIR domain (Figure 2A), in comparison to analysis of protein structure prediction using TLR3 gene sequence of references fishes from GenBank are *L. crocea* [KKF15845.1], *E. coioides* [AEX01718.1], *T. rubripes*. [AAW69373.1] and *D. rerio* [AAT37633.1]. Based on analysis of protein structure prediction using SMART, it is known that the partial cds of gouramy TLR3 homologous to part TIR2 domain superfamily of fish *L. crocea*, *E. coioides* and *D. rerio*. *Takifugu rubripes*, is a fish that does not have a transmembrane region in the TLR3 gene. In Figure 2, it can be seen that there are differences in the

results of the phylohenetic tree using the TLR3 and IL1β genes. This difference is due to the use of partial gene, rather than the complete gene. Although different, the gouramy is still one group with *T. rubripes, L. crocea*, *E. coioides*.

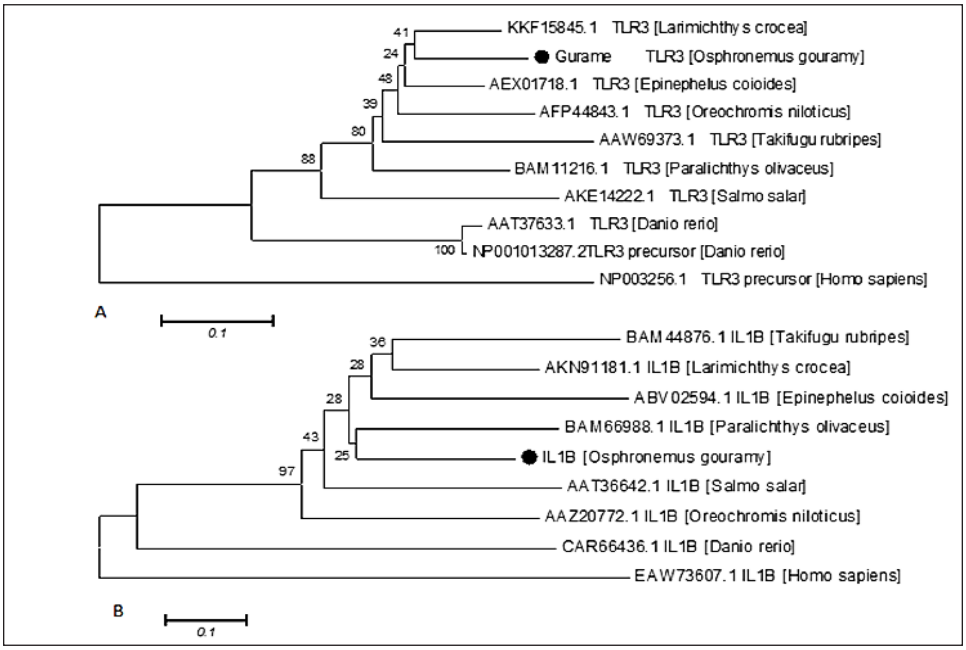


Figure 1. Phylogenetic tree of *O. Gouramy* partial TLR3 (A) and IL-1β (B)

The tree was constructed by the Neighbor-joining method using the CLUSTALW in MEGA 6 and was bootstrapped 5000 times.

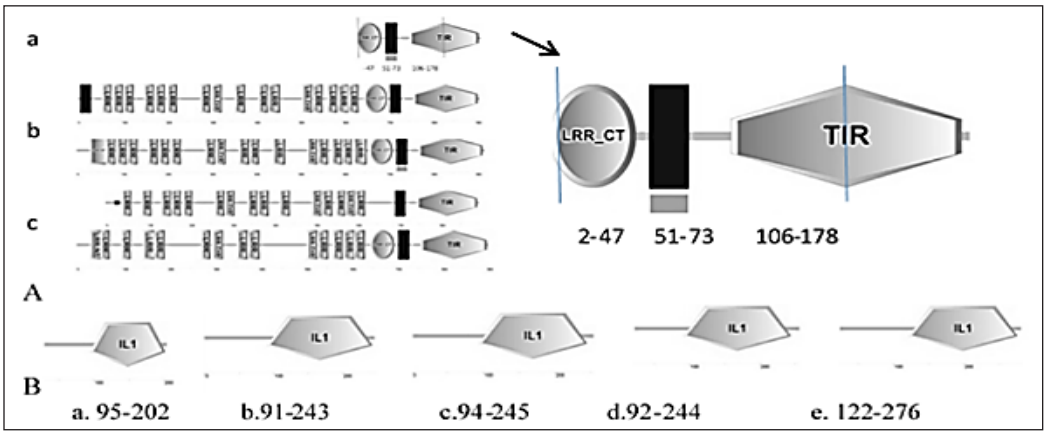


Figure 2. Structure prediction of *O. Gouramy* partial ORF TLR3 Partial (A) and partial ORF IL-1β (B). TLR3: a. *O. gouramy*, b. *L.crocea* [KKF15845.1], c. *E.coioides* [AEX01718.1], d. *T.rubripes*. [AAW69373.1], e. *D.rerio* [AAT37633.1] IL1β: a. *O. gourami,* b. *P.olivaceus* [BAM66988.1], c. *E.coioides* [ABV02594.1], d. *T. rubripes* [BAM44876.1], e. *D. rerio* [CAR66436.1]

Analysis of protein structure prediction OgIL-1β partial gene using the SMART programme found that amino acids 95-202 region is part of the area IL1 superfamily (Figure 2B). For comparison amino acid sequence of the fish *P. olivaceus* [BAM66988.1], *E. coioides* [ABV02594.1], *T. rubripes* [BAM44876.1] and *D.rerio* [CAR66436.1] were analysed using SMART. The analysis of protein structure using SMART showed that all species of fish reference derived from the NCBI GenBank had a similar structure which had a domain IL1 at 91-245 amino acid regions except the *D. rerio* domain IL1 area on 122-276.

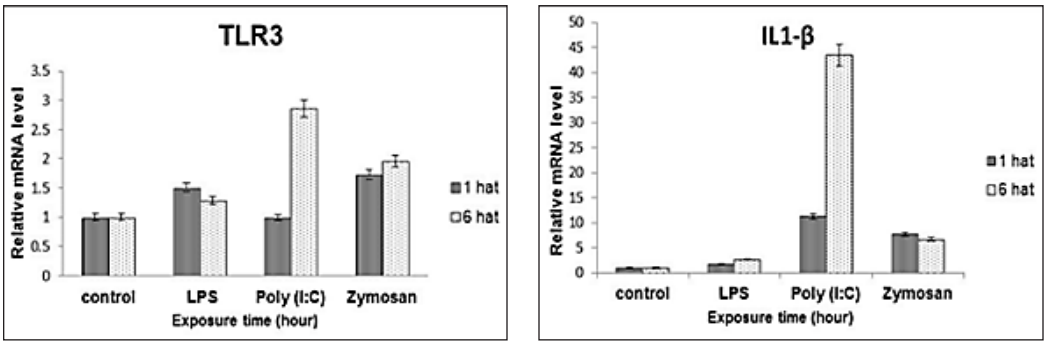
Expression Analysis of IL-1β and TLR3 Genes

The results of TLR3 and IL1-β gene expression analysis on gouramy kidney cell post stimulation (LPS, Zymosan and Poly I: C) can be seen in Figure 3. The relative expression of the genes TLR3 and IL1- β is normalised by the expression of house keeping genes Ef1α and gapdh. One hour after treatment (hat) to six hours after treatment (hat) kidney cell post treated with LPS shows to increase expression of interleukin-1β and downregulate expression of TLR3. Poly (I:C) as an antigen that responds to antiviral induces an increase in the transcription both of Toll-like receptor 3 and IL1β. At the same time, Zymosan in kidney cells increased expression of TLR3, but downregulated expression of IL-1β. This shows that TLR3 was activated not only by Poly I:C but also by LPS and Zymosan.

Expression of OgTLR3 showed up-regulation after treatment with lipopolysaccharide (LPS) in gouramy. This shows that OgTLR3 in gouramy also plays a role in defense against gram negative-bacteria**.** This research is in line with in mice (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Kadowaki et al., 2001), after infection with the Gram-negative *Edwardsiella ictulari* in a channel and *Edwardsiella tarda* in zebrafish (Phelan, Mellon, & Kim, 2005) and after infection large yellow croaker, (*Pseudosciaena crocea*) with *Vibrio parahemolyticus* (Huang, Wang, & Yao, 2011).

PBS was used as a control expression levels which are normalised to the expression level of EF-1α and Gapdh (hat: hour after treatment). However, the IL-1β and TLR3 have significantly increased transcript in the kidney cell six hours after treatment with Poly I:C. Poly I:C induced labor IL-1β and TLR3 expressed higher than LPS and Zymosan. Therefore, this research has showed the TLR3 response to Poly I:C is more dominant than Zymosan and LPS. This research contrasts with other research projects which have shown LPS to be more dominant than poly I:C to induce expression of IL-1β in Japanese flounder (Taechavasonyoo et al., 2013). The results of this study indicate that gouramy potency against viral infection is higher than gram negative bacteria like *Aeromonas hydrophila*.

Genetic information pertaining to the innate defense system in gouramy is required to understand the level of gene



(a) (b)

Figure 3. Expressions of: (a) OgTLR3; and (b) OgIL-1β in Gouramy after Stimulation LPS, Poly IC and Zymosan.

expression as a molecular response in the innate defense system in gouramy infected with pathogenic microbes. Such information is required to anticipate management of disease control in gouramy in a more targeted future at the molecular level whose application may include (i) development of bacteriosides aimed at the intervention of pathogen virulence, (ii) the development of supplements, feeds or drugs that can be ascertained molecules increase the immune response in fish, especially in gouramy and (iii) the development of gouramy strains of certain pathogens through conventional crosses or through the development of transgenic fish.

CONCLUSION

Phylogenetic tree, BLAST and structure predictive analysis show that OgIL-1β and OgTLR3 like those found in other fish such as *L. crocea, E. coioides, T. rubripes, P. olivaceus* and *D. rerio*. OgTLR3 in kidney cell transcript was identified in response to poly I:C, Zymosan, and LPS. These results indicate that OgTLR3 in gouramy also plays a role in defense against bacterial fungi and viral infection. However, the highest expression of IL-1β and TLR3 post-treatment with poly IC shows the main functions of OgTLR3 against viral infection.

ACKNOWLEDGMENTS

The authors would like to extend their gratitude to the support received from Doctoral Scholarship Programme by DIKTI (Directorate General of Higher Education Indonesia) and to Professor Ikuo Hirono, from Laboratory of Genome Science, Tokyo University of Marine Science and Technology, for providing LPS, Poly IC and Zymosan.

REFERENCES

Alexopoulou, L., Holt, A. C., Medzhitov, R., & Flavell, R. A. (2001). Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature, 413*(6857), 732.

Christien, H., Djayus, Y., & Ezraneti, R. (2014). The effectiveness of mahkota dewa (*Phaleria macrocarpa*) leaf extract as antibacterial to

prevent the *Aeromonas hydrophila* bacteria attack against gouramy (*Osphronemus gouramy*). *Aquacoastmarine*, *3*(2), 120-132.

Engelsma, M. Y., Stet, R. J. M., Schipper, H., & Verburg-van Kemenade, B. L. (2001). Regulation of interleukin 1 beta RNA expression in the common carp, *Cyprinus carpio* L. *Developmental & Comparative Immunology*, *25*(3), 195-203.

Fierro-Castro, C., Barrioluengo, L., López-Fierro, P., Razquin, B. E., Carracedo, B., & Villena, A. J. (2012). Fish cell cultures as in vitro models of pro-inflammatory responses elicited by immunostimulants. *Fish & Shellfish Immunology*, *33*(2), 389-400.

Gadberry, M. D., Malcomber, S. T., Doust, A. N., & Kellogg, E. A. (2004). Primaclade - A flexible tool to find conserved PCR primers across multiple species. *Bioinformatics*, *21*(7), 1263-1264.

Hsieh, J. C., Pan, C. Y., & Chen, J. Y. (2010). Tilapia hepcidin (TH) 2-3 as a transgene in transgenic fish enhances resistance to vibrio vulnificus infection and causes variations in immune-related genes after infection by different bacterial species. *Fish & Shellfish Immunology*, *29*(3), 430-439.

Huang, X. N., Wang, Z. Y., & Yao, C. L. (2011). Characterization of Toll-like receptor 3 gene in large yellow croaker, Pseudosciaena crocea. *Fish & Shellfish Immunology, 31*(1), 98-106.

Kadowaki, N., Ho, S., Antonenko, S., de Waal Malefyt, R., Kastelein, R. A., Bazan, F., & Liu, Y. J. (2001). Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *Journal of Experimental Medicine*, *194*(6), 863-870.

Kusumawaty, D., Suhandono, S., Pancoro, A., & Aryantha, I. N. P. (2017, March). Induction of Toll-like receptor (TLR) 2 and Myeloid88-dependent TLR-signaling response to aeromonas hydrophila infections in gouramy (osphronemus gouramy). *IOP Conference Series: Materials Science and Engineering*, *180*(1), 012270.

Lu, D. Q., Bei, J. X., Feng, L. N., Zhang, Y., Liu, X. C., Wang, L., ... & Lin, H. R. (2008). Interleukin-1β gene in orange-spotted grouper, Epinephelus coioides: Molecular cloning, expression, biological activities and signal transduction. *Molecular Immunology*, *45*(4), 857-867.

Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., & Beyaert, R. (2008). Stimulation of Toll-like receptor 3 and 4 induces interleukin-1β maturation by caspase-8. *Journal of Experimental Medicine*, *205*(9), 1967-1973.

Matsumoto, M., Oshiumi, H., & Seya, T. (2011). Antiviral responses induced by the TLR3 pathway. *Reviews in Medical Virology*, *21*(2), 67-77.

Oppenheim, J. J., Kovacs, E. J., Matsushima, K., & Durum, S. K. (1986). There is more than one interleukin 1. *Immunology Today*, *7*(2), 45-56.

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

Rauta, P. R., Nayak, B., & Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: A model for higher organisms. *Immunology Letters*, *148*(1), 23-33.

Scapigliati, G., Buonocore, F., Bird, S., Zou, J., Pelegrin, P., Falasca, C., ... & Secombes, C. J. (2001). Phylogeny of cytokines: molecular cloning and expression analysis of sea bass Dicentrarchus labrax interleukin-1β. *Fish & Shellfish Immunology*, *11*(8), 711-726.

Simanjuntak, S. B. I., Wibowo, E. S., & Indarmawan, I. (2016). Stimulation of deprivation cycles with spirulina platensis feed supplementation on

osphronemus gouramy physiological responses. *Biosaintifika: Journal of Biology and Biology Education*, *8*(3), 377-384.

Taechavasonyoo, A., Kondo, H., Nozaki, R., Suzuki, Y., & Hirono, I. (2013). Identification of novel interleukin 1 beta family genes in Japanese flounder paralichthys olivaceus. *Fish & Shellfish Immunology*, *34*(1), 393-396.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution*, *28*(10), 2731-2739.

Tort, L., Balasch, J. C., & Mackenzie, S. (2003). Fish immune system. A crossroads between innate and adaptive responses. *Inmunología*, *22*(3), 277-286.

Zou, J., Grabowski, P. S., Cunningham, C., & Secombes, C. J. (1999). Molecular cloning of interleukin 1β from rainbow trout Oncorhynchus mykiss reveals no evidence of an ICE cut site. *Cytokine*, *11*(8), 552-560.